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Drying Stress and Survival of *Shigella* and *Salmonella* in Food Derived Model Systems

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Dipl.–Ing. Stephan Flessa

Zusammenfassung

Jedes Jahr erkrankt weltweit eine hohe Anzahl von Personen durch Lebensmittelinfektionen. Diese führen zu hohen Kosten für den Gesundheitssektor. Von großer Bedeutung dabei sind Lebensmittelkontaminationen durch kleine, Bakterien enthaltende Tropfen.

Um die Anzahl der Lebensmittelinfektionen aufgrund von Tropfenkontaminationen reduzieren zu können, wurden die Einflüsse auf Bakterien in 10 µl Tropfen während der Trocknung und die Mechanismen während der Trocknung im Detail untersucht. Die untersuchten Hauptgebiete können in drei Bereiche unterteilt werden.

Als erstes wurden die Einflüsse des Trägermediums und der Zelldichte während einer Trocknungszeit von 1,5 h untersucht. Die größte Reduktion an Zellpopulationen wurde immer kurz vor der kompletten Austrockung des Tropfens beobachtet. Die Zelldichte hatte einen signifikanten Einfluß auf das Überleben von Laktobazillen, *Shigella sonnei*, *Salmonella* Enteriditis und *Escherichia coli* O157:H7. Je höher die verwendete Zelldichte, eine desto bessere Überlebensrate wurde beobachtet. Die Verwendung von MilliQ Wasser, Peptonwasser, Pferdeblutserum und einer Salzlösung als Träger der Bakterien resultierte in signifikant unterschiedlichen Überlebensraten von *S. sonnei*, *S.* Enteriditis und *E. coli* O157:H7. Die Überlebensraten waren am besten bei der Verwendung von Pferdebluserum gefolgt von Peptonwasser, MilliQ Wasser und Salzlösung. Auch ein signifikanter Schutzeffekt von toten Zellen auf lebende während der Trocknung wurde beobachtet.

Zweitens wurden die äusseren Einflüsse auf die Bakteien während der Trocknung untersucht. Kein Unterschied bei der Überlebensrate von *S. sonnei* wurde festgestellt, wenn die Bakteien aerob oder anaerob aufgezogen wurden. Weder eine Änderung der Hydrophobizität der Oberfläche, noch die Verwendung von Glas- oder Stahloberflächen veränderte die Überlebensrate. Ein signifikanter Unterschied der Überlebensrate wurde festgestellt, wenn die Bakterien vor der Trocknung bei verschieden Temperaturen aufgezogen

wurden. Die Bakterien überlebten am besten, wenn sie bei optimaler Temperatur aufgezogen wurden. Eine signifikant bessere Überlebensrate von *S. sonnei* wurde auch auf der Oberfläche von Erdbeeren im Vergleich zu der Oberfläche von Glass festgestellt.

Dittens wurden die intrazellulären Veränderungen während der Trocknung untersucht. Bei *S. sonnei* und *S.* Enteriditis wurde eine vermehrte Produktion von allgemeinen Streßproteinen, Proteinen der äusseren Membran und Proteinen der Energieproduktion festgestellt. Darüberhinaus wurde eine vermehrte Produktion von Trehalose während der Trocknung beobachtet. Eine Veränderung der Fettsäurenzusammensetzung der Zellmembran von *S. sonnei* und *S.* Enteriditis wurde während einer Trocknungsperiode von 3 h festgestellt. Die Menge der 18:0 Fettsäure fiel in dem Mase ab, in dem die Menge der 15:1 Fettsäure stieg.

Die in dieser Arbeit gefundenen Resultate verbessern das Verständnis für Einflüsse auf das Überleben von Bakterien – sowohl pathogenen Bakterian als auch Starterkulturen – in einer Suspension bei der Trocknung. Durch das Verständnis und die Anwendung der Resultate kann eine Verminderung an krankheitserregenden Lebensmittelkontaminationen erzielt werden.

Summary

Foodborne pathogens are responible for a large number of illnesses and even deaths every year. The economic cost of the illnesses is very high, for 1995 an estimated cost of $6.5 – 34.9 billion for the United States of America was reported. Of great comncern is the contamination of food via droplets containing bacteria.

To be able to reduce the number of foodborne illnesses due to droplet contaminations an in depth study on the behavior of pathogens in droplets was needed. Influences on and mechanisms of bacteria in droplets during desiccation were investigated in detail. The areas investigated can be divided into three major groups.

First, influences of carrier medium and cell density during a drying period of 1.5 h were investigated. The biggest decrease of cell population was always seen shortly before the inoculum visibly dried (20 min). Cell density had a significant impact on the survival of lactobacilli, *Shigella sonnei*, *Salmonella* Enteriditis, and *Escherichia coli* O157:H7. The higher the cell density in the inoculum the better the observed survival rate. The use of MilliQ water, 0.1% peptone water, horse serum, and saline solution as carrier media resulted in significantly different survival rates for *S. sonnei*, *S.* Enteriditis, and *E. coli* O157:H7. Survival rates were highest when horse serum was used, followed by peptone water, MilliQ water, and saline solution. Dead bacteria provided significant protection to viable cells.

Secondly, environmaental influences on the bacteria during desiccation were evaluated. No influence on the survival of *S. sonnei* was observed when bacteria were either grown under aerobic or anaerobic conditions. Changes of the hydrophobicity of the surfaces on which the bacteria were dried did not change the survival rate, nor was there an influence if steel or glass as a surface was used. Also a significant better survival was shown for *Shigella flexneri* on the surface of strawberries compared to glass slips. But a significant difference was observed when *S. sonnei* and *S.* Enteriditis were grown at different temperatures prior to desiccation. Bacteria survived best when grown at optimal growth temperature.

Thirdly, intracellular changes during desiccation were determined. For *S. sonnei* and *S.* Enteriditis up-regulated proteins were observed during desiccation, such as general stress proteins, proteins of the outer membrane, and proteins needed for energy production. Furthermore an up-regulated production of trehalose during desiccation was observed. A change of the fatty acid composition of the cell membrane of *S. sonnei* and *S.* Enteriditis was observed during a 3 h drying period. The amount of the 18:0 fatty acid decreased by an amount corresponding to the increase in the 15:1 fatty acid.

All results improve the understanding of and influences on the survival of bacteria - pathogenic bacteria as well as starter cultures - in a suspension during drying. Application of these results may lead to improved reduction of pathogenic bacteria in food processing environments ultimately resulting in reduced morbidity and mortality.

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Chapter One

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Introduction and objectives

Introduction

The most serious food safety problems are of microbial origin (Collins, 1997). Because of the increase of working persons in a family, less time is left to prepare home made foods. Therefore, an increased consumption of ready to eat foods was observed in the last decade. Changing consumer lifestyles from home made food to buying ready to eat or ready to cook food in the supermarket play a major role in the safety of food (WHO, 2004a). With that change, the spectrum and epidemiology of foodborne pathogens has also changed over the last years (Tauxe, 2002; Altekruse et al. 1997). Newly emerging multidrug resistant pathogens *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium DT 104 have increasingly caused illness. Recently a *Salmonella enterica* serotype Choleraesius was discovered in Taiwan, which is resistant to all antimicrobials commonly used to treat salmonellosis (Chiu et al., 2004). Also *Cyclospora* and *E. coli* O157:H7 were newly recognized as foodborne pathogens (Sivapalasingam, 2004). Furthermore *Enterobacter sazakii* was reported to be associated to powdered milk formulas, thus causing meningitis and enterocolitis in premature infants (Muytjens et al., 1983; van Acker et al., 2001).

Worldwide foodborne illness is recognized as an important cause of morbidity and mortality (WHO, 2004b). In the United States of America the cost of the illnesses and death caused by the seven most common foodborne bacteria (*Campylobacter jejuni*, *Clostridium perfringens*, *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus*, and *Toxoplasma gondii*) is estimated at $6.5 – 34.9 billion for the year 1995 (Buzby and Roberts, 1997). In California there were 56,660 reported cases of nontyphodial *Salmonella* infections in the years of 1990 through 1999 with an estimated cost for hospitalization of $200 million (Trevejo et al., 2002). For New Zealand the cost of foodborne diseases was estimated to be $55.1 million annually, with campylobacteriosis as the most costly illness (Scott et al., 2000). An outbreak of *E. coli* O157:H7 associated with school lunches affecting 268 people in Japan had an estimated economic and health cost of $1 million (Abe et al., 2002). In Uppsala,

Sweden, a one-year study of foodborne illnesses was conducted 1998/1999. The most common illnesses were attributed to calcivirus, *Campylobacter* spp., and *Staphyloccus aureus* (Lindqvist et al., 2001). In Table 1.1 these studies are summarized and the approximate average cost per illness is calculated.

Table 1.1: *Cost of foodborne illnesses*

Country	Year(s)	Approximate average cost per illness in US$
USA	1995	2,837
California	1990 - 1999	3,530
New Zealand	Annually	462
Japan	1996	3,731
Sweden	1998 - 1999	303

All over costs for the health system due to foodborne pathogens is very high. Thus prevention of possible contamination of food with pathogenic bacteria could relieve the health sector of many costs.

Foodborne illnesses increased due to the consumption of raw vegetables and fruits in the Unites States of America from 1987 to 1997, in the period of 1973 through 1997 almost half the outbreaks were caused by *Salmonella* (Harris et al., 2003; Beuchat and Ryu, 1997; Tauxe et al., 1997). All together the majority of foodborne illnesses was traced back to *Shigella*, *Salmonella*, and *E. coli* O157:H7 strains (Figure 1.1). Of these three, *Shigella* is the least well studied organism.

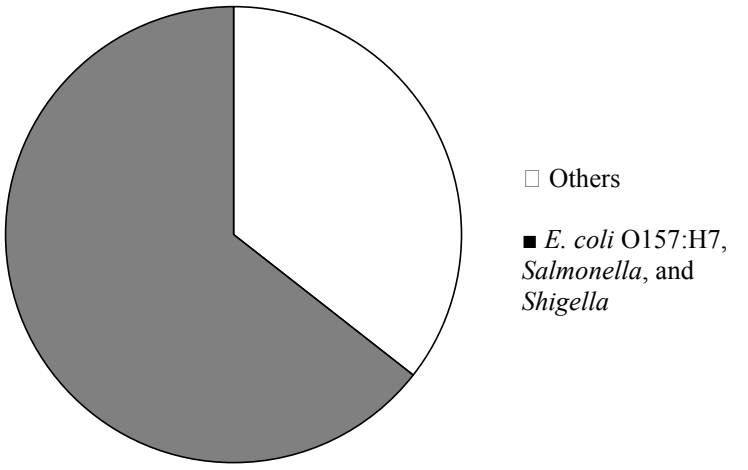

Figure 1.1: *Outbreaks of foodborne illnesses associated with fresh produce 1987-2001 related to Shigella, Salmonella, and E. coli O157:H7 (Harris et al., 2003).*

In 2000/2001 and 2004 outbreaks of salmonellosis associated with California almonds were reported (Issaac et al., 2005). In 1998 a shigellosis outbreak was associated with parsley (CDC, 1999). An *E. coli* O157:H7 outbreak was associated to the consumption of spinach in 2003 and more recently to the consumption of cantaloupes in 2004 (www.about - E. coli.com).

Sources, modes, and levels of natural contamination are generally unknown, but it is hypothesized that contamination occurs from contact with soil, manure, water, hands, or equipment (Beuchat and Ryu, 1997; FDA 2003). Water as potential contamination source is important, since water is used for irrigation, during harvest, packing, and processing and water has major potential to cause large scale cross contamination.

When studying the interaction of pathogens with produce and or the produce processing environment, a number of factors must be taken into consideration, e.g. inoculum size and carrier medium (Beuchat et al., 2001). However the choice for some of these factors

is often determined by technical limitations (e.g. limit of detection with plating methods) rather than a more systematic investigation of their individual impacts.

In general, the avoidance of droplet contamination is part of the risk management strategy in infection control (McCulloch, 1999). Roundy (1967) reported on the danger of contamination by droplet infections of cheese. Droplet contamination also plays an important role for the cross-contamination during defeathering of poultry (Allen et al., 2003). However, little is known about the behavior of pathogens in these suspensions, in particular during drying.

Few papers report about the influence of the inoculum density of bacteria on the survival during desiccation. On strawberries it was shown that a lower inoculum concentration of *Listeria monocytogenes* decreased faster than a higher inoculum concentration (Flessa and Harris, 2005). Wilson and Lindow (1994) noted a similar effect for survival of *Pseudomonas syringae* on bean plant leaves. Kusumaningrum et al. (2003) inoculated steel plates with *Salmonella* Enteriditis, *Staphylococcus aureus*, and *Campylobacter jejuni* with up to three different inoculum densities. For *Campylobacter jejuni* a greater decrease of the high inoculum compared to the low inoculm was observed within 1 h. However, for *Salmonella* Enteriditis and *Staphylococcus aureus* levels dropped faster for the low and moderate inoculum compared to the high inoculum within a drying time of 4 hours.

Drying can be divided into different individual kinds of stresses on the bacteria. The removal of water around the cells concentrates the solutes providing an osmotic stress. As water is removed oxygen can more readily react with the cells, which is an oxidative stress. Furthermore, starvation stress is imposed on the cells, since nutrients are no longer readily available. The same stresses are also imposed on starter cultures when they are either dried for distribution or used in the final product if a drying step is included.

Membranes are one key factor during the desiccation of bacteria. In most cases when bacteria are dried their membrane will undergo a phase change from liquid-crystalline to a gel. When cells are rehydrated and change their phase to their initial phase, ruptures in the membrane take place and solute leakage will occur (Crowe et al., 1998). This effect can be prevented by addition of trehalose (Figure 1.2). Bacteria are known to counteract temperature increases or drops by changing their lipid composition within the membrane to keep the liquid-crystalline phase stable, whereas more unsaturated and short-chained fatty acids are incorporated into the membrane at sub-optimal temperatures and more saturated and long-chained fatty acids are incorporated at supra-optimal growth temperatures (Beney and Gervais, 2001; Crowe et al., 1998).

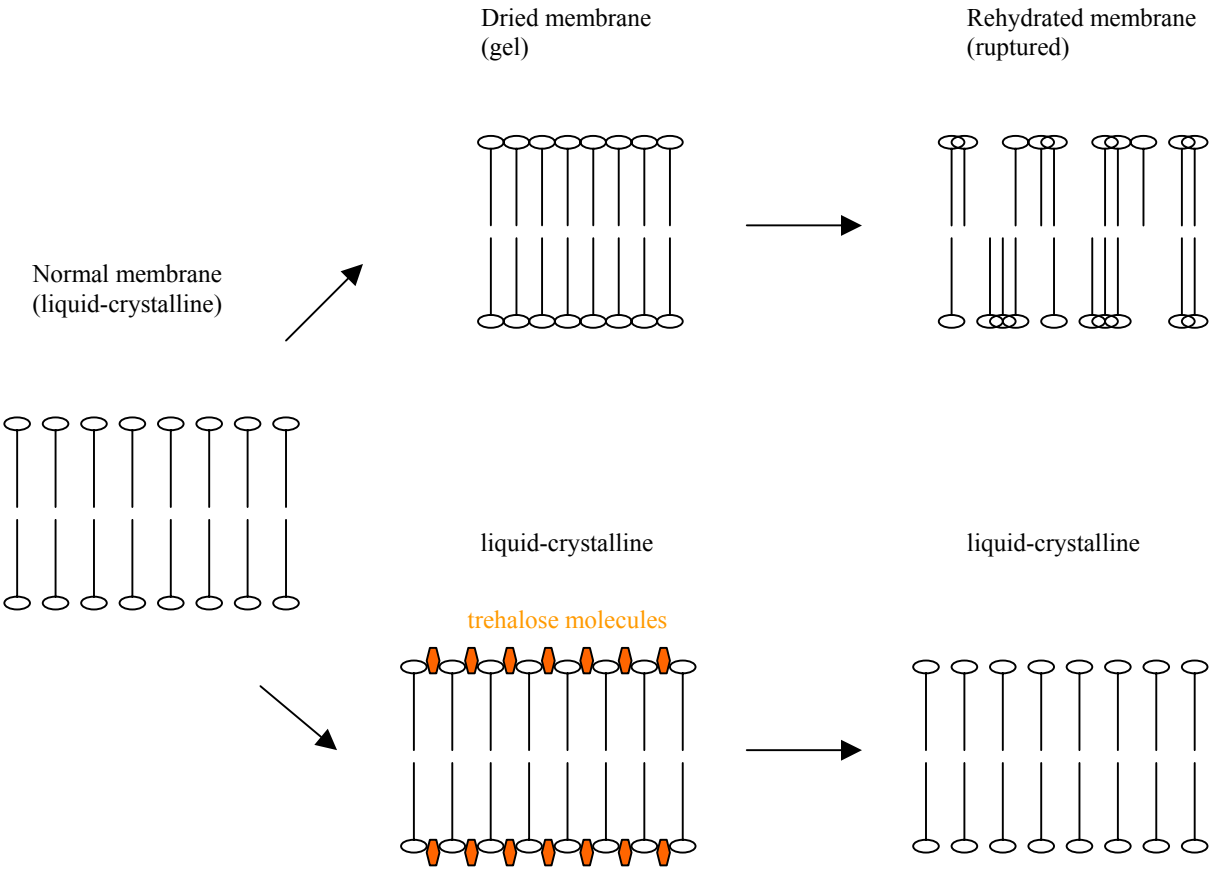

Figure 1.2: *Proposed mechanism of trehalose during drying (Crowe et al., 1998)*

Most of the published studies have focused on influences on the drying process with the goal of enhancing survival of dried starter cultures. Compatible solutes as betaine, carnitine, and especially trehalose were found to enhance survival during freeze- and air-drying (Leslie et al., 1995; Kets et al., 1995; Elbein et al., 2003). The protection effect of trehalose against abiotic stresses as drought, salt, and cold, was also shown in the plant kingdom for transgenic rice plants (Jang et al., 2003). Trehalose is able to act as a water substitute in membranes and proteins and thus prevents phase transition in the cell membrane (Crowe et al., 1998; Beney et al., 2004). As seen in Figure 1.2, when trehalose is present during desiccation the membrane is able to maintain its liquid crystalline phase. In the absence of trehalose the membrane changes to a gel state, which leads to rupture of the membrane after rehydration.

Bacterial populations respond to environmental stress factors intracellularly, i.e., respond with an up-regulation of stress-response proteins. In order to evaluate changes on the transcriptional level, mRNA isolation, transcription into cDNA, and quantification can be employed (Van der Mer-Van Kraaij, 2003). With the help of real time PCR the number of cDNA copies of known sequences of enzymes and proteins can be determined. This can provide information on whether the gene is up- or down-regulated in response to a certain stress on the cell.

To determine the effects on the translational level, two-dimensional SDS polyacrylamide gels can be made of protein extracts of stressed and non-stressed cells. These gels are compared to determine proteins that are up- or down-regulated during the stress. Protein spots can be extracted and the protein can be sequenced to gather information which enzyme or protein is regulated (Cordwell et al., 2001; Liao et al., 2003).

Because of the many influences on bacteria during drying this work is divided into chapters, each focused on a different aspect of drying. Each chapter will provide a more

indepth introduction for each topic. To determine the influences on bacteria during drying this study has the following objectives.

Objectives of this work

In this study the drying process of a droplet with bacteria in suspension on a general surfaces described. The following aspects were studied in detail:

1. The survival of *Shigella flexneri* on whole and cut strawberries stored at temperatures of -20, 4, and 24ºC.
2. The drying mechanics of a droplet on glass slips with a microscope.
3. The survival of *Shigella sonnei*, *Salmonella* Enteriditis, *E. coli* O157:H7, and *Lactobacilli* with regard to inoculum size and carrier medium on steel and glass surfaces.
4. The influence of different parameters as surface hydrophobicity, dead bacteria, and different growth temperatures on the survival of *S. sonnei* and *S.* Enteriditis.
5. Desiccation induced changes of transcription, translation, and bio expression of *S. sonnei* and *S.* Enteriditis.
6. Changes of the membrane of gram-negative bacteria during desiccation.

Chapter Two

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Strains used and basic observations

INTRODUCTION

The drying process of suspensions of bacteria in a droplet is very complex and includes many different aspects such as the physics of the actual drying, the complexity of the drying surface, and the behavior of the bacteria. Therefore this thesis is divided into chapters, each addressing the different aspects of the phenomenon. In the second chapter all bacterial strains used in the study are described in detail.

The basic methods and research on the compatibility of the strains with respect to antibiotic resistance and to the drying surface are discussed. These results apply throughout the whole work.

STRAINS USED

Gram-negative bacteria *Shigella*, *Salmonella*, and *Escherichia coli* O157:H7 were used. All strains belong to the Enterobacteriacae family. *Shigella* and *E. coli* are non-motile, but *Salmonella* are motile. *Lactobacillus* was the gram-positive bacterium used, which is also non-motile.

Shigella. *Shigella flexneri* LJH 607, a non-pathogenic strain by genetic mutation and deletion (2457M) and LJH 613, a variant of LJH 607 resistant to rifampicin (80 μg/ml) were used. LJH 613 was adapted to grow in the presence of rifampicin (80 μg/ml) by step-wise exposure. Briefly, 100 μl of an overnight culture was spread onto tryptic soy agar (Difco, Detroit, MI) containing rifampicin at levels of 5 to 40 μg/ml (Sigma, St. Louis, MO). After incubation for 24 h at 37°C, isolated colonies were selected from the plate containing the highest level of rifampicin and cultured overnight in tryptic soy broth with rifampicin. This procedure was repeated until a variant resistant to 80 μg of rifampicin per milliliter was obtained. Growth curves of the parent and variant strain in tryptic soy broth were similar (data not shown).

S. sonnei LJH 664 (wild type) and its rifampicin-resistant variant LJH 661 (Figure 2.1) were used. *S. sonnei* strains were kindly provided by Dr. T. V. Suslow, University of California, Davis.

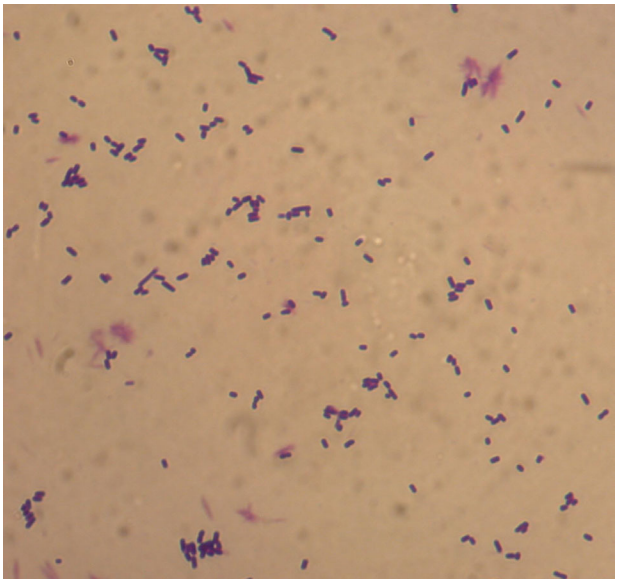

Figure 2.1: *Violet red stained* Shigella sonnei *cells, LJH 613.*

Salmonella. *Salmonella enterica* serovar Enteriditis PT30 (LJH 608) (Figure 2.2) and its nalidixic acid-resistant (50 µg/ml) variant LJH 637 were used.

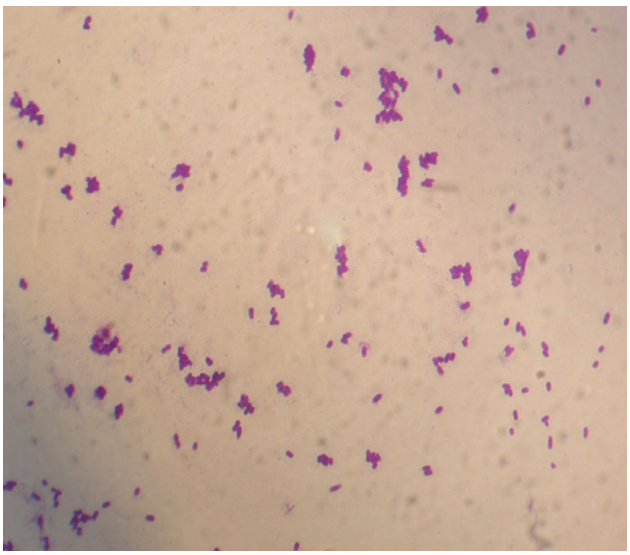

Figure 2.2: *Violet red stained* Salmonella *Enteriditis cells, LJH 608.*

***E. coli* O157:H7.** A nalidixic acid-resistant (50 µg/ml) *E. coli* O157:H7 (LJH 537) isolated from human feces associated with a lettuce outbreak was used in the experiments (kindly provided by Dr. Beuchat, University of Georgia).

Lactobacilli. *Lactobacillus sanfranciscensis* TMW 1.52 (Figure 2.4) and TMW 1.362 were used, as well as a mutant of TMW 1.362 where the gene for the production of exo-polysaccharids (EPS) was deleted (kindly provided by M. Tieking, Technical University of Munich). A strain of *L. reuteri* TMW 1.656 was included in some experiments.

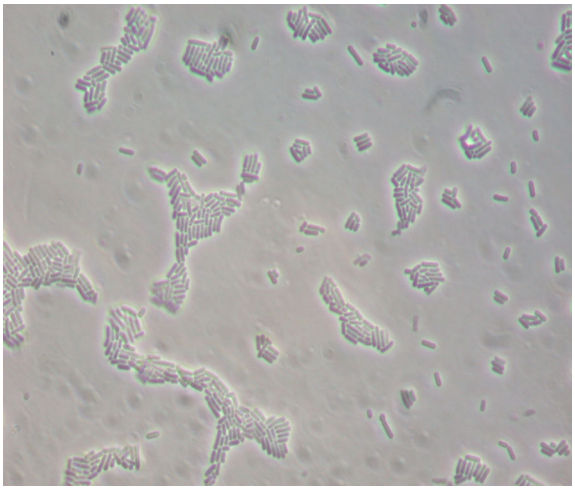

Figure 2.4: *Non-stained* Lactobacillus sanfranciscensis *cells, TMW 1.52.*

BASIC METHODS AND GENERAL RESULTS

Strawberries. Fresh, ripe, unblemished strawberries were purchased at a local supermarket either the day before or the day of an experiment. The strawberries were stored at 4°C until approximately 1 h before the experiment when they were transferred to ambient temperature (24 ± 2°C).

Test strains and media. *S. sonnei* LJH 664, non-resistant and LJH 661 rifampicin-resistant were used, as well as the rifampicin-resistant *S. flexneri* LJH 613. The non-resistant *S.* Enteriditis strain LJH 608 and the nalidixic acid-resistant strain LJH 637 were also used.

To enumerate viable populations of non-antibiotic resistant strains tryptic soy agar (Difco, Detroit, MI) (TSA) was used as recovery media. The recipe for TSA is shown in Appendix 1. For strains resistant to rifampicin or nalidixic acid, tryptic soy agar with rifampicin (80 μg/ml) (TSAPR) or nalidixic acid (50 μg/ml) (Sigma) (TSAPN), respectively were designated the recovery media.

Inoculum preparation. Before each experiment, frozen stock culture was streaked onto TSA, TSAPN or TSAPR and an isolated colony was subcultured in tryptose soy broth without supplement for the non-resistant strains, or supplemented with rifampicin (80 μg/ml) for *S. flexneri* and *S. sonnei*, or nalidixic acid (50 μg/ml) for *S.* Enteriditis (37°C for 24 h). Single loop transfers were prepared at two consecutive 18 to 24-h intervals. Overnight cultures were collected by centrifugation (12,000 rpm for 2 min) and suspended in an equal volume 5% horse serum (Gibco, BRL, Grand Island, NY) or MilliQ water. The culture was washed twice and further diluted in horse serum or MilliQ water to produce the desired inoculum concentration.

Inoculation procedure. A 10 μl drop of the prepared inoculum was spot inoculated onto strawberries, glass slips (No.1, 18 mm sq., Cover Glass, Corning Glass Works) or onto food grade steel slips (304 steel) (1cm x 1cm, custom made). Berries and slips were stored in a biological hood with the fan running at 28 ± 1ºC for up to 24 h.

Recovery of pathogens. Pathogens on whole strawberries were recovered by stomaching (Stomacher 400, Seward Medical, London, UK) for 90 s in 100 ml 0.1 M phosphate buffer (0.1M KH_2PO_4, 0.04 M Na_2CO_3, BBL, Becton Dickinson, Cockeysville, MD) (Knudsen et al., 2001).

Inoculated glass and steel slips were placed into a 50 ml plastic tube (Becton Dickinson Labware, Franklin Lakes, NJ) with 5 ml of 0.1% peptone water and 15 glass beads (3 mm solid, Fisher). Tubes were vortexed (Vortex Genie 2, Fisher) for 20 s at high speed.

Sample rinse or homogenate was subsequently diluted 10-fold in 0.1% peptone and spot plated (25 µl) onto media. Presumptive *S.* Enteriditis, *S. flexneri* and *S. sonnei* were counted after incubation at 37°C for 24 h.

Statistics. Anova models were employed using the SAS software package (ver. 6.0, Cary, NC). Data for antibiotic resistance and surface influences were log transformed. To compare the differences between the resistant and non-resistant strain, linear contrast models were used. Differences were considered significant at $P \leq 0.05$.

RESULTS

Influence of the antibiotic resistance. To determine the influence of the antibiotic resistance regarding survival during desiccation, cells were inoculated onto glass slips with MilliQ water as carrier. For *S. sonnei* a significantly better survival was seen for the rifampicin resistant strain ($P \leq 0.05$). In contrast non-resistant *S.* Enteriditis survived significantly ($P \leq 0.05$) better during the 1.5-h drying period (Table 2.1).

Table 2.1: *Influence of antibiotic resistance on the survival of* S. sonnei *and* S. *Enteriditis over time on glass slips (log (CFU ± SD)) (n = 6)*

	Drying time				
Strain	20 min	30 min	60 min	90 min	Δ decrease[a]
S. sonnei$^{rif-,b}$	5.75 ± 0.08 A[c]	4.49 ± 0.23 A	3.23 ± 0.28 A	3.23 ± 0.35 A	2.52
S. sonnei^{rif+}	5.80 ± 0.07 A	4.86 ± 0.23 B	3.84 ± 0.42 A	3.69 ± 0.25 B	2.11
S. Enteriditis$^{nal-,d}$	6.01 ± 0.13 A	4.85 ± 0.39 A	3.81 ± 0.32 A	3.43 ± 0.13 A	2.58
S. Enteriditis^{nal+}	5.93 ± 0.08 A	3.95 ± 0.33 B	3.22 ± 0.45 B	2.87 ± 0.38 B	3.06

[a]: log (CFU) decrease between 20 min and 90 min

[b]: rif indicates rifampicin, + indicates resistant, - indicates non resistant

[c]: different letters are indicating a significant difference among resistant and non-resistant strains at each tome point

[d]: nal indicates nalidixic acid, + indicates resistant, - indicates non resistant

Influence of the surface. When the survival of *S. sonnei* on strawberries and glass slips was compared, the rifampicin-resistant strain was inoculated onto the surfaces with 5% horse serum as carrier. After 1.5 h, populations declined significantly on berries and on glass slips ($P \leq 0.05$). No significant difference was seen for the decrease between the two surfaces (Table 2.2). Populations further decreased significantly for *S. sonnei* on strawberries and on glass slips from 1.5 to 24 h ($P \leq 0.05$). At 24 h numbers of *S. sonnei* were significantly lower on glass slips than on berries ($P \leq 0.05$).

Table 2.2: *Survival of* S. sonnei *on strawberries and glass slips with horse serum as carrier inoculated onto glass slips (log (CFU ± SD)) (n = 6)*

		Time after inoculation				
Inoculum	Surface	0 h	0.5 h	1.5 h	24 h	Δ decrease[a]
10^7	strawberry[b]	6.73 ± 0.08 A	6.43 ± 0.22 A	5.82 ± 0.26 A	4.71 ± 0.22 A	2.02
	glass	6.75 ± 0.04 A	6.03 ± 0.06 B	5.94 ± 0.05 A	4.25 ± 0.14 B	2.50

[a]: log (CFU) decrease between 0 h and 24 h

[b]: type of surface

[c]: different letters are indicating a significant difference among resistant and non-resistant strains at each time point

When glass slips and steel slips were compared, the non-resistant strain of *S. sonnei* was used with MilliQ water as carrier. Inocula decreased by approximately 2.6 log over the 90 min drying period. No significant difference in the survival could be observed (Table 2.3).

Table 2.3: *Survival of* S. sonnei *inoculated on glass and steel slips with MilliQ water as carrier (log (CFU ± SD)) (n = 6)*

		Time after inoculation				
Inoculum	Surface	15 min	30 min	60 min	90 min	Δ decrease[a]
10^6	glass[b]	5.70 ± 0.07 A	4.20 ± 0.39 A	3.52 ± 0.56 A	3.23 ± 0.38 A	2.47
	steel	5.71 ± 0.08 A	4.55 ± 0.27 A	3.37 ± 0.40 A	2.94 ± 0.52 A	2.77

[a]: log (CFU) decrease between 15 min and 90 min

[b]: type of slip used

DISCUSSION

These results investigate the basic influences of bacterial strains and surfaces during drying.

Caution has to be used to determine if an antibiotic resistant strain was used or not, since opposing influences of the antibiotic resistance could be shown. For *S. sonnei* the antibiotic resistance led, actively or not has yet to be determined, to an increased survival during desiccation, for *S.* Enteriditis it had the opposite effect.

For this work it could be determined that there is a influence of the surface on the survival of the bacteria during desiccation. Survival on glass slips and on the surface of whole strawberries was significantly different starting after storage of 24 h. It is possible that the epiphitic bacteria on the surface of berries had a positive effect on the survival of *S. sonnei*. It is also possible that the surface of the berries could provide to some extent nutrients to the cells and provide a more humid environment, in which cells survive better (Flessa et al., 2005).

When the survival of *S. sonnei* was compared for glass and steel slips no significant difference could be observed within 1.5 h, therefore results in this work which were seen on glass slips can be directly compared to results when steel slips were used. In contrast, Nakamura (1962) saw a survival of *S. sonnei* on glass for 1-6 days in comparison to metal surfaces for 0-4 days. However, Rose at al. (2003) showed a better survival of *Yersinia pestis* on stainless steel (1.42 CFU after 72 h) compared to glass (0.35 CFU after 72 h).

Chapter Three

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Survival of *Shigella flexneri* on strawberries

INTRODUCTION

Over the past decade, fresh fruits and vegetables have become increasingly important vehicles of foodborne illnesses in the United States of America (Altekruse et al., 1997; Beuchat 1997). Numerous surveys designed to determine the presence or absence of various foodborne pathogens are available for a wide variety of fruits and vegetables (Harris et al., 2003; Johannessen et al., 2002; Viswanathan and Kaur, 2001). The generally low rate of contamination (Harris et al., 2003) and perishability (7 days – 4 weeks (Cantwell, 2002)) of these products has made it impossible to quantify pathogen concentrations in naturally contaminated products.

Beuchat et al. (2001) noted a number of factors that were important to consider when inoculating fruits and vegetables with pathogens. Factors included in the inoculation procedures were carrier composition, inoculation procedure, and cell level in the inoculum. Low levels of inoculum may be desirable for challenge studies that assess the ability of the pathogen to survive or grow. Much higher levels are necessary to allow demonstration of several log reductions of an antimicrobial treatment or if death is expected upon drying of the inoculum. With detection limits of 100 to 1000 CFU in most cases, inoculum levels of greater than 6 log CFU per unit are often used (Beuchat, 1997; Rafi, 1997; Knudsen et al., 2001). Although this approach is valid given the limitations of microbial techniques, it assumes similar behavior at all inoculum levels, which may or may not be the case. Understanding the impact of inoculum density on survival or growth and on sensitivity to antimicrobial treatments is important for assessing the risks of these products.

There are very few published studies that have compared the influence of different inoculum levels on fresh fruits and vegetables. Survival and growth patterns of *Shigella sonnei* inoculated onto chopped and whole parsley were similar when the initial inoculum level was 10^3 or 10^6 CFU/g (Wu et al., 2000). Richert et al. (2000) reported a greater decrease of *Escherichia coli* O157:H7 on sliced cucumbers for the lower inoculum, when inoculated at

at levels of 10^3 and 10^6 CFU/g. Also, when *Listeria monocytogenes* was inoculated onto strawberries at 10^6 CFU and 10^8 CFU per berry, significantly higher reductions in population were observed with the lower inoculum levels when strawberries were stored at 24ºC (Flessa et al., 2005).

Monier and Lindow (2003) made a similar observation, that on leaf surfaces cell aggregates show better survival compared to single cells. Wilson and Lindow (1994) showed that *Pseudomonas syringae* survives better on bean leaves when inoculated at a level of 10^9 CFU/ml compared to a level of 10^6 CFU/ml.

No data is available for the survival of *Shigella flexneri* on strawberries. Therefore, the objective of this chapter was to evaluate the influence of the inoculum level on the survival of *S. flexneri* inoculated onto fresh strawberries stored at 24, 4, and -20°C.

MATERIALS AND METHODS

Strawberries. Fresh, ripe, unblemished strawberries were purchased at a local supermarket either the day before or the day of an experiment. The strawberries were stored at 4°C until approximately 1 h before the experiment when they were transferred to ambient temperature (24 ± 2°C).

The strawberries used in this study were left whole or cut into 0.6 cm slices (Knudsen et al., 2001). Whole strawberries were used unhulled and unwashed. When strawberries were stored frozen, they were hulled by aseptically removing the leafy part of the fruit (calyx) and cut into approximately 0.6 cm slices.

Test strains and media preparation. A non-pathogenic strain of *S. flexneri* (2457M) LJH 613 was obtained from Dr. Gary Acuff, Texas A & M University. Both selective and recovery media were used to enumerate viable populations of *S. flexneri*. Tryptic soy agar (Difco, Detroit, MI) with sodium pyruvate (1 g/l) (Fisher, Fair Lawn, NJ) and 80 µg of rifampicin per ml (TSAPR) was designated the recovery medium and MacConkey (Difco)

agar with rifampicin (80 µg/ml) (Sigma) (MACR) was used as the selective medium. Recipes for media are given in Appendix 1.

Inoculum preparation. Before each experiment, frozen stock culture was streaked onto TSAPR and an isolated colony was subcultured in tryptose soy broth supplemented with rifampicin (80 µg /ml). The subculture was incubated at 37°C for 24 h and single loop transfers were prepared at two consecutive 18 to 24-h intervals. Overnight cultures were collected by centrifugation and suspended in 5% horse serum (Gibco, BRL, Grand Island, NY). The culture was further diluted in 5% horse serum to produce the desired inoculum concentration. Inoculum levels were confirmed by plating onto both recovery and selective media. Horse serum was chosen as carrier to mimick contamination with organic material. Survival of cells in horse serum was highest of the 4 carriers evaluated (MilliQ water, 0.1% peptone water, horse serum, and 0.85% saline solution) (Flessa et al., 2003). Therefore using horse serum predicts the worst-case scenario.

Inoculation procedure. The intact surface of samples (one strawberry) was spot inoculated with 10 µl (2 to 4 drops) of the prepared *S. flexneri* inoculum. Strawberries were held at room temperature for 1.5 h in a biological hood with the fan running to facilitate drying. In previous studies the initial population fell approximately 1 log during the drying period but remained relatively consistent thereafter regardless of storage temperature (Knudsen et al., 2001). Strawberries were either immediately analyzed or transferred to closed plastic containers and incubated at 4 ± 2 or 24 ± 2°C for up to 5 or 3 days, respectively.

Sliced strawberries (25 ± 2 g) were placed into 15 x 23 cm filter bags (Nasco, Inc., Modesto, CA). Prepared bags were inoculated with 10 µl of the *S. flexneri* culture and gently massaged to ensure even dispersion of the bacteria. Samples were placed flat in a single layer in a freezer (-20 ± 2°C). Frozen samples were thawed by immersing the bag in a 25 ± 2°C water bath for 8 ± 2 min.

Recovery of pathogens. Pathogens on whole strawberries were recovered by the "shake-rub-shake" method. Inoculated whole strawberries were placed in a plastic bag with 25 ml of 0.1% peptone water. The bag was closed and shaken 15 times in a 30-cm arc, strawberries were gently rubbed by hand for 1 min and shaken an additional 10 times. Sample rinse was subsequently diluted 10-fold in 0.1% peptone. Samples were plated using an Autoplater 4000 Spiral Plater (Spiral Biotech, Inc, Bethesda, MD). Presumptive *S. flexneri* were counted after incubation at 37°C for 48 h.

When counts below the detection limit were expected for *Shigella*, sample rinse was enriched in double strength S*higella* broth (DSSB) made as described in the BAM manual (Andrews et al., 1998) (see Appendix 1). After enrichment, the presence or absence of *Shigella* was determined by PCR analysis as described by Flessa and Harris (2002a).

PCR to detect *Shigella*. Sample rinse was inoculated into DSSB (1:1, v/v) and incubated overnight at 37°C in a shaking water bath. DNA was extracted from 1.5 ml of enrichment broth by washing the cells in MilliQ water three times and boiling the cell suspension for 10 min. PCR primers targeting a 602-bp region of the *IapH* loci (Hartman et al., 1990) were used. The PCR-Mix is shown in Table 3.1. The sequence of the primers are listed in Appendix 2.

Table 3.1: *PCR-Mix (all chemicals were purchased from Qiagen (Hilden, Germany))*

10x PCR buffer with 15 mM $MgCl_2$	2.5 µl
DNTP mix 10 mM (2.5 mM each)	0.3 µl
Primer *IapH* (forward) 10 µM	2.5 µl
Primer *IapH* (reverse) 10 µM	2.5 µl
Millipure water	14.0 µl
Hotstartaq (5u/µl)	0.3 µl
DNA extract	2.5 µl

The PCR was run in a Gen Amp PCR System 2400 (Perkin Elmer, Foster City, CA) with a program adopted by FDA (FDA/CFSAN) and modified by (Flessa and Harris, 2002a) as shown in Table 3.2.

Table 3.2: *PCR program*

1 cycle at 94°C for 15 min
60 cycles (94°C 1 min, 60°C 1 min, 72°C 1 min)
1 cycle at 72°C for 10 min
hold at 4°C

Gel-electrophoresis. Agarose (Bio Rad Laboratories, Hercules, CA) gels (1.5%) were made with 1X TBE buffer (Bio Rad Laboratories, Hercules, CA). Sample (10 µl) and 8 µl of the 1000 bp ladder (Bioline Inc., Reno, NV) were loaded into the gel. The gel was run for 1.5 h at 72V (Power Pac 300, Bio Rad Laboratories, Hercules, CA). Preliminary experiments determined that this method was capable of detecting (after enrichment) an initial population of 10 to 100 cells inoculated onto the strawberry surface (Flessa and Harris, 2002b). An example gel is shown in Figure 3.1.

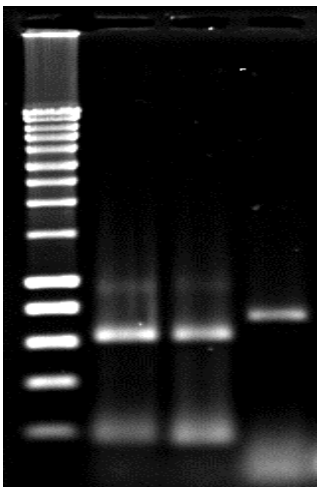

Figure 3.1: *Gel electrophoresis of PCR products recovered from the surface of strawberries after enrichment. Lanes from left to right: Ladder,* Shigella *positive,* Shigella *positive,* Shigella *negative.*

Relative humidity. Relative humidity in the biological hood and storage refrigerator was measured with a humidity sensor (TempTale H, Sensitech Inc., Beverly, MA).

Statistics. All experiments were repeated at least three times with duplicate samples. Statistical analysis was performed using the SAS software package (ver. 6.0, Cary, NC). Data were compared using Anova models. Data for *Shigella* at 24 and 4°C were log transformed, data for *Shigella* at -20°C were transformed to the 4^{th} root. To compare the differences in between the inoculum densities, linear contrast models were used. Differences were considered significant at $P \leq 0.05$.

RESULTS

Survival of *S. flexneri* on whole strawberries stored at 24°C. For berries stored at room temperature the relative humidity was 27 ± 2%. Whole strawberries were inoculated with approximately 7.8 log CFU (high), 5.7 log CFU (moderate), and 3.8 log (low) per sample. Berries were stored at 24 ± 2°C for 72 h in loosely closed plastic containers. Populations on whole berries declined significantly ($P = 0.0493$) by 0.6 log CFU within the first 1.5 h on TSAPR for the high inoculum, by 1.0 log for the moderate inoculum ($P < 0.0008$), and by > 1.2 log for the low inoculum ($P < 0.0001$). The high inoculum decreased significantly by a total of 2.2 log ($P < 0.0001$) over the 72 h period. Whereas the moderate inoculum dropped below the detection limit within 48 h on TSAPR (> 3.4 log) and within 24 h on MACR (> 3.5 log). The low inoculum on the berries decreased below the detection limit within 24 h. The better recovery rates for the recovery compared to the the selective media after 24 h indicated the presence of injured cells (Figure 3.2).

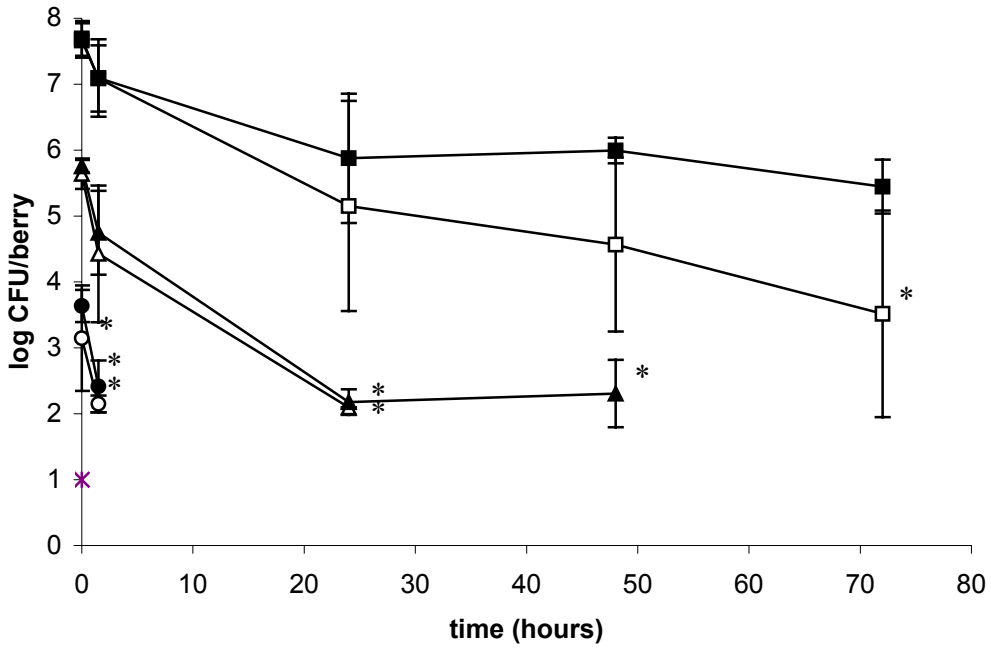

Figure 3.2: *Survival of* S. flexneri *on whole strawberries inoculated with* 10^8 *(square symbols),* 10^6 *(triangular symbols), and* 10^4 *(round symbols) CFU/berry stored at 24°C and plated onto TSAPR (closed symbols) and MACR (open symbols). * indicate that one or more samples were below the detection limit (2.1 log CFU/berry). Error bars represent the standard deviation (n = 6).*

Survival of *S. flexneri* on whole strawberries stored at 4°C. The relative humidity was 52 ± 2% for strawberries stored at refrigeration temperature. Whole berries were inoculated with a high (6.8 log), moderate (4.7 log), or low (3.0 log) level and stored for up to 5 d at 4°C. At the high inoculum level, an insignificant (P = 0.1361) reduction of 0.6 log was observed during the drying period (1.5 h). At the moderate inoculum level, a significant reduction of 0.9 log (P = 0.0151) after 1.5 h was observed. At the low inoculum the population decreased significantly by 1.2 log to 2.6 log (P = 0.0026) in the same time frame and dropped below the detection limit for all samples within the first day. For the high inoculum a significant decrease of approximately 1.8 log (P < 0.0001) was observed over a 3-

d storage period whereas the moderate inoculum population levels dropped below the detection limit (> 3.5 log decrease). For the high inoculum a significant decrease of approximately 3.9 log ($P < 0.0001$) was observed over the 5-d storage period. Beginning at 24 h an increasing difference was observed between the selective and the recovery medium indicating the presence of injured cells (Figure 3.3).

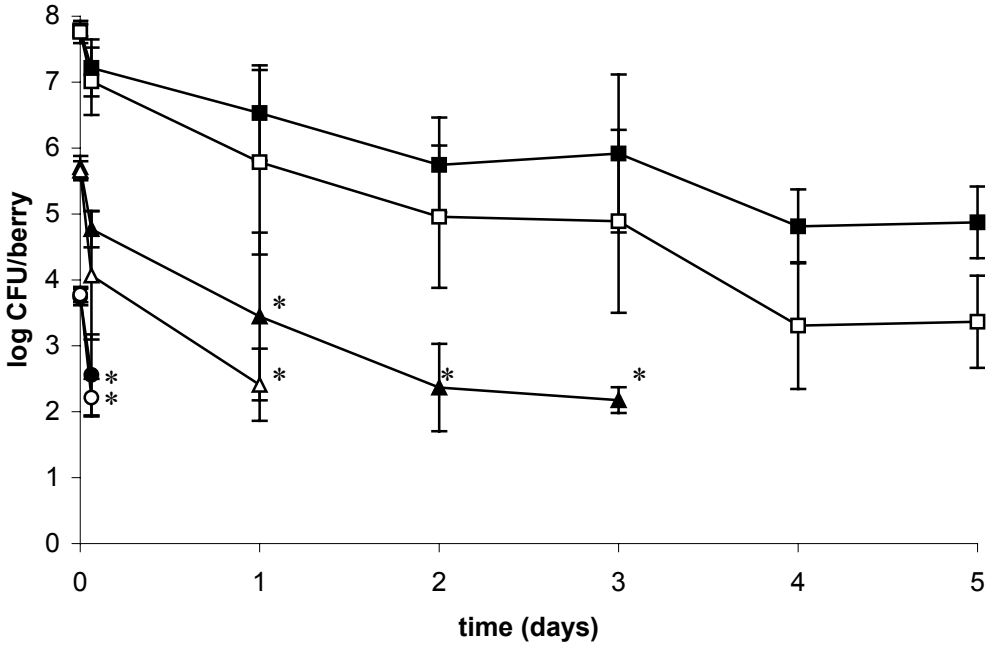

Figure 3.3: *Survival of* S. flexneri *on whole strawberries inoculated with* 10^8 *(square symbols),* 10^6 *(triangular symbols), and* 10^4 *(round symbols) CFU/berry stored at 4°C and plated onto TSAPR (closed symbols) and MACR (open symbols). * indicate that one or more samples were below the detection limit (2.1 log CFU/berry). Error bars represent the standard deviation (n = 6).*

Survival of *S. flexneri* on cut strawberries stored at -20°C. Sliced strawberry samples were inoculated with approximately 7.5 log CFU and 5.7 log CFU of *S. flexneri* culture. No significant drop was seen in the first 1.5 h, while holding under the biohazard hood. Populations of *S. flexneri* dropped significantly by log 2.3 for the high and 1.7 log for the low inoculum within 24 h ($P < 0.0001$). After the 24-h period the population declined significantly another 2 log cycles for the high inoculum after 30 days ($P < 0.0001$) (TSAPR). After 1.5 h a higher proportion (0.28 to 1.46 log) of *S. flexneri* was recovered on the recovery medium than the selective one, indicating the presence of injured cells (Figure 3.4).

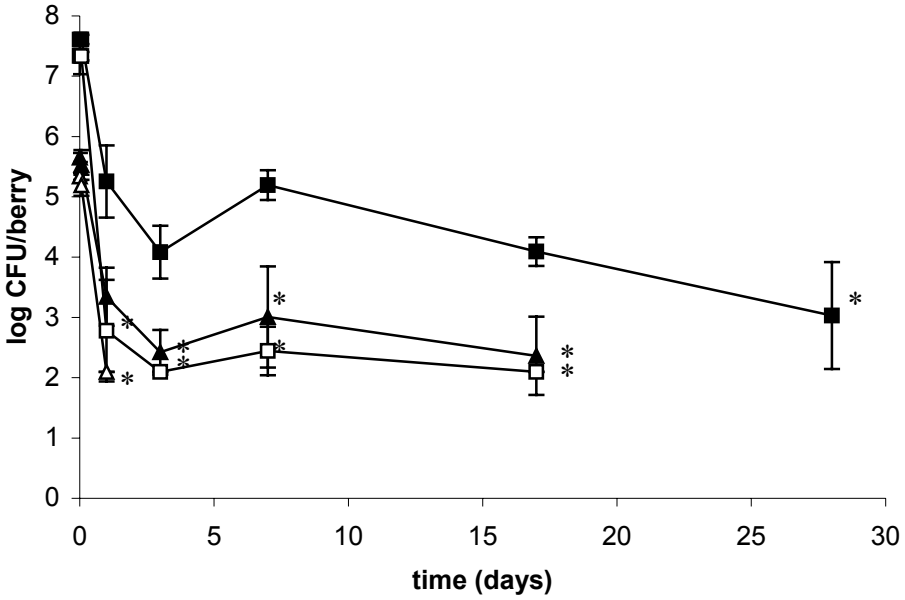

Figure 3.4: *Survival of* S. flexneri *on cut strawberries inoculated with 10^8 (square symbols) and 10^5 (triangular symbols) CFU/berry stored at -20°C and plated onto TSAPR (closed symbols) and MACR (open symbols) * indicate that one or more samples were below the detection limit (2.1 log CFU/berry). Error bars represent the standard deviation (n = 6).*

In Table 3.3 the reduction of *S. flexneri* corresponding to the storage temperature after 1.5 h and 24 h are shown.

Table 3.3: *Reduction of* S. flexneri *(log CFU/berry) on whole strawberries after a 1.5-h drying period and after 24 h storage plated onto TSAPR. Data shown are the difference between the log initial population and log population at the given time.*

	-20°C		4°C		24°C	
Inoculum	1.5 h	24 h	1.5 h	24 h	1.5 h	24 h
10^7	0.00 A[a]	2.35 A	0.57 A	1.25 A	0.57 A	1.79 A
10^5	0.12 A	2.31 B	0.94 A	>2.27 B	1.00 A	>3.58 B
10^3	nd[b]	nd	>1.18 A	nd	>1.22 A	nd

[a]: different letters are indicating a significant difference among time points within each inoculum level

[b]: not done

DISCUSSION

Much research has been done to understand the survival of *Shigella* in food. Growth was observed only at temperatures above 20ºC. When *Shigella* spp. was inoculated onto the surface of fresh sliced jicama, papaya, and watermelon for up to 6 h at 26ºC an average growth of 1.5 log was shown for the pathogen populations (Escartin et al., 1989). *S. sonnei* was able to grow on chopped parsley when stored at 21ºC (Wu et al., 2000). Growth of *S. flexneri* was seen in traditional Bangladesh foods for up to 72 h when stored at 25 and 37ºC. When the same food was stored at 5ºC populations remained almost stable over 72 h (Islam et al., 1993). A stable population was also observed when *S. sonnei* was inoculated onto whole parsley at 21ºC (Wu et al., 2000). Also *S. sonnei* and *S. flexneri* were able to survive on mixed lettuce peppers under equilibrium modified atmosphere storage at 7 and 12ºC for 7 days (Bagamboula et al., 2002). But on grated carrots and chopped bell peppers populations declined when held under the same conditions. Decreases of *S. flexneri* and *S. sonnei* below the detection limit were seen on sauerkraut within 23 and 19 days for storage at 4 and 22ºC,

respectively. On butter *S. sonnei* decreased below the detection limit within 63 days at 4ºC and 22ºC (Siegmund, 1960). Furthermore *S. sonnei* decreased at 4ºC for 14 days on parsley (Wu et al., 2000).

In this study good survival of *S. flexneri* on whole and sliced strawberries was observed for all temperatures. Decreases of 0.2 to 2.3 log, 0.8 log, and 1 to 2 log of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*, respectively, were observed on whole berries stored at 24 ºC within one day of storage (Knudsen et al., 2001; Yu et al., 2001; Flessa et al., 2005). When stored at 4 ºC *E. coli*, *Salmonella* and *L. monocytogenes* decreased between 1 and 3 log cycles over a time period of 7 days respectively (Knudsen et al., 2001; Flessa et al., 2005). When cut berries were stored at room or refrigeration temperature, populations of *Salmonella*, *E. coli* and *L. monocytogenes* remained stable over 2 and 7 days, respectively. When cut berries were stored at -20ºC decreases of 1.2 log (*E. coli* O157:H7) were reported within 32 days (Knudsen et al., 2001) and *L. monocytogenes* declined by 1.2 log cycles on sliced strawberries in 28 days stored at -20ºC (Flessa et al., 2005).

Few studies have attempted to evaluate the survival of pathogens on fruits and vegetables when inoculated at lower levels. Survival patterns of *S. sonnei* inoculated onto chopped and whole parsley were similar when the initial inoculum level was 10^3 or 10^6 CFU/g (Wu et al., 2000). Richert et al. (2000) inoculated broccoli, cucumber, and green pepper with *E. coli* O157:H7 at levels of 10^3 and 10^6 CFU/g. In contrast to Wu et al. (2000) on sliced cucumbers a decrease of less than one log was observed on sliced cucumbers for the high inoculum in 14 days at 4ºC, however for the low inoculum the population dropped below the detection limit (detection limit 1.9 log CFU) after 10 days. On strawberries a greater decrease of *L. monocytogenes* was observed when inoculated at 10^5 CFU/berry when compared with 10^7 CFU/berry (Flessa et al., 2005).

Similar results were observed in the current study. A significant difference between the decrease of the high and moderate inoculum for *S. flexneri* ($P < 0.0001$) was observed

when inoculated on the intact surface of strawberries stored at 24ºC within 24 h (Figure 3.2). The biggest difference between the inoculum levels was observed within the first day of storage.

A significant difference in survival could be seen when strawberries were stored at 4ºC after 72 h. But the overall differences in cell death were smaller than compared to stored at room temperature. The same behavior was observed when cut berries were stored at -20ºC.

Similar patterns were observed when *L. monocytogenes* and *E. coli* O157:H7 were inoculated onto strawberries at moderate (10^6 CFU) and high levels (10^8 CFU). Significantly higher reductions in population were observed with the moderate inoculum levels when whole strawberries were stored at 24 but not at 4°C (Flessa et al., 2005).

In general better survival was observed under refrigerated storage. The higher humidity observed when berries were stored in a refrigerator may have enhanced the survival. Better survival of *Pseudomonas syringae* on bean leaves and *Acinetobacter* spp. on dry surfaces was reported when stored at higher relative humidity (Monier and Lindow, 2003; Jawad et al., 1996). Similar results were observed for the survival of *L. monocytogenes* and *E. coli* O157:H7 on strawberries, where populations of cells inoculated onto the cut surface of strawberries at a high and moderate inoculum remained almost stable (Flessa et al., 2005; Knudsen et al., 2001). These results suggest that the actual drying of the inoculum on the surface might be responsible for the differences. This would explain why there was no difference when the inoculum did not dry on the cut surface of the strawberry.

More research needs to be done to fully understand the reasons for the significantly different decrease rates of more or less concentrated inocula.

The impact of drying on cell physiology is a relatively understudied area. Drying is considered to be stressful to cells and it is possible that stress response mechanisms are initiated (Breewer et al., 2003).

Quorum sensing substances are produced by bacteria when certain cell densities are achieved. They are known to initiate the formation of biofilms, (Eberl, 1999; Parsek and Greenberg, 2000). For *Bacillus subtilis* quorum sensing was shown also for low cell densities in the case of starvation and transition into the stationary phase was initiated (Lazazzera, 2000). Both cases, biofilms and being in the stationary phase, will allow bacteria to survive better under stress conditions.

However, quorum sensing is an unlikely explanation for the different survival rates of the different inoculum densities. Because cells were washed twice before experiment all quorum sensing signals should have been removed. And quorum sensing molecules (acylated homoserine lactones for gram negative bacteria) concentrations high enough to trigger effects was not seen before 4 h (Surette and Bassler, 1998). Furthermore cells should not show a strong metabolism, including the production of quorum sensing signals, during desiccation or when cells are dried.

Chapter Four

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Drying mechanisms of a droplet with bacteria in suspension

INTRODUCTION

The evaporation of small amounts of liquid such as droplets and thin films with suspended particles is an often studied phenomenon in physics (Deegan, 2000a; Routh and Russel, 1998; Winnik and Feng, 1996). Daily examples are the formation of a coffee stain, or the drying of water color paints. In the literature, two different modes of evaporation are described. One is the evaporation of a droplet with a non-pinned contact line where the particles are deposited randomly during drying. The other is the pinned contact line, where, due to the evaporation at the edge, a capillary flow will result in depositing particles in a ring structure (Deegan, et al., 2000b). Nagayama (1995) describes this flow towards the boundary in liquid films as a convective flow. The schematics of a drying droplet with a pinned contact line are shown in Figure 4.1.

In this chapter, the drying behavior of bacteria in a droplet is followed under a microscope. Because of the better survival rates of higher inocula compared to lower inocula (Chapter Three), in this chapter it was investigated if the location of the cells during the drying period is correlated to survival.

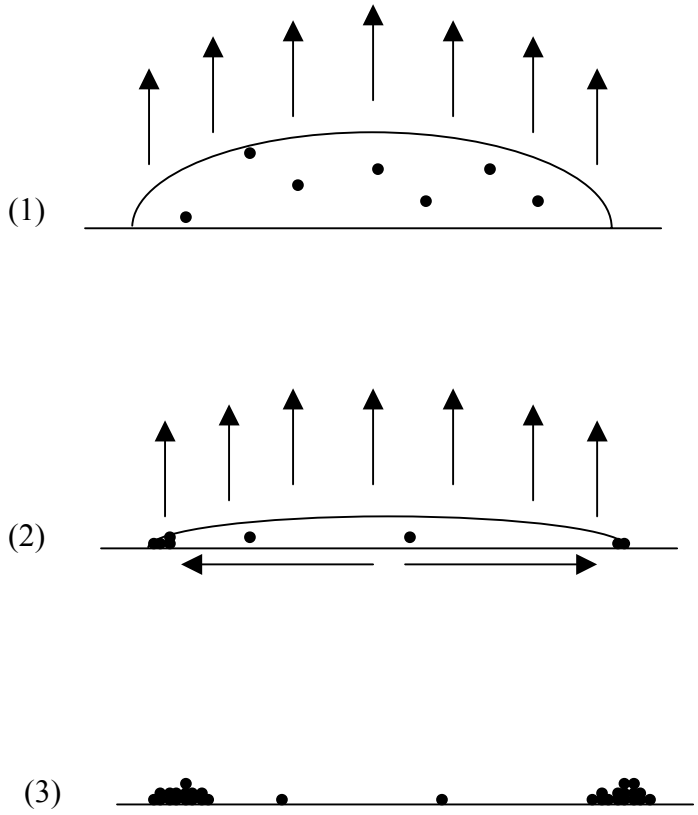

Figure 4.1: *Drying of a droplet: (1) Bacteria are still dispersed within the droplet and evaporation takes place on the surface. (2) Contact line is pinned to the surface, evaporation is still equal over the surface, thus water has to be replaced at the edges and a capillary flow will result in depositing bacteria at the edges. (3) Droplet has completely dried and most bacteria are deposited at the edge with only a few remaining in the middle.*

In order to evaluate the location of viable and dead cells within the dried spots, cells were stained with tetrazolium. Viable cells use the electron transport system of their respiration system for reduction of tetrazolium (Roslev and King, 1993). Thus tetrazolium salts will be reduced to colored formazan products (Altman, et al., 1976), which remain intracellular and can be observed under the microscope.

MATERIALS AND METHODS

Test strains and media preparation. A human isolate of *Shigella sonnei* LJH 664 was obtained from Dr. Trevor Suslow, UC Davis. *Salmonella enterica* serovar Enteriditis Phage Type 30 LJH 608 was isolated from raw almonds associated with an outbreak of salmonellosis (Isaacs et al., 2005).

Inoculum preparation. Before each experiment a frozen stock culture (-80°C) was streaked onto TSA and an isolated colony was subcultured in tryptose soy broth (Difco) (37°C for 24 h). Single loop transfers were prepared at two consecutive 18 to 24 h intervals. Overnight cultures were collected by centrifugation and suspended in either 0.1% peptone water (Difco), MilliQ water, or 0.85% saline solution. The saline solution was prepared by dissolving 0.85 g sodium chloride (Fisher, Fair Lawn, NJ) in 100 ml of MilliQ water. Cells were washed for two times in corresponding carrier medium to ensure complete removal of the growth medium.

Inoculation procedure. A 10 μl drop of the prepared inoculum was spot inoculated, in one drop, onto glass slips (No.1, 18 mm sq., Cover Glass, Corning Glass Works). Slips were held in a biological hood at 28 ± 1ºC for up to 2 h.

Staining with tetrazolium. *S. sonnei* cells were dried on glass slips. A staining method similar to Ulmer et al. (2000) was used. Briefly, phosphate buffer with glucose was made consisting of 50 mM H_2KPO_4, 0.1 g/l $MgSO_4$ X $7H_2O$, 0.05 g/l $MnSO_4$ X H_2O, and 4 g/l glucose with a final pH of 6.5 (all chemicals were purchased at Merck, Darmstadt, Germany). In this buffer 2-(-iodophenyl)-3-(p-nitrophenyl)5-phenyltetrazolium chloride (Molecular Probes, Eugene, Oregon) was dissolved until a final concentration of 2 mmol was reached. After 45 min of drying (cells were visibly dry) tetrazolium buffer was gently applied to the dried spot. Solution was allowed to sit for 15 min and cells were observed under microscope.

Microscopy. Drying was observed with a Zeiss microscope (Zeiss, Oberkochen, Germany).

RESULTS

Evaporation of droplet. The droplet of 10 µl needed approximately 20 min to appear completely dry. The contact line always remained pinned to the glass surface. Therefore, due to evaporation, the medium had to flow from the middle of the droplet to the rim. This flow is called a capillary flow (Deegan, 1997). This flow transported bacteria to the rim of the droplet and deposited them there (Figure 4.1). These bacteria formed a multi-layer ring. For the inocula of 10^8 cells per 10 µl a much bigger ring was formed compared to the inocula of 10^6 cells per 10 µl (Figure 4.2 and 4.3). In these experiments, when MilliQ water was used as carrier only a few single bacteria were observed to remain inside of the formed ring for the inocula of 10^6 cells per 10 µl (Figure 4.4 a), whereas for the inocula of 10^8 cells per 10 µl an almost complete single layer inside the ring was observed (Figure 4.4 b).

These principles are visible in the following photographs. Figures 4.2 a and b show the movement of *Shigella* cells in a drying droplet. Figures 4.3 a and b show the growth of the deposited bacteria ring. Whereas figures 4.4, 4.5 and 4.6 show the portion of the droplet, away from the ring itself, of the droplet in a dried state with initially 10^6 cells per 10 µl cells (Figure 4.4 a, 4.5 a, and 4.6 a) and 10^8 cells per 10 µl (Figure 4.4 b, 4.5 b, and 4.6 b), dried in MilliQ water, peptone water, and saline solution, respectively.

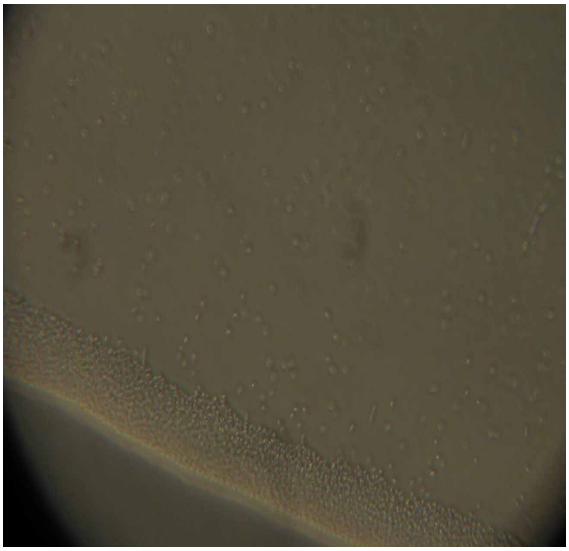
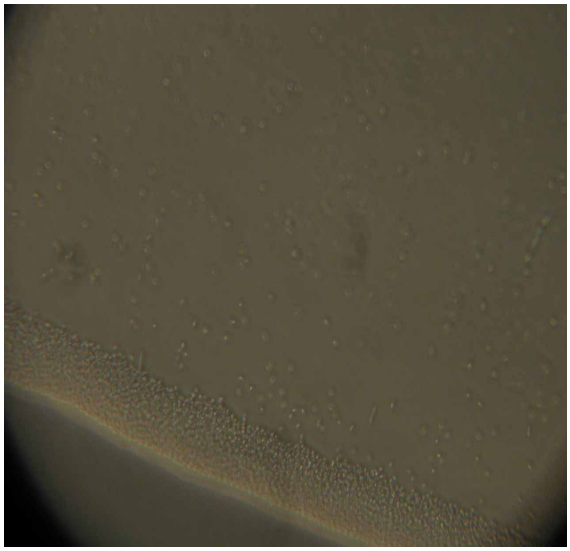

Figure 4.2 a: *Drying* S. sonnei *cells with MilliQ water as carrier, 10^6 CFU / 10 µl inoculum, time 15.0 min (not completely dry)*, b: *same sample as Figure 4.2 a, time 15.5 min (not completely dry)*

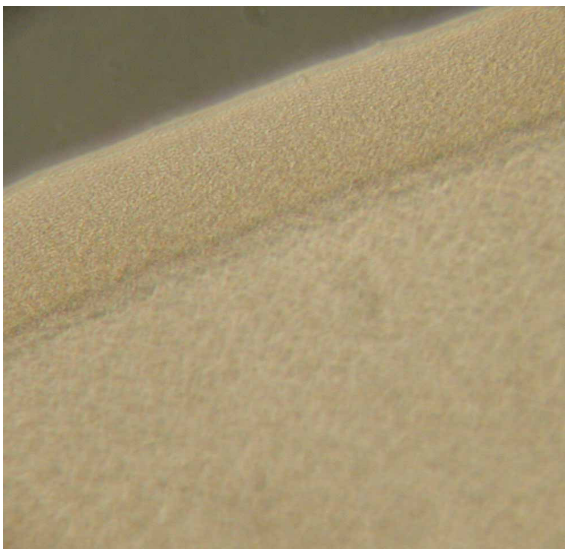
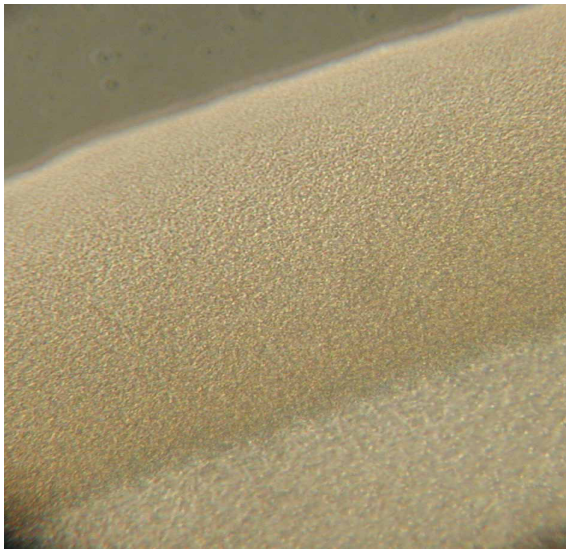

Figure 4.3 a: *Drying* S. sonnei *cells with MilliQ water as carrier, 10^8 CFU / 10 µl inoculum, time 15.0 min (not completely dry)*, b*: same sample as Figure 4.3 a, time 15.5 min (not completely dry)*

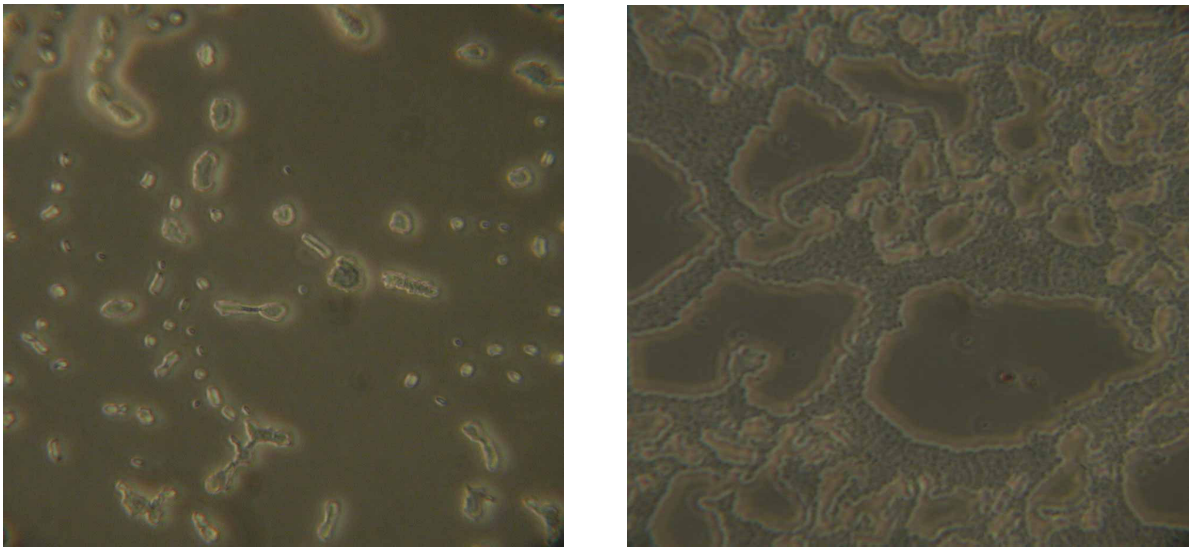

Figure 4.4 a: *Picture of completely dried* S. Enteriditis *cells with MilliQ water as carrier, 10^6 CFU / 10 µl inoculum, away from the deposited ring*, b: *same sample as Figure 4.4 a, but with 10^8 CFU / 10 µl*

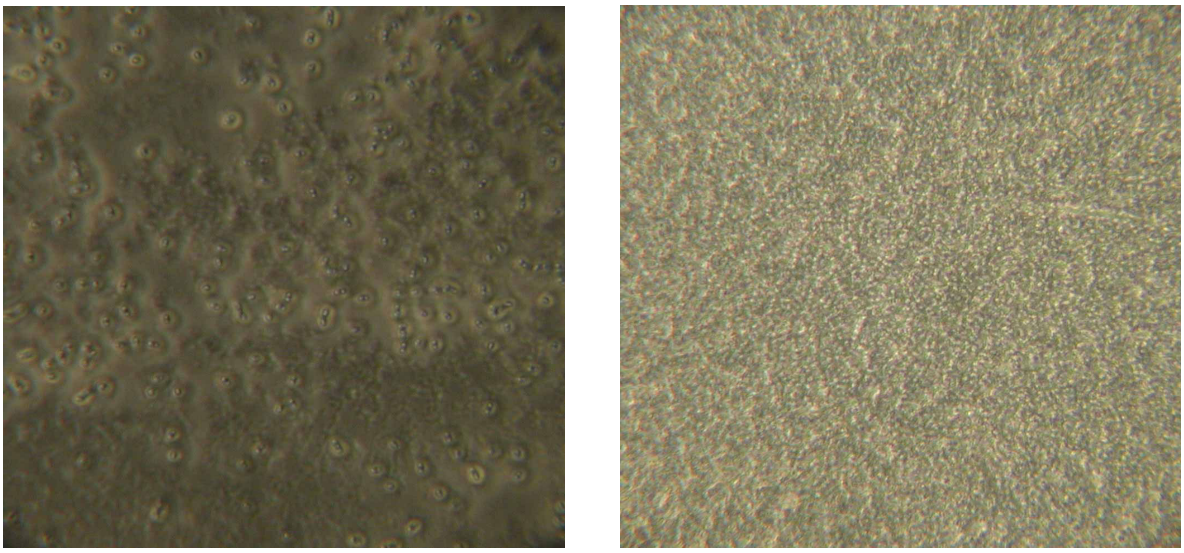

Figure 4.5 a: *Picture of completely dried* S. Enteriditis *cells with peptone water as carrier, 10^6 CFU / 10 µl inoculum, away from the deposited ring,* b: *same sample as Figure 4.5 a, but with 10^8 CFU / 10 µl*

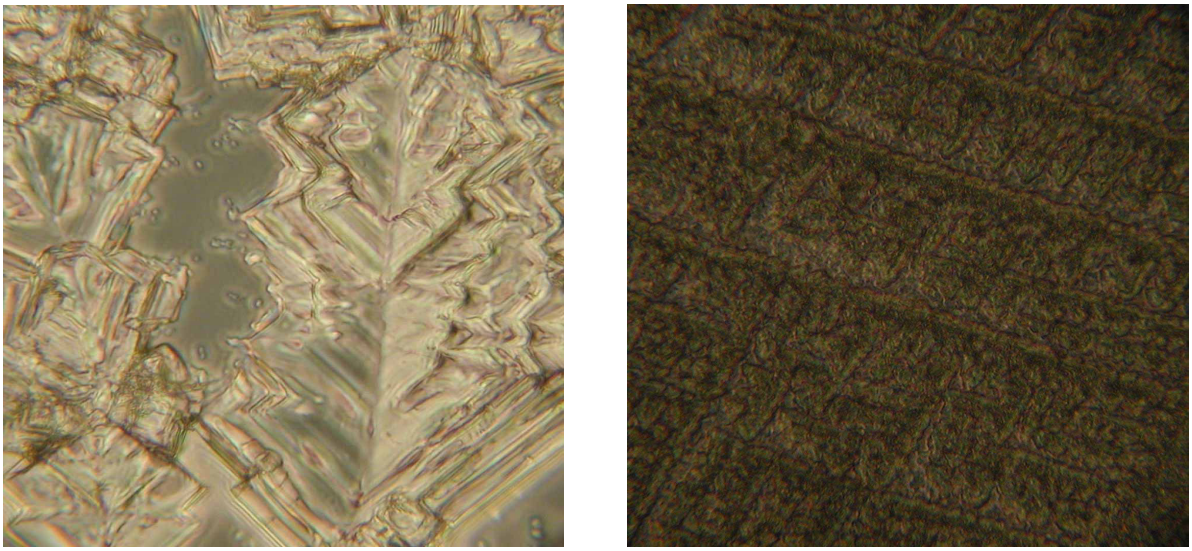

Figure 4.6 a: *Picture of completely dried* S. sonnei *cells with saline solution as carrier, 10^6 CFU / 10 µl inoculum, away from the deposited ring,* b: *same sample as Figure 4.6 a, but with 10^8 CFU / 10 µl*

Staining with tetrazolium. Figure 4.7 shows a part the ring of 10^8 CFU/10 µl dried *S. sonnei* when MilliQ water was used as carrier. Surface cell layers are colored in a darker red outside compared to the lower layers. This indicated that the cells on the top were dead, because they were not able to modify the tetrazolium to the bright orange formazan. The surviving cells appear to be below the layers of dead cells and shine in a bright orange color due to the reduction of the tetrazolium salt.

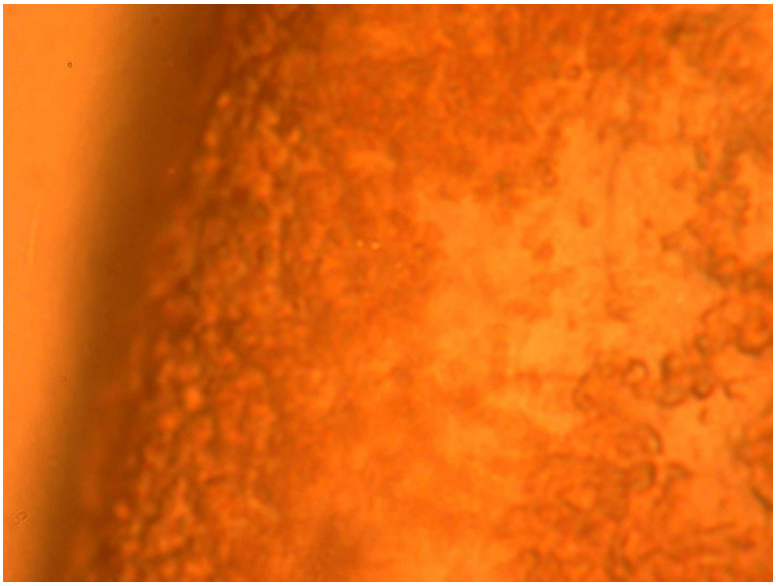

Figure 4.7: *Tetrazolium stained* S. sonnei *cells (10^8 CFU / 10 µl) after complete drying with MilliQ water as carrier*

DISCUSSION

Drying patterns of droplets with bacteria are similar to those with latex particles with a pinned contact line (Deegan, 2000b). The results were the same with the motile *Salmonella* and non-motile *Shigella* strains indicating that motility did not influence drying patterns.

Wilson and Lindow (1994) assumed in their study, when *Pseudomonas syringae* was inoculated onto the phyllosphere of bean leaves, that the bacteria would be dispersed evenly over the surface. Which could be shown is not the case in this study (Chapter Four).

Dead bacteria were located mostly on the outer layer of the cell ring. Outer layers of bacteria would be most exposed to environmental stresses, e.g. oxygen and starvation, which might be the explanation for the lower survival compared to the protected, inner layers of bacteria. Bozoğlu et al. (1987) concluded that the layers of bacteria protect the inner ones from stresses such as oxidation during freeze drying.

Even with the same volume of carrier there is a noticeable difference in the size of the outer ring with different cell densities (see Figure 4.2 and 4.3). After drying the outer ring has a greater volume when the cell density in the inoculum is higher. When considering the

deposited external ring as a ring torus (Figure 4.8), theoretically a greater number of cells should be protected during desiccation, because the surface and volume of the deposited ring follow the mathematical equation shown in Equations 4.1 and 4.2.

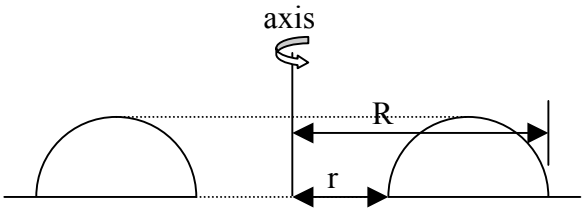

Figure 4.8: *Cut of a half ring torus*

$$\textbf{Surface} = \tfrac{1}{2}\,\Pi^2(R^2-r^2)$$

Equation 4.1: *Equations for the surface of a half ring torus which would touch the air when laying on a plane surface, R and r are explained in Figure 4.8.*

$$\textbf{Volume} = \tfrac{1}{8}\,\Pi^2(R+r)(R-r)^2$$

Equation 4.2: *Equations for the volume of a half ring torus, R and r are explained in Figure 4.8.*

If the difference between R and r increases, which means the number of cells increases, the surface will increase by a lower rate than the volume. Therefore the ratio of protected cells (volume) to cells on the surface (surface) is growing with the increase of (R – r) and the survival rate should increase. This may explain that higher inocula level result in greater survival rates as shown in Chapter Three.

Chapter Five

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Influence of inoculum density and carrier medium on survival of pathogenic, gram-negative bacteria on steel and glass slips

INTRODUCTION

In recent years several different outbreaks of shigellosis, salmonellosis, and *Escherichia coli* O157:H7 infections have been associated to produce such as parsley (Crowe et al., 1999), almonds (Isaacs et al., 2005), and lettuce (Hilborn et al., 1999). Survival of foodborne pathogenic bacteria on produce is an important factor for the ability of these products to transmit disease. The outer surface of produce can vary considerable from smooth and waxy to highly complex and rough, and from dry to relatively moist.

E. coli O157:H7 survived well in acidic conditions, for up to one day at room temperature (Tsai and Ingham, 1997), on produce such as peppers or broccoli (Richert et al., 2000), and on strawberries a decrease of 0.7 to 2.3 log cycles was seen within 24 h at room temperature (Yu et al., 2001; Knudsen at al., 2001). *Shigella* survived for more than 3 days on strawberries at room temperature (Chapter Three).

Beuchat et al. (2001) mention that inocula sizes, carrier media, and inoculation methods need to be considered when designing experiments to evaluate the survival or removal of pathogens from produce.

De Caesare et al. (2003) also reported the enhanced survival of bacteria when the carrier medium included various organic compounds. Leslie et al. (1995) showed that trehalose and sucrose enhanced the survival of *E. coli* and *Bacillus thuringiensis* during freeze-drying. When lactic acid bacteria accumulated betaine an enhanced survival was observed (Kets et al., 1996).

Not much work has evaluated the influence of the inoculum level on the survival of bacteria. Usually a generally high level of $>10^5$ CFU (Bagamboula et al., 2002; Islam et al., 1993) is used, to facilitate recovery after storage or treatment. Only a few papers mention the use of low or more than one inoculum level (Richert et al., 2000; Wu et al., 2000; Kusumaningrum et al., 2003).

The effect of inoculum density dependent survival was also studied in relation to the phyllosphere. When Wilson and Lindow (1994) studied the colonization of bean leaves by *Pseudomonas syringae*, an influence of the inoculum level on the survival during desiccation could be shown, where higher inocula survived better than lower inocula.

Produce and the phyllosphere can be difficult to study because of the perishibility of these foods and plants and the variability among samples. A standardized inert surface might be used as a model to evaluate the effects of drying on the survival of pathogens.

As early as 1962, the first studies on the survival of *Shigella sonnei* on surfaces as cotton, wood, glass, paper, and metal were done (Nakamura, 1962). Survival times of 1 to 6 and 0 to 4 days were seen on glass and steel, respectively at a temperature of 37°C. More recently a study on the survival of *Yersinia pestis* on stainless steel, polyethylene, paper, and glass surfaces was published (Rose et al., 2003). The best survival was seen on paper (>120 h) when BHI was used as carrier, a shorter survival time (>48 h) was seen when phosphate buffer was used as a carrier. When *Salmonella enterica* spp. were inoculated onto stainless steel with tryptic soy broth as carrier an approximate decline of 3.2 log cycles was observed within 24 h (de Cesare et al., 2003).

The objective of this study was to work with surfaces that do not influence the survival of bacteria, such as stainless steel or glass and determine the influence of inoculum density and inoculum carrier on the survival of *Salmonella* Enteriditis, *Shigella sonnei*, and *E. coli* O157:H7. This chapter concentrates on the survival within first 90 min of drying, because as shown in Chapter Three, within that time frame the biggest decrease was observed.

MATERIALS AND METHODS

Test strains and media preparation. A human isolate of *Shigella sonnei* LJH 664 was obtained from Dr. Trevor Suslow, UC Davis. *Salmonella enterica* serovar Enteriditis Phage Type 30 LJH 608 was isolated from raw almonds associated with an outbreak of salmonellosis (Isaacs et al., 2005). *E. coli* O157:H7 LJH 537 was an human outbreak isolate associated with lettuce (Hilborn et al., 1999). This strain was adapted to grow in the presence of 50 µg/ml nalidixic acid.

Tryptic soy agar (TSA) (Difco, Detroit, MI), was used as recovery medium for *S. sonnei* and *S.* Enteriditis, TSA with 50 µg/ml nalidixic acid for *E. coli*.

Inoculum preparation. Inoculum was prepared as described in Chapter Three. Briefly, before each experiment a sample from a frozen stock culture was grown on TSA and an isolated colony was subcultured in tryptose soy broth (Difco) (37°C for 24 h). Single loop transfers were prepared at two consecutive 18-to 24-h intervals. Overnight cultures were collected by centrifugation and suspended in either 5% horse serum (Gibco, BRL, Grand Island, NY), 0.1% peptone water (Difco), MilliQ water, or 0.85% saline solution. The saline solution was made by dissolving 0.85 g sodium chloride (Fisher, Fair Lawn, NJ) in 100 ml MilliQ water. Cells were washed for one to five times in the corresponding carrier to ensure complete removal of the growth medium. No significant difference ($P > 0.05$) was seen for the different number of washing cycles (Table 5.1).

Table 5.1: *Survival of* S. sonnei *log (CFU ± SD) washed two and five times prior to inoculation of cover slips with MilliQ water as carrier (n = 6)*

		Drying time			
Inoculum	Number of wash cycles	15 min	30 min	60 min	90 min
10^6	2	5.61 ± 0.15 A[a]	3.96 ± 0.47 A	3.38 ± 0.25 A	3.32 ± 0.44 A
	5	5.61 ± 0.14 A	3.82 ± 0.27 A	3.45 ± 0.32 A	3.34 ± 0.38 A

[a]: different letters are indicating a significant difference between the number of wash cycles at each time point

For the following experiments culture was washed twice prior to use. The culture was further diluted in the chosen carrier to produce the desired inoculum concentration. Samples of the inoculum were plated onto TSA to confirm the inoculum levels.

To produce non-viable populations, cells were washed twice in MilliQ water and pasteurized for 5 min in a water bath at 80ºC. After heating, cells were washed an additional time in MilliQ water. Pasteurized cells were plated on TSA to confirm that viable cells were below the detection limit (2.1 log CFU).

Inoculation procedure. A 10 µl drop of the prepared inoculum was spot inoculated onto glass slips (No.1, 18 mm sq., Cover Glass, Corning Glass Works) or onto food grade steel slips (304 steel) (1cm x 1cm, custom made). Slips were stored in a biological hood at 28 ± 1ºC for up to 2 h. No significant difference was seen between the survival on steel and glass slips when MilliQ water was used as carrier (see Chapter Two). Glass slips were chosen for further experiments.

Recovery of pathogens. Inoculated glass slips were placed into a 50 ml plastic tube (Becton Dickinson Labware, Franklin Lakes, NJ) with 5 ml of 0.1% peptone water and 15 glass beads (3 mm solid, Fisher). Tubes were vortexed (Vortex Genie 2, Fisher) for up to 60 s at medium power. Experiments showed that there was no significant difference ($P > 0.05$) in recovery during drying between 20 and 60 s of vortexing when MilliQ water was used as carrier (Table 5.2).

Table 5.2: *Survival of* S. sonnei *log (CFU ± SD) vortexed 20 and 60 s to recover from glass slips when MilliQ water was used as carrier (n = 6)*

		Drying time				
Inoculum	vortex time	15 min	30 min	60 min	90 min	Δ[a]
10^6	20 s	5.57 ± 0.24 A[b]	4.10 ± 0.39 A	3.30 ± 0.26 A	3.13 ± 0.37 A	2.44
	60 s	5.59 ± 0.26 A	3.99 ± 0.27 A	3.34 ± 0.44 A	3.23 ± 0.48 A	2.36

[a]: decrease between 25 and 90 min.
[b]: different letters are indicating a significant difference between the vortex times at each time point

The sample rinse was subsequently diluted in 0.1% peptone water. Samples were plated using an Autoplater 4000 Spiral Plater (Spiral Biotech, Inc, Bethesda, MD). Colonies were enumerated for 24 h at 37°C.

Relative humidity. Relative humidity in the biological hood was measured with a TempTale H (Sensitech Inc., Beverly, MA).

Statistics. All experiments were repeated at least three times with duplicate samples. Statistical analysis was performed using the SAS software package (ver. 6.0, Cary, NC). Data were compared using Anova models. Data for *Shigella* were log transformed, data for the high inoculum of *Salmonella* were square root transformed, for the low inoculum log transformed, and data for *E. coli* O157:H7 were also log transformed. To compare carrier media, linear contrast models were used.

Data for the vortex, washing, and protection with dead cells experiments were log transformed. To compare the differences between the methods, linear contrast models were used.

Differences were considered significant at $P \leq 0.05$.

RESULTS

Relative humidity. Relative humidity through out the experiments under the biohazard hood was 27 ± 2%.

Behavior of inoculum within the first 20 min. Inoculum levels remained constant as long as the droplet on the slip was wet, approximately 20 min. After visible drying began, inoculum levels began to decline (Table 5.3). For subsequent experiments, initial populations were determined at 20 min.

Table 5.3: *Survival of* S. sonnei *log (CFU ± SD) on cover slips during drying at 28 ± 2 °C with MilliQ water as carrier (n=9)*

	CFU/slip
0 min	5.77 ± 0.03
20 min	5.78 ± 0.07
30 min	5.22 ± 0.11

Horse serum and peptone water as carrier. When inoculated at a level of 10^8 CFU/slip, *S. sonnei* (Figure 5.1)*, S.* Enteriditis (Figure 5.2), and *E. coli* O157:H7 (Figure 5.3) declined by approximately 0.50 to 0.75 log within 90 min. For the 10^6 inoculum, horse serum proved to be the most protective carrier for *S. sonnei* and *S.* Enteriditis, where 1 log decrease was observed. Horse serum was also most protective for *E. coli* O157:H7, resulting in a 1.5 log decrease. For all inocula the biggest decrease was observed between 20 and 30 min after inoculation. After that the decrease leveled out.

Peptone water as carrier. The high inoculum of all bacteria declined by approximately 1 log for peptone water after 90 min drying (Figures 5.1, 5.2, and 5.3). The lower inoculum showed a bigger decrease compared to the higher one. The inocula of *S. sonnei, S.* Enteriditis, and *E. coli* O157:H7 decreased by approximately 2 log in 90 min. For all inocula the biggest decrease was observed between 20 and 30 min after inoculation. After that the decrease leveled out.

MilliQ water as carrier. Decreases for *S. sonnei, S.* Enteriditis, and *E. coli* O157:H7 inoculated at the high level were approximately between 1 and 1.5 log cycles after the whole drying period of 90 min. In contrary, for the low inoculum a decrease of 2.5 log, 3.5 log, and 3.2 log cycles were observed for *S. sonnei, S.* Enteriditis, and *E. coli* O157:H7, respectively.

Saline as carrier. When saline solution was used as carrier the high inoculum declined by approximately 2.6 log cycles for all used bacteria during the drying period. The low inocula for saline solution decreased by 2 log, 2.7 log, and 2.0 log cycles for *S. sonnei, S.* Enteriditis, and *E. coli* O157:H7, respectively within 90 min.

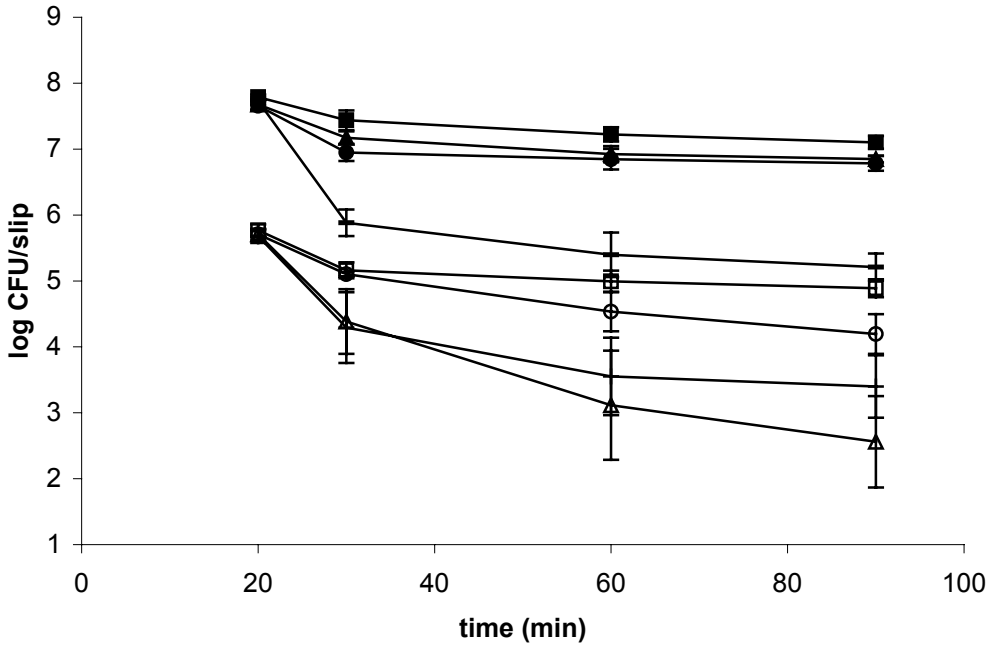

Figure 5.1: *Survival of* S. sonnei *on glass slips inoculated with 10^8 (closed symbols), 10^6 (open symbols) CFU at 28℃ with 5% horse serum (square symbols), 0.1% peptone (round symbols), MilliQ water (triangular symbols), and 0.85% saline solution (line symbols) as carrier. Error bars represent the standard deviation (n = 6).*

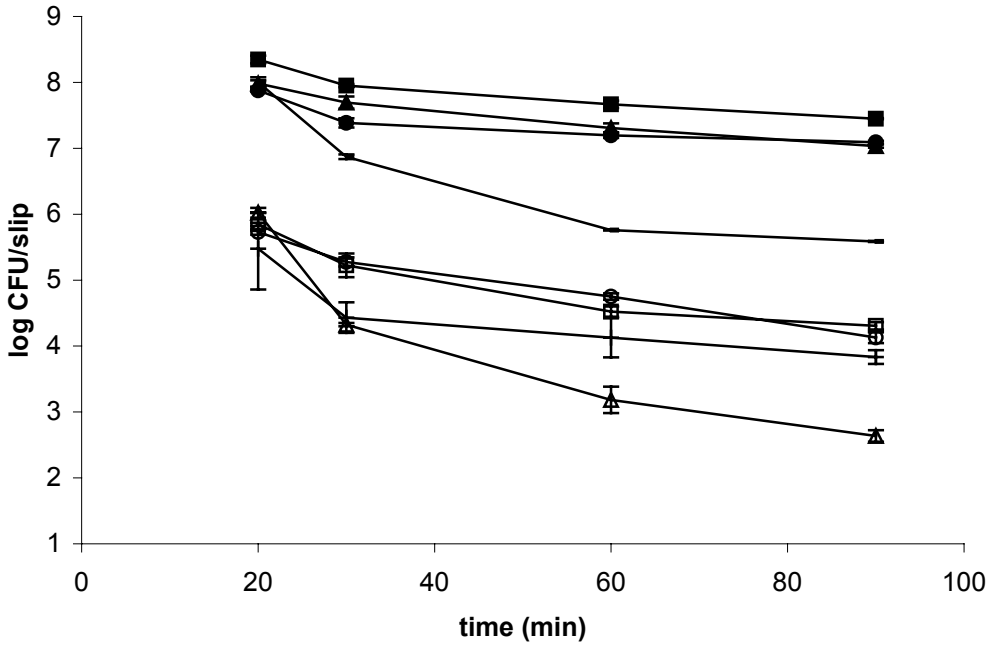

Figure 5.2: *Survival of* S. Enteriditis *on glass slips inoculated with* 10^8 *(closed symbols),* 10^6 *(open symbols) CFU at 28℃ with 5% horse serum (square symbols), 0.1% peptone (round symbols), MilliQ water (triangular symbols), and 0.85% saline solution (line symbols) as carrier. Error bars represent the standard deviation (n = 6).*

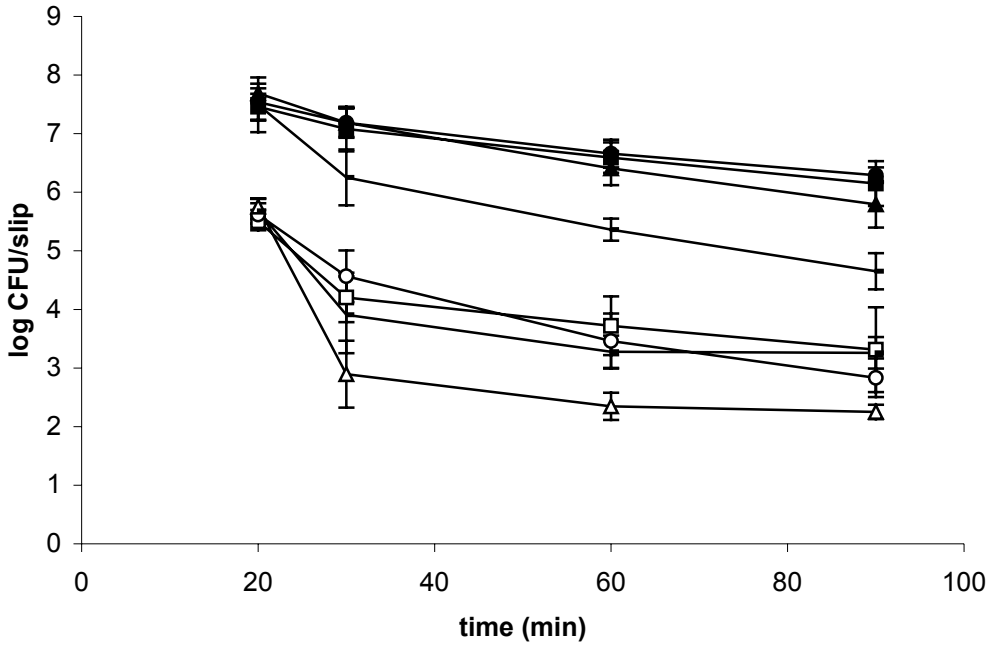

Figure 5.3: *Survival of* E. coli *O157:H7 on glass slips inoculated with 10^8 (closed symbols), 10^6 (open symbols) CFU at 28℃ with 5% horse serum (square symbols), 0.1% peptone (round symbols), MilliQ water (triangular symbols), and 0.85% saline solution (line symbols) as carrier. Error bars represent the standard deviation (n = 6).*

Statistical observations. The decline during 90 min drying for all bacteria, all carrier, and all inoculum densities was significant ($P < 0.0001$). The biggest drops during the drying period were observed between 20 and 30 min as the drops went visibly dry. After that the decrease leveled out.

For *S. sonnei* at the high inoculum the decrease rates for MilliQ water, horse serum, and peptone water were not significantly different to each other, but the decrease rate of saline was significantly different from MilliQ water ($P < 0.0001$). At the low inoculum the rate of decrease in peptone water, horse serum, and saline were significantly different from the rate of decrease in MilliQ water ($P < 0.014$).

When drying *S.* Enteriditis for 90 min at the high inoculum the decrease rate in horse serum and peptone water was not significantly different from MilliQ water, but the decrease

rate of saline was significantly different to MilliQ water ($P < 0.0001$). At the low inoculum the decrease rates when using peptone water, horse serum, and saline as carrier were significantly different from the decrease rate of MilliQ water ($P < 0.0001$).

E. coli O157:H7 showed a similar behavior, that at the high inoculum the decrease rates for saline, horse serum, and peptone water as carrier were significantly different to MilliQ water as carrier ($P < 0.0001$, $P = 0.0031$, and $P = 0.0027$, respectively). At the low inoculum the decrease rates when using peptone water, horse serum, and saline as carrier were significantly different from the decrease rate of MilliQ water ($P < 0.0029$).

When high and low inocula were compared for the different carriers, a significantly faster decrease ($P \leq 0.05$) for the low inocula was observed, except for *E. coli* O157:H7 where no significantly different decrease between the two inocula was observed when horse serum was used as carrier.

Combination of survival rates of *S. sonnei* at different cell densities. Survival experiments were done with *S. sonnei* at different inoculum sizes of approximately 10^4, 10^6, 10^7, 10^8 and 10^9 CFU/slip with MilliQ water as carrier. An increased survival with increasing cell density in the inoculum was observed after a drying time of 90 min (Figure 5.4). When a polynomial trend line to the second order was applied, the corresponding R^2 value was 0.9921.

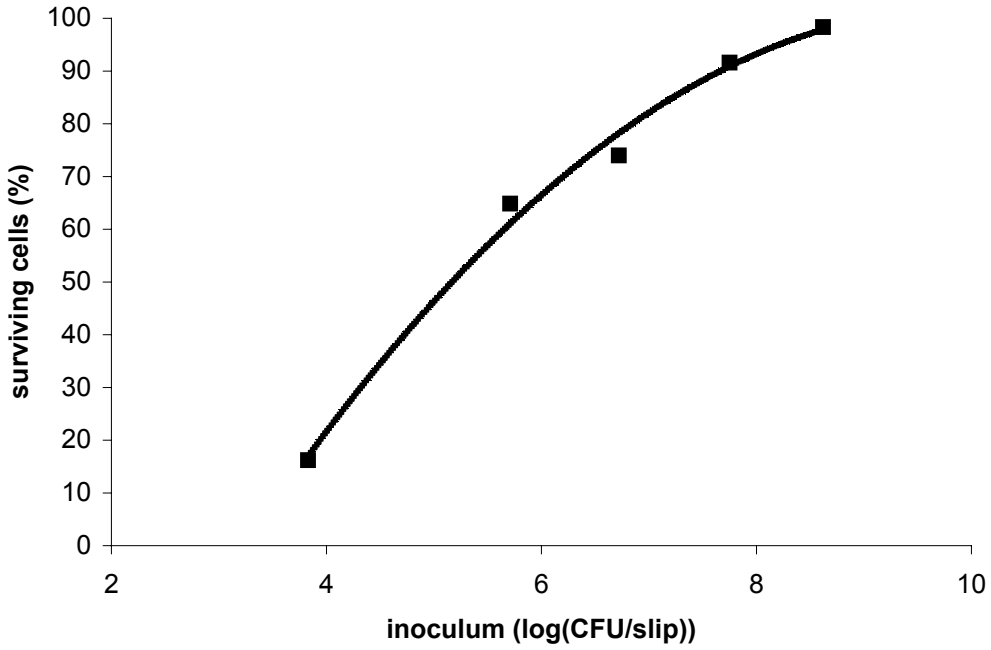

Figure 5.4: *The proportion of* S. sonnei *surviving after drying for 90 min with MilliQ water as carrier at different inoculum concentrations including trendline.*

Protection of viable cells by dead cells. To demonstrate the influence of dead cells on the viable ones a glass slip was inoculated with a combination of 10^6 viable and 10^8 pasteurized cells using MilliQ water as carrier. The survival was compared to glass slips spot inoculated with a 10^6 CFU droplet of *Shigella sonnei* with MilliQ water as carrier. The inoculum of viable cells dropped by 3 log in 1.5 h. In comparison the inoculum of the mixture of viable and dead cells showed a significantly better survival ($P < 0.0001$) by dropping approximately 0.7 log in the same time (Figure 5.5). Further it could be shown that the protection effect can be seen for dead *Salmonella* Enteriditis protecting viable *Salmonella* Enteriditis and also for dead *Salmonella* Enteriditis protecting *Shigella sonnei* and vice versa (results not shown).

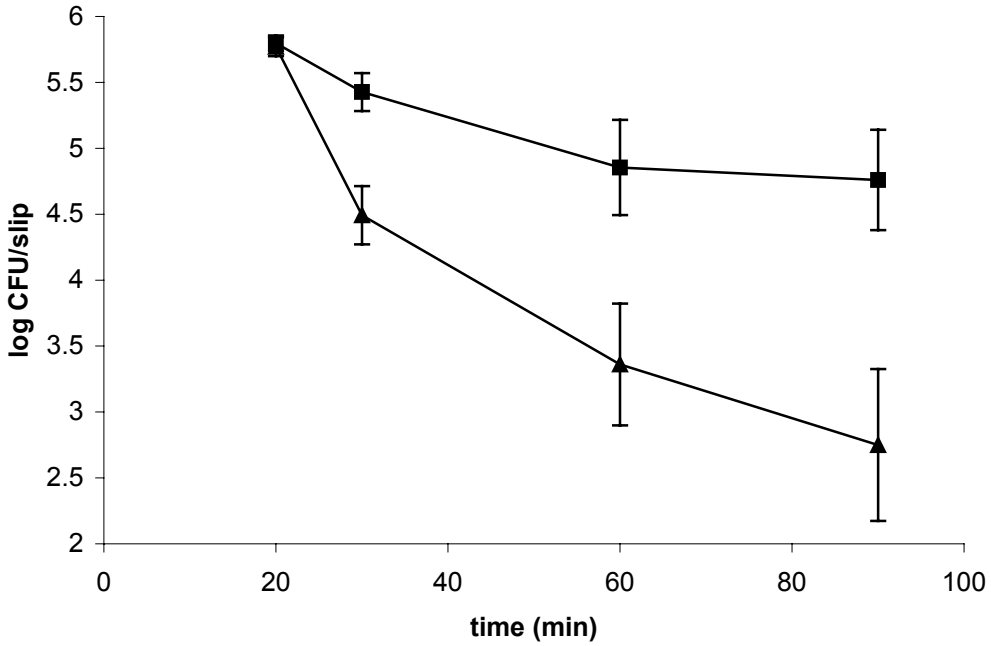

Figure 5.5: *Survival of* S. sonnei *on glass slips with MilliQ water as carrier, 10^6 CFU combined with 10^8 pasteurized cells inoculated (square symbols) and 10^6 CFU (triangular symbols) inoculated at 28°C. Error bars represent the standard deviation (n = 6).*

DISCUSSION

All observed declines over the drying period were significant. As discussed earlier in Chapter Three, no quorum sensing effect should be responsible for the observed effects due to the washing of the cells, the short experiment duration, and the low metabolism of the cells during the experiment.

All observed decreases were highest between 20 and 30 min of drying time and leveled out after that. A reson for that steep decrase might be that dehydration resulted in an osmotic stress for the cells and that this resulted in a combined effect of phase separation of the membrane and the volumetric contraction of the cells, which are responsible for cell death Beney at al. (2004) showed this effect for *E. coli*.

The biggest decreases were seen when saline was used as carrier at the high inoculum. During the drying process at low and high inoculum levels the salt concentrates and imposes an osmotic stress on the bacteria that can be lethal (Shadbolt et al., 1999; Beney et al., 2004).

When horse serum and peptone water are used at the low inoculum level, a comparable decrease to the high inoculum was seen within 1.5 h. The reason for the similar survival rate might be the composition of the carrier. Peptone water and especially horse serum have a high amount of organic material. In accordance to these data, de Cesare et al. (2003) reported at a reduction time for *Salmonella* on stainless steel of 476 and 4,565 min for phosphate buffer and tryptic soy broth carriers, respectively. Leslie (1995) and Welsh and Herbert (1999) reported a protection effect of sugars, especially trehalose, on *E. coli* and *Bacillus thuringensis* during air and freeze drying. The mode of protection may be a replacement of the evaporated water molecules with the sugar components (Crowe, et al., 1998).

When MilliQ water was used as carrier, the high inoculum survival was comparable to that in horse serum and peptone water. But for the low inoculum significantly poorer survival was observed with MilliQ water. Wilson and Lindow (1994) saw a similar effect when *Pseudomonas syringae* was inoculated onto the phyllosphere of bean leaves with potassium phosphate buffer as carrier.

Wilson and Lindow (1994) used un-washed cells for their experiments and attribute a better survival to the cell produced exo-polysacarides (EPS). Having used washed cells in these experiments, it could be shown that EPS production is not necessary for a better survival at higher densities. *Staphylococcus aureus* and *S.* Enteriditis inoculated onto steel surfaces decreased at greater rate when a lower inoculum of 10^5 CFU/100 cm^2 was used compared to 10^7 CFU/100 cm^2 (Kusumaningrum et al., 2003) within 2 h, but within the next 42 h of drying no more difference between the inoculum densities was observed.

It can be hypothesized that the better survival at a higher cell density is due to the volume surface ratio increase as discussed in Chapter Three, since an increasingly better survival with increasing cell density could be shown (Figure 5.4).

The protection effect of dead cells on viable cells was also shown by Wilson et al. (1994) where dead *P. syringae* cells protected viable ones during desiccation. There is also evidence that dead *E. coli* cells were able to protect viable cells from acid stress (Rowbury, 2000).

Chapter Six

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The influence of inoculum density on survival of gram-positive starter cultures

INTRODUCTION

In the food industry starter cultures are often used for the production of fermented foods. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are used for dairy products such as yogurt, *Lactobacillus curvatus* and *sake* for meat products such as sausages, for vegetable products such as sauerkraut *Lactobacillus bavaricus* and *plantarum* are used, and for sour dough products *Lactobacillus sanfranciscensis* and *brevis* are used (Doyle, et al. 1997). Most starter cultures can be purchased in a dried (primarily freeze dried) state. Therefore, it is of interest to determine how starter cultures survive freeze drying, and during drying under ambient temperatures, e.g. in the process of producing dried sausages.

Research has been done intensively on the mechanisms and survival of such starter bacteria during freeze drying (Carvalho et al., 2004; Gardiner et al., 2000; Bozoglu et al., 1987). Higher cell densities were able to survive freeze drying better than lower cell densities (Bozoglu et al., 1987).

Not much data is available about the behavior of different inoculum densities during desiccation at room temperature. Pre-stressing of lactobacilli increases the survival rate. Prasad et al. (2003) determined that pre-stressed (heat and osmotic) *Lactobacillus rhamnosus* cells would survive drying stress better than non pre-stressed cells.

Lactobacilli are gram-positive bacteria and thus have a different membrane structure than *Salmonella*, *Shigella*, and *Escherichia coli* O157:H7. After determining the significant influence of the inoculum density on gram-negative bacteria as *Salmonella*, *Shigella*, and *E. coli* O157:H7 (see Chapter Two and Four), in this chapter the influence of the inoculum density on the survival of lactobacilli on the model surface of glass slips is demonstrated.

MATERIAL AND METHODS

Test strains and media preparation. Two different strains of *Lactobacillus sanfranciscensis* TMW 1.52 and TMW 1.392 were used, and a mutant of TMW 1.362 where the gene for the production of exo-polysaccharids (EPS) was deleted, provided by Markus Tieking, Technical University of Munich. *Lactobacillus reuteri* TMW 1.656 was also evaluated. MRS4 (details see Appendix 1) was used as growth medium.

Inoculum preparation. Bacteria were streaked from a frozen stock culture stored at –80°C onto the MRS4. All bacteria except *L. reuteri* were incubated anaerobically at 37°C whereas *L. reuteri* was incubated anaerobically at 30°C. A single colony was transferred after 24 h to the same media broth and incubated overnight until the culture reached the stationary phase. A 10 µl loop of this culture was again transferred to 10 ml of the broth and incubated overnight. The loop transfer was repeated once more. For lactobacilli, an overnight incubation was not enough to reach the stationary phase, so the incubation period was prolonged to 3 days. Cells were harvested by centrifugation, washed twice in Li Chrosolv water (Merck KGaA, Darmstadt, Germany) and washed cells were diluted or concentrated to reach the desired inoculum density. Li Chrosolv water was used as carrier.

Inoculation procedure. Approximately 10^4, 10^6 or 10^8 CFU of inoculum (10 µl) was spot-inoculated onto a 18 x 18 mm glass cover slip in single spots. Slips were stored in a biohazard hood with the fan running at room temperature (26 ± 2°C).

Recovery of bacteria. At each time point (shortly before the droplet was visibly dry t = 20 min, when droplet was visibly dry t = 30 min, t = 60 min, and t = 90 min) cover slips were placed in a test tube containing 5 ml of 0.1% peptone water and approximately 15 sterile 3-mm glass beads. After vortexing (Vortex Genie 2, Fisher Scientific, Pittsburg, Pensylvania) at high speed for 20 s the sample was further diluted in 0.1% peptone water and plated onto MRS4.

Enumeration. Lactobacilli samples were spot plated (25 µl) onto MRS4 and incubated anaerobically at 37°C for *L. sanfranciscensis* and at 30°C for *L. reuteri*.

pH measurement. Lactobacillus cells at a concentration of 10^8 CFU/ml were washed twice in Li Chrosolv water. The final cell pellet was resuspended in 1 ml Li Chrosolv water. The pH was measured after 0, 20, 30, and 60 min.

Statistics. All experiments were repeated at least three times with duplicate samples. Statistical analysis was performed using the SAS software package (ver. 6.0, Cary, NC). Data were compared using Anova models. Data for *L. sanfranciscensis* 1.52 and 1.392 EPS mutant were log transformed, data for *L. reuteri* and *L. sanfranciscensis* 1.392 were log to the second power transformed. To compare the differences in between the inoculum densities, linear contrast models were used. Differences were considered significant at $P \leq 0.05$.

RESULTS

To verify that only the drying process and not a rapidly decreasing pH value of the medium, due to the built up of acids from the metabolism of the cells, is responsible for the decrease of viable cells, the pH of 10^8 CFU in 1 ml Li Chrosolv water was followed for 60 min (Table 6.1). No significant drop of the pH could be observed.

Table 6.1: *pH during drying of lactobacilli*

Time	
0 min	6.61
20 min	6.62
30 min	6.62
60 min	6.64

Survival of *Lactobacillus sanfranciscensis* TMW 1.52. Cells were inoculated at a high level of approximately 7.5 log CFU/slip, a moderate level of 5.5 log CFU/slip, and a low level of 4.4 log CFU/slip. The population at the high inoculum remained stable, no significantly decrease was observed within the 90 min drying period (P = 0.5235). The

biggest drop in populations was seen for the moderate and low inoculum between 20 and 30 min (P = 0.0049 and P < 0.0001, respectively). After that the cells numbers remained relatively stable. The moderate inoculum decreased significantly (P = 0.0145) by 0.3 log cycles within 90 min. A significant (P < 0.0001) decrease of approximately 2.0 log cycles was also seen for the low inoculum within 90 min (Figure 6.1).

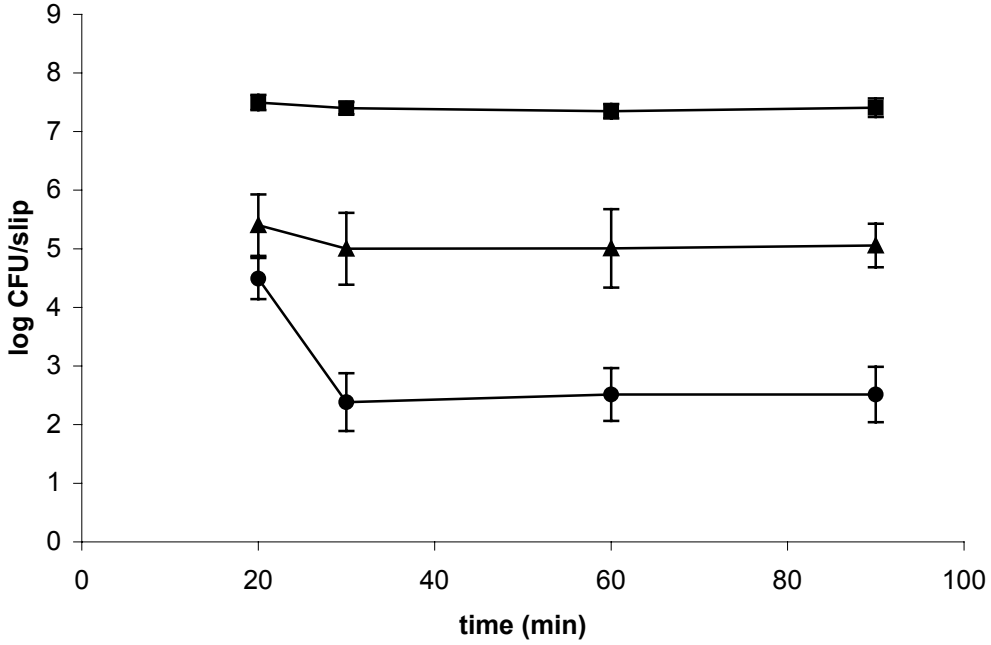

Figure 6.1: *Survival of* L. sanfranciscensis *TMW 1.52, high inoculum (square symbols), moderate inoculum (triangular symbols), and low inoculum (round symbols). Error bars represent the standard deviation (n = 6).*

Survival of *Lactobacillus sanfranciscensis* TMW 1.392 and EPS mutant. Cells were inoculated at a high level of approximately 8.5 log CFU/slip, a moderate level of 6.5 log CFU/slip, and a low level of approximately 4.0 log CFU/slip and 4.5 log CFU/slip for parent and mutant, respectively. Both strains behaved similarly when dried for 90 min. For the parent and the mutant strain the high inoculum stayed stable, no significant decrease was observed within the drying period of 90 min (P = 0.0801 and 0.0556, respectively). The moderate inoculum showed a significant decrease for the parent strain (P = 0.0176), but not for the

mutant ($P = 0.1306$). For both strains at the low inoculum, the biggest significant decrease ($P < 0.0001$) was observed between 20 and 30 min of the drying time. A significant ($P < 0.0001$) decrease of approximately 2.0 log cycles was seen for the low inoculum within 90 min for both strains (Figure 6.2 and 6.3).

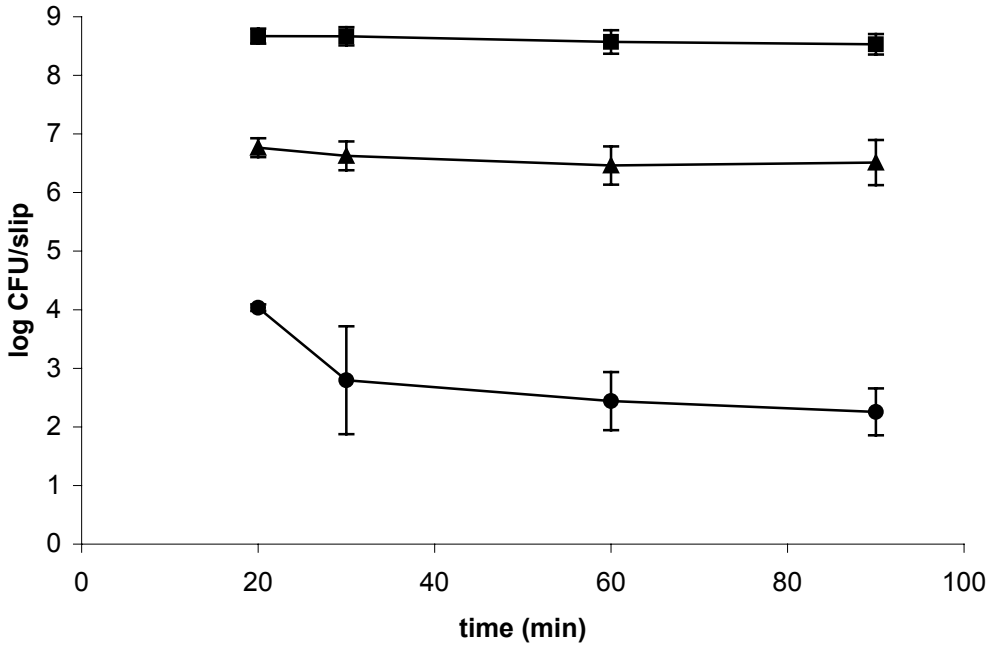

Figure 6.2: *Survival of* L. sanfranciscensis *TMW 1.392, high inoculum (square symbols), moderate inoculum (triangular symbols), and low inoculum (round symbols). Error bars represent the standard deviation (n = 6).*

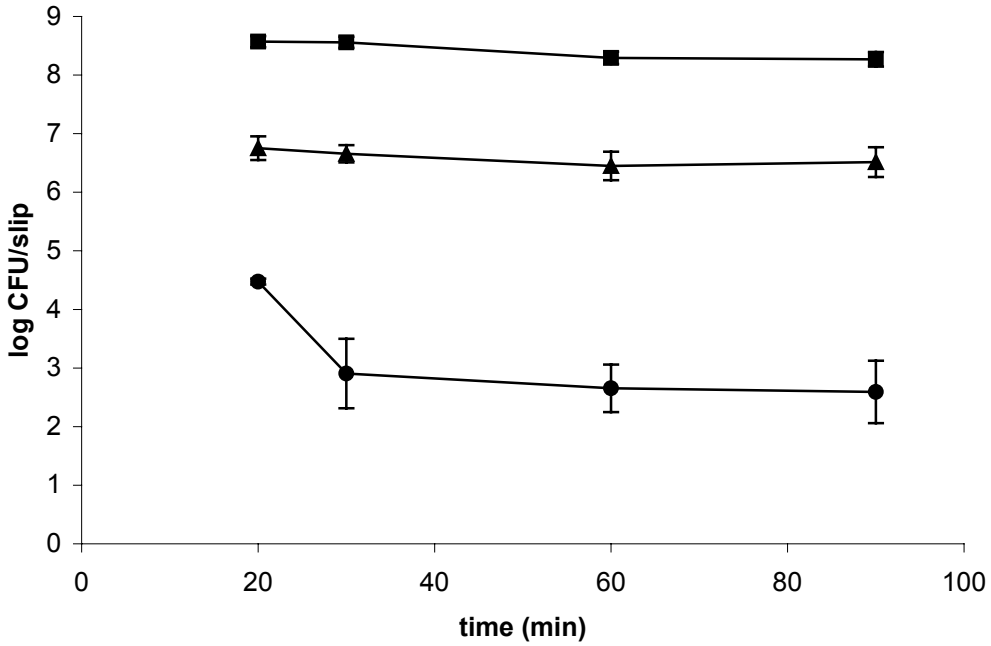

Figure 6.3: *Survival of* L. sanfranciscensis *TMW 1.392 mutant, high inoculum (square symbols), moderate inoculum (triangular symbols), and low inoculum (round symbols). Error bars represent the standard deviation (n = 6).*

Survival of *Lactobacillus reuteri* TMW 1.656. Cells were inoculated at a high level of approximately 6.5 log CFU/slip and a low level of approximately 4.8 log CFU/slip. The high inoculum decreased significantly ($P = 0.0251$) by 0.5 log cycles within the drying period of 90 min. For the low inoculum a significant ($P < 0.0001$) decrease of approximately 1.7 log cycles was observed within 90 min, where the inoculum declined continuously over the drying period (Figure 6.4).

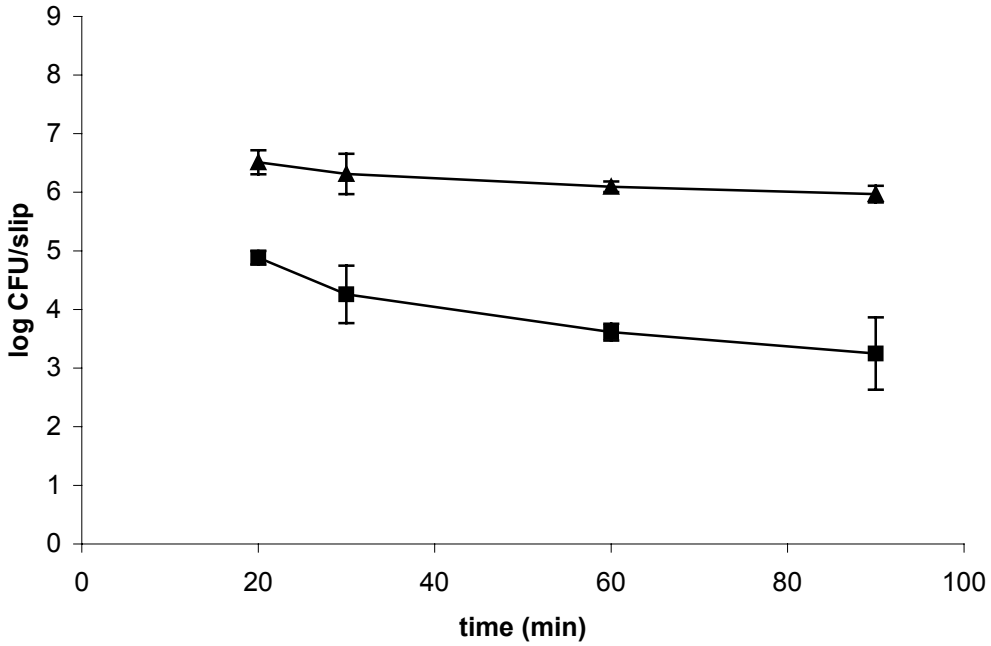

Figure 6.4: *Survival of* L. reuteri *TMW 1.656, high inoculum (triangular symbols) and low inoculum (square symbols). Error bars represent the standard deviation (n = 6).*

Comparison of decrease rates depended on the inoculum density. As shown in Table 6.2 all low inocula decreased significantly faster that the moderate or high inocula for each strain. No significant differences were observed between the high and the moderate inocula.

Table 6.2. *Reduction of lactobacilli (log CFU/slip) after a 90-min drying. Data shown are the difference between the log initial population and log population at 90 min*

Inoculum	*L. reuteri*	*L. sf* 1.52	*L. sf* 1.392	*L.sf* 1.392 mutant
High	nd[a]	0.09 A[b]	0.14 A	0.30 A
Moderate	0.54 A	0.34 A	0.25 A	0.24 A
Low	1.64 B	1.98 B	1.78 B	1.88 B

[a]: not done
[b]: different letters are indicating a significant difference between the decrease for inoculum levels for each lactobacillus

DISCUSSION

All three strains of lactobacilli showed the same general survival pattern. At a high inoculum level of 6.5 to 8.5 log CFU/slip no significant decrease during the drying period was seen. At lower inoculum levels, significant decreases were observed. Decreases of up to 2 log cycles were seen when inoculated at approximately 4.0 CFU/slip. No difference in the behavior of the strain TMW 1.362 and the EPS mutant was observed.

For the *L. sanfranciscensis* strains the biggest decrease for the moderate and the low inoculum was observed between 20 and 30 min, when the droplet visibly dried. After that the decrease leveled out. But for *L. reuteri* at the moderate inoculum a steady decline over the 90 min period was observed.

Bacteria producing EPS often have better surviving rates because of protection against dehydration, bacteriophages, and antibiotics (Whitfield, 1988; Weiner et al., 1995; Roberts, 1996). Wilson and Lindow (1994) speculated that the production of EPS increases the survival of *Pseudomonas syringae* at high densities. In this study it could be shown that the production of EPS is not necessary to see an increased survival rate at higher densities. Since in this experiment cells were washed prior to experiment and all EPS should have been removed and not have influenced the survival.

The survival of gram-positive *Lactobacilli* was very similar to that of *Salmonella*, *Shigella*, and *E. coli* O157:H7 as shown in Chapter Five. When MilliQ water was used and *Salmonella* and *Shigella* were inoculated at a high inoculum of approximately 8.0 log CFU/slip, decrease rates of 0.5 log cycles were observed within 90 min, compared to up to 0.3 log cycles for *Lactobacilli*. For *E. coli* O157:H7 a slightly higher decrease of approximately 1 log cycle was observed.

When *Salmonella*, *Shigella*, and *E. coli* O157:H7 were inoculated at a lower inoculum level of approximately 5.7 log CFU/slip a reduction between 2.5 and 3.0 log cycles could be observed. At this initial inoculum *Lactobacilli* levels decreased slower by approximately only

0.4 log. Only below an initial inoculum level of 4.0 log CFU/slip a decrease of approximately 2 log cycles was observed. Significantly faster decreases were seen for the low inoculum compared to the high and moderate inoculum, but not for the moderate inoculum compared to the high inoculum. Thus it seems that *Lactobacilli* have somewhat better survival rates during drying compared to the gram-negative bacteria.

In this study all cells were taken from the stationary phase. However the phase of the cells in the life cycle plays an important role in the survival during drying. Corcoran et al. (2004) showed the highest survival rate of 50% of spray dried lactobacilli when cells of the stationary phase are used. When cells from the log phase or the lag phase were used, survival rates of 20% and 10%, respectively, were achieved.

As observed, cells survive drying better when higher cell concentrations are present. Therefore, cells should be concentrated before drying for maximum survival. Concentration of samples by evaporation is uneconomical (Linders et al., 1998). Instead a centrifugation of the cells might be an economical solution to reach high cell densities.

Chapter Seven

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Factors influencing the survival of gram-negative bacteria on surfaces

INTRODUCTION

In Chapter Four and Five the influence of carrier medium and inoculum density on the survival rate of *Shigella sonnei*, *Salmonella* Enteriditis, *Escherichia coli* O157:H7, and *Lactobacillus sanfranciscensis* and *reuteri* was evaluated. Significant influences of both carrier and inoculum density could be observed.

In general it is well known that prestressed cells will survive additional stresses better than non-prestressed cells. This effect was shown in the case of acid stress for *E. coli* by Goodson and Rowbury (1989), and for *Salmonella typhimurium* by Foster and Hall (1990). In this study cells were "pre-dried" as a pre-stress.

Earlier studies showed that growth temperature had an effect on the optimal growth rate of *Vibrio costicola* at different saline concentrations (Adams and Russell, 1992).

Anaerobically grown cultures of *E. coli* K12 and *S. flexneri* were equally acid resistant as aerobically grown cultures (Small et al., 1994). Kwon and Ricke (1998) reported a higher acid resistance of *S.* Typhimurium after an anaerobical adaptation to short-chain fatty acids compared to an aerobical adaptation. Anaerobic growth was connected to a greater expression of virulence, adhesion, and penetration of host cells in *S.* Typhimurium (Singh et al., 2000).

Furthermore, it was reported that positively charged biomaterial surfaces have an antimicrobial effect on adhering of gram-negative bacteria, but not on gram-positive bacteria (Gottenbos, et al., 2001).

In this chapter, growth temperature, anaerobical vs. aerobical incubation, and the hydrophobicity of surfaces on the survival rate of *S. sonnei* and *S.* Enteriditis were investigated.

MATERIALS AND METHODS

Test strains and media preparation. A human isolate of *S. sonnei* LJH 664 was obtained from Dr. Trevor Suslow, UC Davis. *S. enterica* serovar Enteriditis Phage type 30 LJH 608 was isolated from raw almonds associated with an outbreak of salmonellosis (Isaacs et al., 2005).

Preparation of inoculum. The inoculum was prepared as described in Chapter Three. In short, before each experiment a sample from a frozen stock culture was grown on TSA and an isolated colony was subcultured in tryptose soy broth (Difco) (37°C for 24 h). Single loop transfers were prepared at two consecutive 18 to 24 h intervals. Overnight cultures were collected by centrifugation and suspended MilliQ water. Cells were washed two times in MilliQ water to ensure complete removal of the growth medium.

When the influence of a lower growth temperature was determined, a culture from a TSA plate of *S. sonnei* and *S.* Enteriditis cells was transferred to TSB and incubated in a waterbath at 13°C. Cells needed 4 days to reach the stationary phase at this temperature. When the influence of a higher growth temperature was determined, one colony of *S. sonnei* and *S.* Enteriditis from TSA plates was transferred to TSB and cells were grown in a waterbath at 45°C overnight. Cultures were collected by centrifugation and suspended MilliQ water. Cells were washed for two times in MilliQ water to ensure complete removal of the growth medium and cells were adjusted to the same optical density (OD = 0.8) to achieve a similar initial inoculum.

To grow cells anaerobically *S. sonnei* and *S.* Enteriditis from TSA plates were transferred to TSB in a 15 ml plastic tube with screw cap (Benton Dickinson and Company, Franklin Lakes, NJ) and incubated at 37°C. Single loop transfers were prepared at two consecutive 18 to 24 h intervals. To grow cells aerobically *S. sonnei* and *S.* Enteriditis from TSA plates were transferred to TSB in a 50 ml Erlenmeyer flask and flask was closed with a

cotton stopper. Flasks were incubated on a rotary shaker (220 rpm) at 37°C. Single loop transfers were prepared at two consecutive 18 to 24 h intervals. Cells were washed for two times in MilliQ water to ensure complete removal of the growth medium.

Inoculation of cover slips and surfaces. Approximately 10^6 or 10^8 CFU of inoculum (10 µl) was spot-inoculated in one droplet onto a 18 x 18 mm glass cover slip (Menzel, Braunschweig, Germany), or onto parafilm (American National Can, Menasha, WI) when a hydrophobic surface was used. For the low temperature experiments, washed cells were inoculated onto glass slips at room temperature and onto glass slips which were put onto a metal sheet sitting on ice. Glass slips and parafilm were stored under a biohazard hood with the fan running at room temperature (28 ± 2°C).

Enumeration. At each time point (shortly before the droplet was visibly dry t = 20 min, shortly afterwards t = 30 min, t = 60 min, and t = 90 min) glass slips or parafilm were placed in a test tube containing 5 ml of 0.1% peptone water and 15 sterile 3 mm glass beads. After vortexing (Vortex Genie 2, Fisher Scientific, Pittsburg, Pensylvania) at high speed for 20 s the sample was further diluted in 0.1% peptone water and spot plated on TSA.

Statistics. All experiments were repeated at least three times with duplicate samples. Statistical analysis was performed using the SAS software package (ver. 6.0, Cary, NC). Data were compared using Anova models. In order to normalize the results, data for *Shigella* grown at different temperatures were log transformed, whereas data for *Salmonella* comparing optimal and cold growth were square root transformed and comparing optimal and hot growth were minus 3/8 power transformed. In order to normalize the results data for *Shigella* grown aerobically and anaerobically were log transformed. To compare differences between growth temperatures, linear contrast models were used. Differences were considered significant at $P \leq 0.05$.

RESULTS

Growth of *Shigella sonnei* and *Salmonella* Enteriditis at different temperatures. One colony of *S. sonnei* and *S.* Enteriditis were inoculated from plate to broth and incubated at temperatures ranging from 4 to 53°C. Growth was determined visibly, by looking for cloudiness of medium. Incubation time for 4°C was 3 weeks, and for 47 and 53°C 5 days, after which possible cell growth was determined. In Table 7.1 it can be seen that *Salmonella* was able to grow at 4°C, whereas *Shigella* was unable to grow at this temperature. Both strains grew from 13°C to a maximum temperature of 45°C. Therefore, temperatures of 13°C (cold) and 45°C (hot) were established for *Salmonella* and *Shigella* (Table 7.1).

Table 7.1: *Growth of* Salmonella *and* Shigella *at different temperatures*

	4°C	13°C	37°C	45°C	47°C	53°C
Salmonella	+[a]	+	+	+	-	-
Shigella	-	+	+	+	-	-

[a]: + indicates growth at set temperature, - indicates no growth

In addition to the minimum and maximum growth temperatures the time to reach the stationary phase at the corresponding temperatures was determined. Again *Shigella* and *Salmonella* showed similar results. Growth curves are shown in Figure 7.1. At the minimum temperature of 13°C both strains required 4 days and at the maximum temperature of 45°C approximately 8 hours to reach the stationary phase, the same as for the optimal growth temperature of 37°C. (Table 7.2).

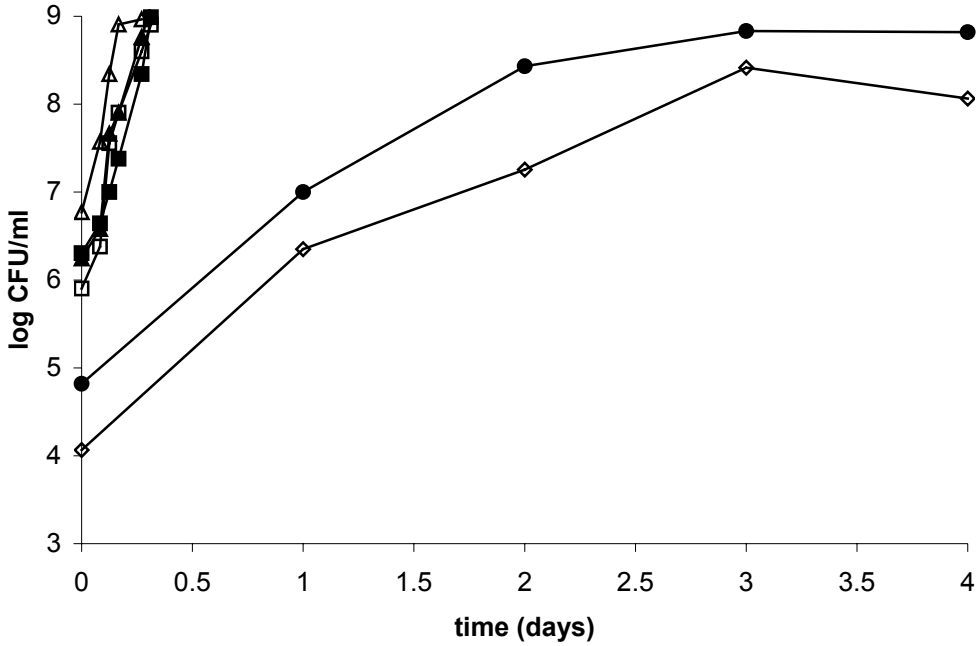

Figure 7.1: *Growth curves of* S. sonnei *and* S. *Enteriditis grown at 13, 37, and 45°C. Closed symbols indicate* Salmonella*, open symbols indicate* Shigella*. Round symbols indicate grown at 13°C, triangular symbols indicate grown at 37°C, whereas square symbols indicate growth at 45°C.*

Table 7.2: *Time for* Salmonella *and* Shigella *to reach stationary phase at set temperatures*

	13°C	37°C	45°C
Salmonella	4 d[a]	8 h[b]	8 h
Shigella	4 d	8 h	8 h

[a]: d indicates days

[b]: h indicates hours

Influence of incubation temperature on survival during desiccation. *Shigella* and *Salmonella* were incubated at 13°C, 37°C, and 45°C until they reached the stationary phase. Upon drying *Shigella* showed an approximate decline of 2.9 log cycles within 90 min when grown at 37°C (with initial inocula of approximately 5.5 log), but a larger decline of 4.3 and 4.0 log cycles when grown at 13°C and 45°C, respectively (Table 7.3). When grown at cold

and hot temperature populations were significantly lower after drying for 90 min ($P = 0.0005$ and $P = 0.0029$, respectively) compared to grown at optimal temperature. For some samples dried for 30 min and more a high standard deviation of >1.0 log was observed.

Table 7.3: *Log (CFU ± SD) of* Salmonella *and* Shigella *during drying on a glass slip, comparing cells grown at 13°C and 37°C, and cells grown at 37°C and 45°C*

	Growth Temperature	20 min	30 min	60 min	90 min
Salmonella					
	13°C	A[a] 6.04 ± 0.22 A[b]	B 3.61 ± 0.63 B	C 2.41 ± 0.22 B	D 1.77 ± 0.65 B
	37°C	A 6.14 ± 0.12 A	BC 4.63 ± 0.24 A	C 3.50 ± 0.09 A	C 3.50 ± 0.09 A
	37°C	A 5.88 ± 0.14 A	B 4.16 ± 0.38 A	C 3.29 ± 0.28 A	C 3.29 ± 0.28 A
	45°C	A 5.43 ± 0.18 A	B 3.26 ± 0.68 A	C 1.50 ± 0.59 B	C 1.50 ± 0.59 B
Shigella					
	13°C	A 5.34 ± 0.22 A	B 3.80 ± 1.04 A	C 1.64 ± 0.77 B	C 1.64 ± 0.77 B
	37°C	A 5.59 ± 0.08 A	B 4.24 ± 0.43 A	C 2.79 ± 0.30 A	C 2.79 ± 0.30 A
	37°C	A 5.61 ± 0.04 A	B 3.40 ± 1.04 A	C 2.53 ± 1.07 A	C 2.53 ± 1.07 A
	45°C	A 5.34 ± 0.36 A	B 2.78 ± 0.91 A	C 1.17 ± 0.35 B	C 1.17 ± 0.35 B

[a]: Capital letters before number indicate a significant difference between time points at each growth temperature for each bacterium.
[b]: Capital letters after number indicate a significant difference between growth at 13°C and 37°C, and 37°C and 45°C at each time point for each bacterium.

Similar results were seen for *Salmonella*, where the decline after a growth temperature of 37°C was approximately 2.6 log cycles within 90 min and 3.7 and 4.2 log cycles when grown at 13°C and 45°C, respectively (Table 7.3). When grown at cold and hot temperature, populations declined significantly faster ($P = 0.0421$ and $P < 0.0001$, respectively) compared to grown at optimum temperature.

Influence of aerobic vs. anaerobic growth of *Shigella sonnei.* *Shigella* cells grown aerobically or anaerobically did not show a significant difference in survival when dried on glass slips. At the inoculum of 10^8 or 10^6 CFU inoculum, a decrease of approximately 0.6 log

or 3.0 log was observed, respectively, for both aerobic and anaerobically grown cells (Figure 7.2).

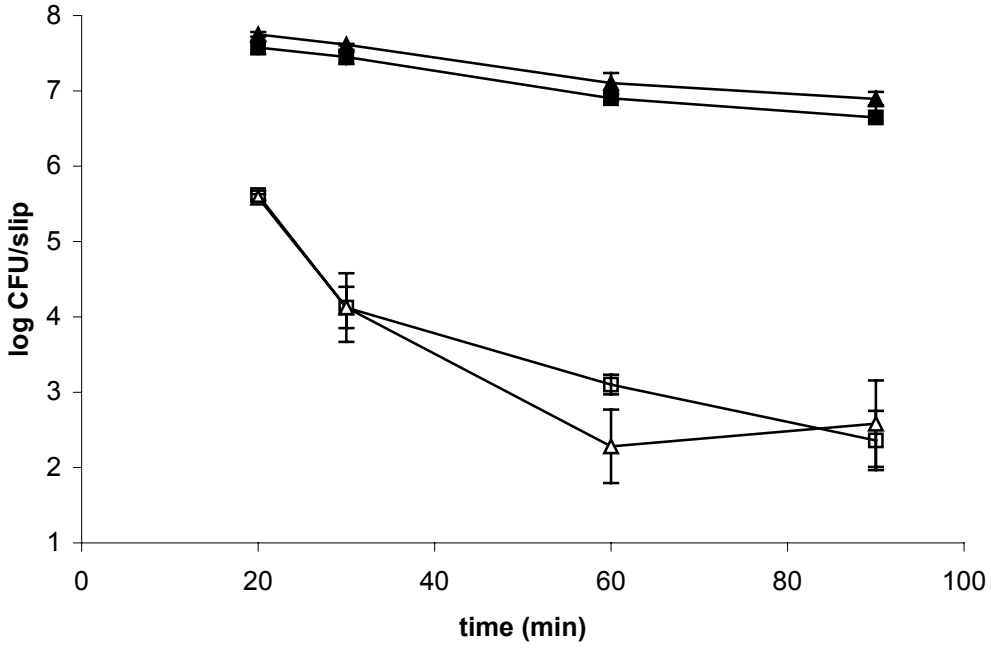

Figure 7.2: *Influence of aerobic (square symbols) and anaerobic (triangular symbols) growth of* S. sonnei *on survival during desiccation. Error bars represent the standard deviation (n = 3).*

Influence of drying stress on survival of *S. sonnei* during drying. When cells were dried at 10^6 CFU and 10^8 CFU per slip (10 µl), grown in TSB, and 10^8 CFU were dried again on a glass slip all three samples declined by approximately 0.5 log cycles. When pre-stressed cells were inoculated at a density of 10^6 CFU per slip all three different samples declined by approximately 2.9 log cycles (Figure 7.3). There was no influence of drying stress on the survival during desiccation.

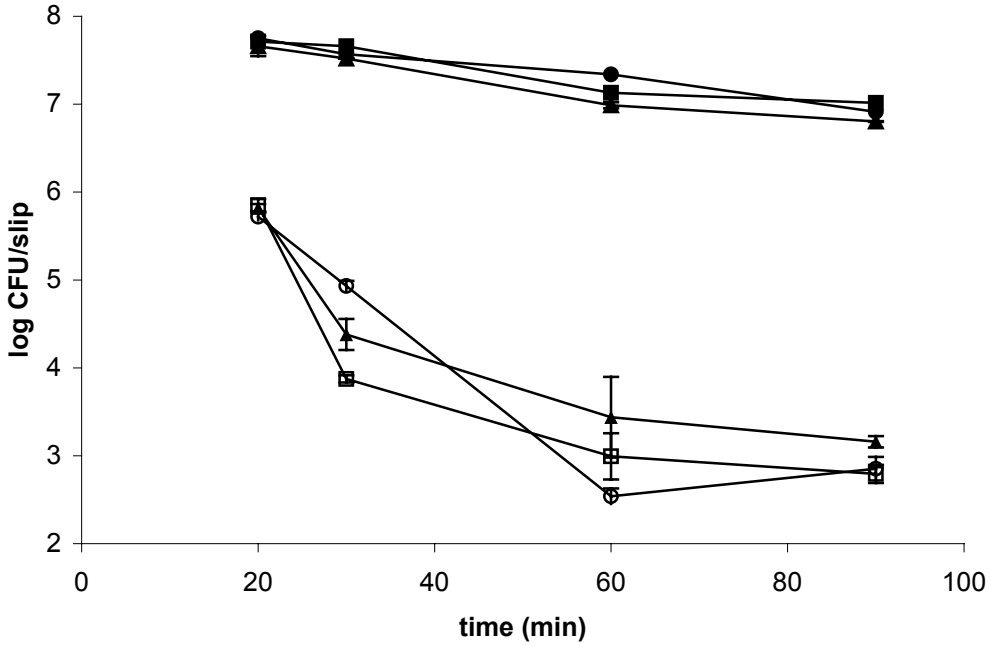

Figure 7.3: *Influence of drying* S. sonnei*, growing the surviving bacteria again and repeat the drying. Square symbols show the survival of freshly grown and non pre-dried cells, triangular symbols the survival of cells where 10^6 CFU were pre-dried and surviving cells re-grown, and round symbols the survival of cells where 10^8 CFU were pre-dried and surviving cells re-grown. Error bars represent the standard deviation (n = 3).*

Influence of hydrophilic and hydrophobic surfaces. Cells were inoculated (10 µl) on glass slips (control) and parafilm (hydrophobic) at high (8.5 log CFU/slip), moderate (7.7 log CFU/slip), and low (6.8 log CFU/slip) inoculum levels.

When inoculated onto parafilm droplets needed approximately 30 min longer (50 min in total) than glass slips to be visibly dry.

For the high inoculum, decreases of approximately 0.1 log cycles were observed for parafilm, whereas on glass no decrease was observed. The moderate inoculum on the parafilm decreased by 0.5 log cycles, for the glass surface the inoculum declined by 0.2 log cycles. At

the low inoculum level both populations decreased by approximately 1.8 log cycles (Figure 7.4).

In these experiments no influence of the hydrophobicity of surfaces on the survival of *S. sonnei* was seen.

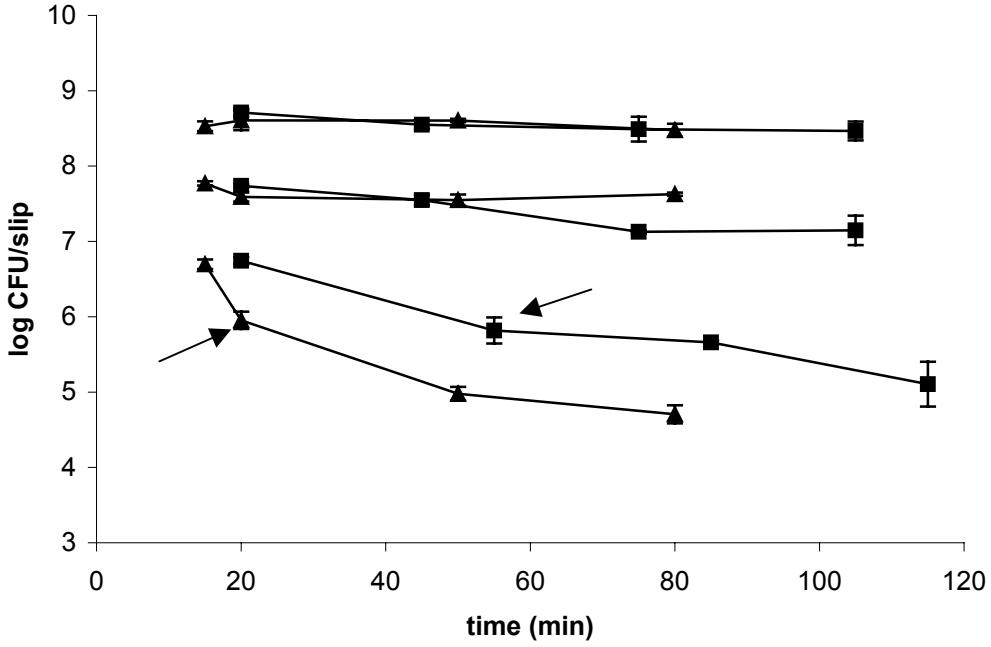

Figure 7.4: *Influence of different surface hydrophobicity on the survival of* S. sonnei*. Square symbols indicate the survival on a hydrophobic surface (parafilm), triangular symbols show the survival on untreated glass slips. Error bars represent the standard deviation (n = 3). Arrows indicate when inocula were visibly dry for the low inoculum.*

DISCUSSION

For growth experiments at different temperatures a cold growth temperature for *Salmonella* and *Shigella* of 13°C, and a hot growth temperature of 45°C were established. In this study no difference of the growth rates between 37°C and 45°C were observed. These results are very similar to reported maximal growth temperatures for *Salmonella enterica* of 45°C with near optimal growth rate at this temperature (Bronikowski et al., 2001). Minimal

growth temperatures for these organisms of 5 to 8°C are reported (Fehlhaber and Kruger, 1998; Matches and Liston, 1968).

Bacteria must keep their membranes in a liquid-crystalline state to ensure biological activity (Denich et al., 2002). Growing bacteria in a cold environment results in a relatively higher amount of unsaturated, short chained fatty acids in the membrane, whereas more saturated and long chained fatty acids are incorporated into the membrane at above optimum growth temperatures (Denich et al., 2002).

In this study it is hypothesized that the altered composition of the membranes, when grown at non-optimal temperatures, results in a significantly lower survival rate. The decrease rates at sub-optimal, optimal, and supra-optimal temperatures are shown in Table 7.4. Significant differences between the temperatures were seen for *S. sonnei* only after 90 min drying time, whereas significant differences for *S.* Enteriditis were seen after 30 min for the low temperature and no difference at all for the high temperature.

Table 7.4: *Reduction (log CFU) of bacteria grown at 13°C and 37°C, and at 37°C and 45°C after spotting (10 µl) onto glass slips and drying for 90 min (for 13°C and 37°C n = 6, for 37°C and 45°C n = 4)*

	13°C	37°C	37°C	45°C
Salmonella	4.27	2.64	2.59	3.94
Shigella	3.70	2.80	3.08	4.17

Many studies show a better survival of cells when pre-stressed. Alkali pre-stressed *E. coli* cells were more tolerant to alkali stress (Rowbury et al., 1996). Mildly acidic pre-stressed *S. typhimurium* cells were more resistant to subsequent acid stress than non-stressed cells (Foster and Hall, 1990). When *L. sanfranciscensis* was pre-stressed with salt, cold, and pressure surviving cells were more tolerant of pressure and heat (Scheyhing et al., 2003). In the current study no influence of drying pre-stress on the survival rate during desiccation was observed for *S. sonnei*.

Considering the growth under aerobic conditions also as a pre-stress, all the imposed pre-stresses in this study did not have an effect on the survival of *S. sonnei* during desiccation. It is hypothesized, if it was possible to use the dried cells directly again – without any growth phase in between – for a subsequent drying experiment, that these cells would be somewhat more resistant to the drying process. More research is necessary to determine if classic stresses, as heat, cold, or pressure stress can influence the survival during desiccation.

When the influence of the surface on the survival was evaluated, a longer drying time for the more hydrophobic surface was observed than for the more hydrophilic one. The effect of a faster evaporation, corresponding to a smaller contact angle for a pinned droplet was also observed by Sefiane (2003). No difference of the survival rate between the hydrophobic and the hydrophilic surface was observed in contrary to the hypothesis, that with increased hydrophobicity the droplets on the surface become more spherical in shape and the deposited bacteria should be more concentrated in a smaller area - compared to droplets dried on a non hydrophobic surface. When the layers are higher the surface to volume ratio of the deposit should become smaller (see Chapter Three) and more bacteria are protected by the outer layer and the survival rate should be better. This effect did not change the survival rate in these experiments. A reason for that could be that this effect is so small that it could not be observed within this experiment's parameters.

Chapter Eight

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Desiccation induced changes of transcription, translation, and bio expression

INTRODUCTION

Drying stress includes osmotic stress, due to the concentration of solutes, oxidative stress where, oxygen radicals can directly influence the cell, and starvation stress, since molecules are not readily available for the dried cells.

Garay-Arroyo et al. (2000) were able to show the up-regulation of highly hydrophilic proteins in *Escherichia coli* during conditions of water deficit resulting in osmotic stress.

Monitoring of gene expression via the technologies encompassed under the term 'proteomics' allows proteins of significance to be related to phenotypes associated with strain variability, environmental influences and the effects of genetic manipulation (Cordwell, et al., 2001). In the literature a protein map of *Shigella flexneri* (Liao, et al., 2003) is described, but no protein maps related to drying stress could be found.

It is reported that cells can survive drying stress better when saccharides (e.g. trehalose (Figure 8.1)) or compatible solutes are produced by bacteria or are added to the bacteria (Kempf and Bremer, 1998; Welsh and Herbert, 1999; Hincha et al. 2002; Leslie et al., 1995; Zavaglia et al., 2003). An improved survival was reported for gram-positive and gram-negative bacteria, e.g. *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Bacillus subtilis*, and *E. coli*. In the plant kingdom trehalose – a disaccharide - was found to be accumulated in the desiccation tolerant resurrection plant (*Myrothamnus flabellifolia*) (Müller at al., 1995). Garcia de Castro and Tunnacliffe (2000) observed that trehalose improves osmotolerance but not desiccation tolerance in mammalian cells.

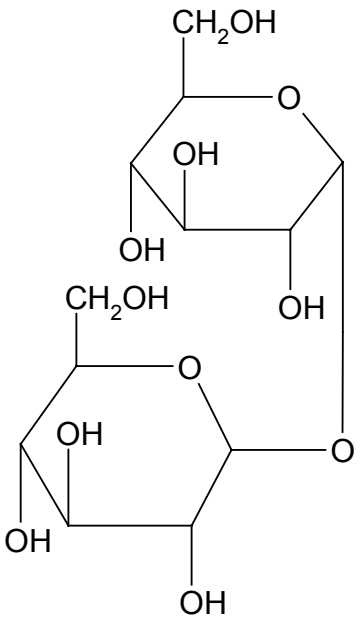

Figure 8.1: *Structure of trehalose*

The mechanisms by which trehalose protects cells from stress were discussed by Crowe et al. (1998). Sucrose and trehalose are both able to substitute evaporated water in the membrane and thus prevent fusion of the membrane (Figure 1.2). Furthermore, trehalose was able to lower the phase transition temperature in membranes, so that membranes stay in a liquid crystalline phase. Trehalose also showed a protection effect on the cell proteins of *E. coli* and *Bacillus thuringensis* (Leslie et al., 1995). For *Enterobakter sazakii* it was shown that cells up regulate their trehalose production when dried (Breeuwer et al., 2003).

The trehalose pathway described by Padilla et al. (2004) for *E. coli* is shown in Figure 8.2.

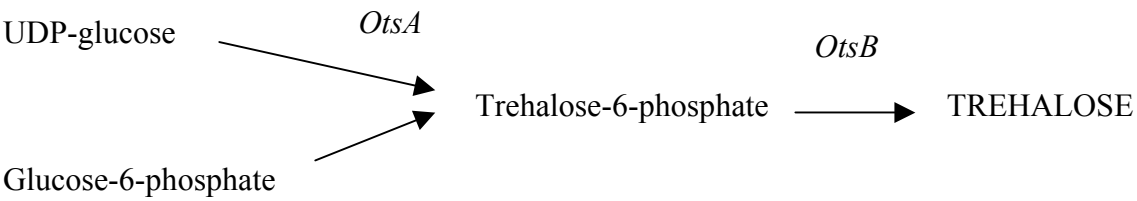

Figure 8.2: *Trehalose pathway*

Shigella virulence parameters for invasion and intercellular spreading are coded on a virulence plasmid, but further regulatory genes are also located on the chromosome (Maurelli and Sansonetti, 1987). The *Shigella* virulence is regulated by a cascade where the VirF gene is the activator of the InvE gene (Dorman et Porter, 1998). No literature was found about any connection between desiccation and the expression of virulence parameters.

In the current study the impact of drying on *Shigella sonnei* and *Salmonella* Enteriditis was evaluated using differential proteomics of drying-stressed and non-stressed cells, an evaluation of mRNA levels for *VirF* and *InvE*, and measurement of the accumulation of trehalose.

MATERIAL AND METHODS

Test strains. A human isolate of *S. sonnei* LJH 664 and the antibiotic resistant strain LJH 661 were obtained from Dr. Trevor Suslow, UC Davis. *Salmonella enterica* serovar Enteriditis Phage type 30 LJH 608 was isolated from raw almonds associated with an outbreak of salmonellosis (Isaacs et al., 2005), also the antibiotic resistant strain LJH 637 was used.

Preparation of inoculum. Inoculum was prepared as described in Chapter Three. In short before each experiment a sample from a frozen stock culture of *S. sonnei* or *S.* Enteriditis was grown on TSA, TSAR or TSAN and an isolated colony was subcultured in tryptose soy broth (Difco) (37°C for 24 h), when needed with the appropriate antibiotics. Single loop transfers were prepared at two consecutive 18 to 24 h intervals. Overnight cultures were collected by centrifugation (12,000 rpm for 2 min)

Experiment procedure and protein extraction. Proteins were extracted from cells grown under normal conditions (in TSB, TSBR, or TSBN at 37°C) and from stressed cells. For the non stressed cells, cells were harvested from 10 ml overnight culture (approximately 10^{10} cells of *S.* Enteriditis or *S. sonnei*) and were washed twice in Li Chrosolv water. Proteins were extracted as shown in Table 8.1.

To stress the bacteria, cells were applied in 10 µl droplets (approximately 10^8 CFU) onto glass slips (9 per slip). 100 drops then were dried for 3 h under the biohazard hood with the fan running. To remove cells, slips were put into a 50 ml plastic tube containing 5 ml of 0.1% peptone water and approximately 15 glass beads (3 mm diameter, Fisher). Tubes were vortexed on high for 20 s and bacteria solutions were combined and centrifuged at 10,000 rpm for 2 min. Proteins were extracted from the resulting cell pellet as shown in Table 8.1.

Table 8.1: *Protein extraction procedure (buffers recipes see Appendix 4)*

Resuspend cell pellet in 100 µl tris buffer
Add 100 µl lysozyme solution
Incubate at 37°C for 30 min
Centrifuge at 7000 rpm for 3 min
Resuspend pellet in 200 µl SDS buffer
Ultrasonic treatment 3 x 30 s at 90% power
Heat at 95°C for 5 min
Cool to room temperature
Mix with 500 µl DDM lysis buffer
Vortex for 20 min on medium
Centrifuge at 14000 rpm for 30 min
Store supernatant at -80°C

Isoelectric focusing of protein extract. The extracted proteins were first separated by isoelectrical focusing on IPG strips (Amersham Biosciences, Uppsala, Sweden). Strips were swelled with a swelling solution (see Appendix 5) for approximately 12 h. To focus the proteins approximately 160 µl of the protein extract was loaded onto a swollen pH strip. Strips

of pH ranges of 4.0 to 5.0, 4.5 to 5.5, 5.0 to 6.0, and 5.5 to 6.7 were used. The program used to focus proteins is shown in the Appendix 7.

Acrylamide Gels. The strips with focused proteins were loaded onto an acrylamide gel and the gel was run in a buffer solution (Appendix 6) with the program shown in Appendix 7.

Staining of protein gels. Gels were either silver stained (modified from Blum et al., 1987) or stained with coomassie blue. Silver staining is the more sensitive method, but proteins can not be analyzed by MALDITOF after staining, therefore the coomassie blue method was used when proteins were sent for identification. The silver staining and coomassie blue staining method are described in Appendix 8 and 9, respectively. After staining, gels were vacuum packed in plastic bags and stored at 4°C.

Protein identification. Protein maps were analyzed and compared using "Image Master 2D" (Amersham Biosciences, Uppsala, Sweden). Differences were reported when seen in all three repetitions of experiment.

Spots were cut out of coomassie blue stained gels and peptides were sent for identification via MALDITOF analysis to the Central Lab of Protein Analytics, Institute of Cell Biology at the Ludwig-Maximilians-Universität of Munich.

Cell treatment to determine trehalose content and mRNA extraction. For trehalose and mRNA extraction, cell pellets of non-stressed and stressed cells were prepared as described above for the protein extraction.

Trehalose extraction. Cell pellet was suspended in 2 ml of 35% perchloric acid and was allowed to sit for 5 min. Afterwards, the solution was neutralized by adding 2.33 ml of 5 M KOH. Sample was centrifuged for 10 min at 4°C at 5000 rpm. Supernatant was removed and evaporated overnight. Sample was suspended in 500 µl Li Chrosolv water and frozen until analysis. The amount of trehalose was related to the protein content of the sample. To extract protein, samples were washed twice in TE-buffer (Appendix 4) and resuspended in 10 ml

digest buffer (Appendix 4). Samples were treated with an ultrasonic probe (Sonoplus UW 2070, Bandelin electronic, Berlin, Germany) 4 times for 30 s at 50% cycle and 80% power. Prepared extract (10 µl) was mixed with 1 ml protein assay dye reagant (Bio-Rad Laboratories, Hercules, CA). After 5 min of incubation the extinction was measured at 595 nm. Protein content was calculated with a calibration curve made with Albumin V (Merck, Darmstadt, Germany).

HPLC conditions for analysis of sugar extract. A Gynkotek system (Germering, Germany) consisting of a High Precision Pump Model 480 and a Gina 50 autosampler were employed. As columns an AminoPac PA10 analytical column (Dionex GmbH, Idstein, Germany) with an AminoPac PA10 Guard (Dionex) were used. The detector was an ED40 electrochemical detector (Dionex). Reagents used were 250 mM sodium hydroxide (Baker B.V., Denventer, Holland), 1 M sodium acetate (Merck, Darmstadt, Germany), and bi-distilled water. A flow rate of 0.25 ml/min was used. For the gradient see Appendix 3.

Isolation and purification of mRNA. The cell pellet was suspended in 1.5 ml Trizol reagent and transferred to two 1.25 ml microcentrifuge tubes containing 0.25 ml glass beads. Samples were sonicated in a sonicator bath for a total of 6 min with shaking 10 times by hand after each minute. Supernatant was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 12,000 g for 10 min at 4°C. Supernatant was transferred to a new microcentrifuge tube and 300 µl chloroform was added. Sample was shaken for 15 s and put on ice for 15 min. Sample was centrifuged at 12,000 g for 10 min at 4°C and the aqueous phase was transferred to a new tube and 750 µl chilled isopropyl alcohol was added to perticipate mRNA. Sample was put on ice for 10 min and afterwards centrifuged at 12,000 g for 10 min at 4°C. Supernatant was discarded and pellet washed with 1.5 ml chilled 75% ethanol. Sample was centrifuged at 12,000 g for 5 min at 4°C. Supernatant was discarded and pellet was allowed to air dry for 10 min. Pellet was resuspended in 100 µl DEPC treated water. Sample was frozen with liquid nitrogen and stored at –80°C over night. To purify

mRNA, to 3 µl of sample, 4 µl of Rnase-free Dnase buffer, 4 µl of Dnase, and 17 µl of Rnase-free water were added and incubated in a thermocycler for 30 min at 37°C. Afterwards, 4 µl of stop solution were added and again incubated for 10 min at 65°C.

cDNA rewrite. Random primer (1 µl) was added to 3 µl of purified mRNA. Sample was incubated at 70°C for 5 min. Afterwards 2 µl 5xRT buffer, 1 µl dNTP, 0.5 µl reverse transcriptase, and 3.5 µl Rnase-free water were added. Samples was incubated for 5 min at 25°C and then for 1 h at 42°C. Samples were stored at –80°C until use in the PCR reactions.

PCR reaction. A PCR with primers targeting the trehalose-6-phosphatase gene in *Shigella* was developed. To develop primers, genome sequences found in PubMed, a service of the National Library of Medicine (www.pubmed.gov), and primers were prepared. The primer sequences is shown Appendix 2. The PCR-Mix used is described in Table 8.2.

Table 8.2: *PCR-Mix (all chemicals were purchased at Qiagen (Hilden, Germany))*

10 X PCR buffer with 15 mM $MgCl_2$	2.5 µl
DNTP mix 10 mM (2.5 mM each)	0.8 µl
Primer (forward)	0.4 µl
Primer (reverse)	0.4 µl
PCR water	20.2 µl
Taq polymerase	0.2 µl
DNA extract	0.5 µl

PCR program. The PCR was run as follows: 1 cycle at 94°C for 15 min**,** 31 cycles (94°C 30 s, 50°C 30 s, 72°C 45 s), 1 cycle at 72°C for 10 min**,** hold at 4°C.

Determination of up regulated mRNA expression. cDNA was used to compare the concentration of mRNA copies in non-stressed and stressed *S. sonnei* cells. By comparing protein gels of stressed and non-stressed *Shigella* cells a house keeping gene, the alkyl hydroxyperoxide reductase subunit 22C was identified. Samples were run in a light cycler 2.0 (Roche, Switzerland) with the PCR program in Table 8.3.

Table 8.3: *RT-PCR-Mix (all chemicals were purchased at Qiagen (Hilden, Germany))*

10 X PCR buffer with 15 mM $MgCl_2$ (including polymerase and DNTPs)	2.5 µl
Primer (forward)	0.4 µl
Primer (reverse)	0.4 µl
cDNA	0.5 µl

RT-PCR program. The RT program consisted of four parts: Activation, at 95°C for 15 min. Quantification 45 cycles (95°C 15 s, 50°C 15 s, 72°C 20 s). Melting, heating to 95°C (0 s), cooling to 60°C (30 s), and increasing the temperature to 95°C with a rate of 0.1°C per s. Cooling at 40°C for 2 min.

RESULTS

Comparison of protein maps. When the rifampicin resistant *S. sonnei* strain and the parent strain were compared, up- and down- regulated protein spots were observed.

Protein maps of non-resistant desiccation stressed and nonstressed *S. sonnei* were compared using the Image Master 2D. A total of 12 up-regulated spots were identified in the stressed cells. These 12 protein spots were excised and sent for sequencing. The identified proteins were general stress response proteins (spot numbers 9 and 11), enzymes responsible for part of energy production (3, 6, and 8), enzymes responsible for part of trehalose production (10), and proteins of the outer membrane (1, 2, 5, and 12) (Table 8.4).

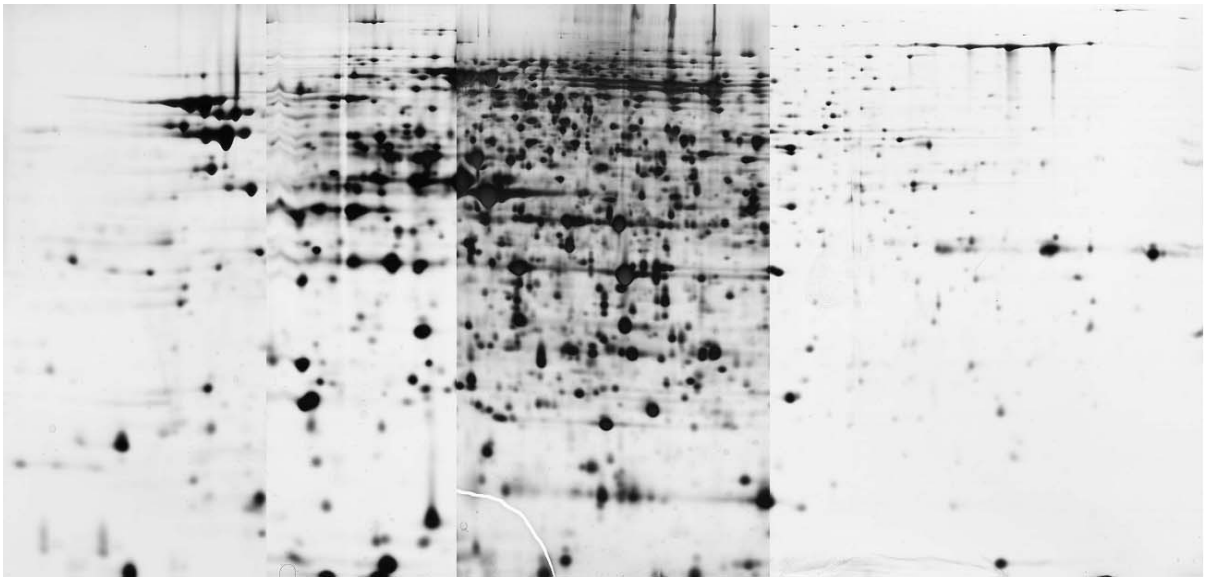

Figure 8.3: *Protein map of* S. sonnei *nonstressed, silver stained. pH range from the left to the right is 4.5 to 6.7. The protein spot mass is approximately 10 to 150 kDa from the bottom to the top.*

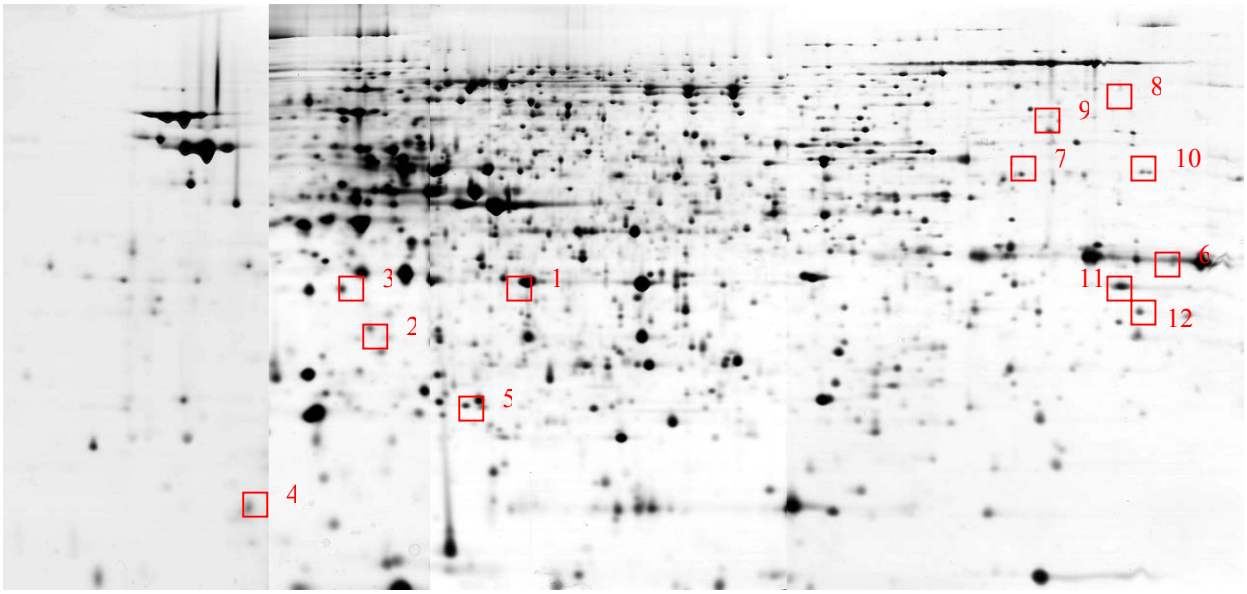

Figure 8.4: *Protein map of* S. sonnei *stressed, silver stained. pH range from the left to the right is 4.5 to 6.7. The protein spot mass is approximately 10 to 150 kDa from the bottom to the top. Up-regulated proteins are marked in boxes with numbers.*

Table 8.4: *Up-regulated proteins during desiccation identified for* S. sonnei *with their approximate mass and IEP*

Spot number		Mass (kDa)	IEP
1	Outer membrane protein 3a	34.4	5.4
2	Outer membrane protein A precursor	29.1	5.1
3	Transaldolase B	34.6	5.1
4	Not identifiable	10.6	4.9
5	Outer membrane protein Slp ECs4377	18.3	5.3
6	Glyceraldehyde-3-phosphate dehydrogenase A	35.8	6.5
7	Pyruvate kinase II	57.6	6.1
8	Nitrate reductase 1, beta subunit	67.9	6.4
9	Solution structure of Apo GroEL	66.9	6.2
10	Trehalose-6-phosphte synthase	53	6.4
11	CbpA	33.5	6.3
12	KDOP	31.9	6.4

As seen for *S. sonnei* up- and down- regulated protein spots were observed when the nalidixic acid resistant strain and the parent strain were compared.

After comparison of the protein maps of non-antibiotic resistant *S.* Enteriditis (Figure 8.5 and 8.6) with the Image Master 2D program, 6 up-regulated spots were identified and sent for sequencing. The identified proteins were a general stress response protein (spot number 4), enzymes associated with energy production pathways (1, and 6), and proteins of the outer membrane (2, and 3) (Table 8.5).

Figure 8.5: *Protein map of* S. *Enteriditis nonstressed, silver stained. pH range from the left to the right is 4.5 to 6.7. The protein spot mass is approximately 10 to 150 kDa from the bottom to the top.*

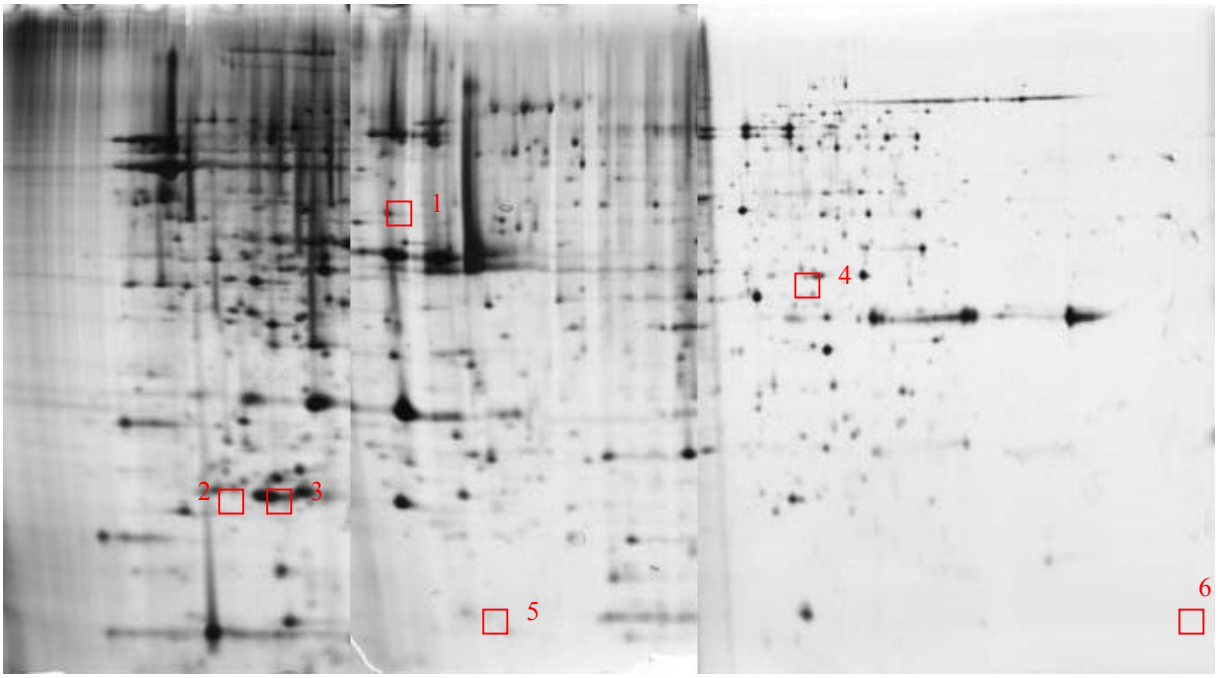

Figure 8.6: *Protein map of* S. *Enteriditis stressed, silver stained. pH range from the left to the right is 4.5 to 6.7. The protein spot mass is approximately 10 to 150 kDa from the bottom to the top. Up-regulated proteins are marked in boxes with numbers.*

Table 8.5: *Up-regulated proteins during desiccation identified for* S. *Enteriditis with their approximate mass and IEP*

Spot number		Mass (kDa)	IEP
1	Aldehyde dehydrogenase	60.4	5.3
2	Outer membrane protein	19.4	4.9
3	Putative periplasmatic protein	19.0	5.0
4	Fumarase C	54.1	6.0
5	Not identifiable	15.8	5.6
6	Glyceraldehyde-3-phosphate dehydrogenase A	15.4	6.7

Trehalose production during desiccation. To estimate the contribution of trehalose to the drying resistance, the trehalose amount in drying-stressed and non stressed cells of *S. sonnei* and *S.* Enteriditis. The retention time of trehalose was approximately 2.7 min (Figure 8.7). When the areas of the trehalose peak of the non-stressed *S. sonnei* was compared to the peak area of stressed *S. sonnei* an approximate 3.7-fold increase of the trehalose production, respectively, was observed (Table 8.6). The trehalose amount in *S.* Enteriditis cells also increased. However, no precise increase factor could be calculated, since the trehalose amount in non-stressed celss was below the detection limit (0.6 µM/mg protein) (Table 8.6). When calculated with the detection limit as trehalose amount for the non-stressed *Salmonella* cells the trehalose content was increased by a factor of 4.8.

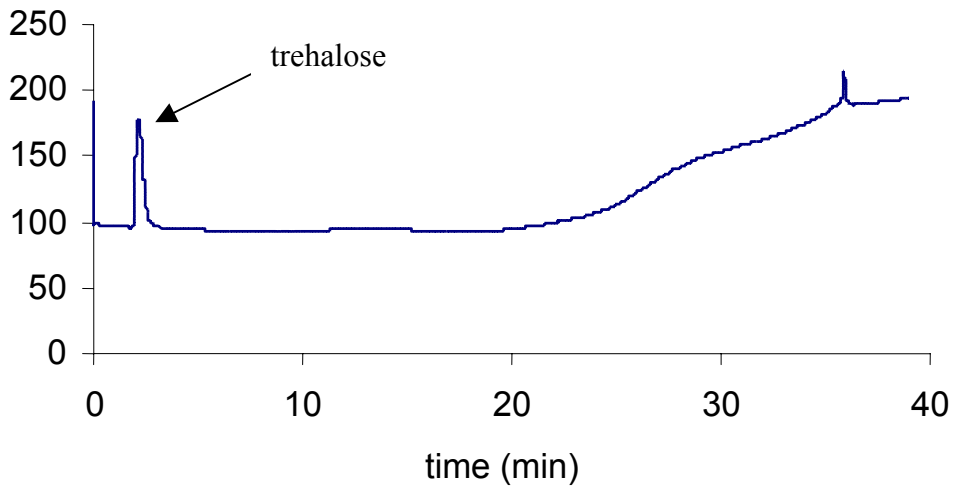

Figure 8.7: *HPLC diagram of trehalose*

Table 8.6: *Trehalose peak area and concentration of stressed cells, nonstressed cells, and trehalose standards*

	Stressed cells[a]	Non stressed cells[a]	0.1mM standard	0.01mM standard
Peak area (mV*min)				
Shigella	11.5	1.5	78.8	11.5
Salmonella	10.9	nd[b]	67.5	157.2
According protein amount per sample				
Shigella	5.2 mg	5.0 mg		
Salmonella	5.1 mg	5.0 mg		
Concentration (µM/mg protein)				
Shigella	10.0	2.7		
Salmonella	2.9	-		

[a]: Extracted from approximately 10^{10} cells
[b]: Below detection limit (0.6 µM/mg protein)

Comparing mRNA levels. The up-regulation of outer membrane proteins and KDOP led to the hypothesis that the virulence parameters would be up regulated during drying stress. Therefore, primers were designed to investigate by PCR analysis the expression of the virulence genes *InvE* and *VirF* of *S. sonnei*. Furthermore primers were designed for the *OtsAB* operon, as the genes responsible for trehalose production. Primers worked for the normal PCR, but not for the RT-PCR.

No significant indication for an up-regulation of the trehalose pathway or the virulence genes could be found, because the RT-PCR method could not be optimized in a way leading to any results.

DISCUSSION

Proteomics. The up-regulated proteins found for *S. sonnei* when stressed and non-stressed cells were compared can be separated into three different groups. The first is the up-regulation of general stress response proteins. General stress proteins are synthesized by cells under stress conditions regardless of the type of stress (Svensaeter et al., 2000). *GroEL* is mainly known in gram-negative bacteria such as *E. coli* and *Enterococcus faecalis* to be an

heat shock response (Bukau, 1993; Laport et al., 2004). *CbpA* in *E. coli* is a *dnaJ* homolog (Chae et al., 2004). It is also a *dnaK* co-chaperone, which is able to bind curved DNA (Ueguchi et al., 1995). *DnaK* is also mainly known as a heat shock protein (McCarthy et al., 1996).

In the second group are proteins responsible for energy production. Nitrate reductase mediates the use of nitrate as an electron acceptor in *E. coli* during anaerobic growth (Tseng et al., 1995; Ruiz-Herrera and De Moss, 1969). Glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase II play a major role in the glycolysis pathway to gain energy in form of ATP. Glycolysis is also directly involved in many biochemical adaptations of plant and nonplant species to environmental stresses such as nutrient limitation, osmotic stress, drought, cold/freezing, and anoxia (Plaxton, 1996).

The sigma factor σ^E is responsible for the membrane integrity during stress (Ades et al., 2003). The sigma factor σ^E was induced by the over expression of the outer membrane porin *ompC* (Ades et al., 1999). Furthermore the outer membrane protein A was one of the most damaged proteins in *E. coli* during oxidative stress (Tamarit et al., 1997). Therefore, the detected up-regulation of outer membrane proteins might be a step in the stress response to induce the sigma factor σ^E and to keep the number of intact outer membrane proteins at a constant level.

No literature could be found about an up-regulation of KDOP enzymes during any kind of stress. 3-Deoxy-D-*manno*-octulosonate-8-phosphate (KDOP) synthase catalyzes the production of KDOP from phosphoenolpyruvate and arabinose-5-phosphate. In gram-negative bacteria KDOP is subsequently dephosphorylated, cytidylylated, and linked to lipid A and is required for lipid A incorporation into the outer membrane (Raetz, 1990). But in combination of the up-regulation of various membrane proteins it might also be a part of an up-regulation of virulence properties to prolong survival during desiccation.

The third group consists of the trehalose-6-phosphate synthase. This is a strong indication that the production of trehalose is up-regulated during desiccation in *S. sonnei*. Trehalose production was directly related to desiccation stress (Breewer et al., 2003).

For *S.* Enteriditis fewer up-regulated proteins could be identified. But most proteins identified were similar to the proteins identified for *S. sonnei*. Fumarase is responsible for the conversion of fumarate to L-malate in the tricarbocylic acid cycle (Hill et al., 1971). Three fumarase genes are known for *E. coli*: *fumA*, *fumB*, and *fumC*. *Fum*C, up-regulated in drying *S.* Enteriditis, is synthesized optimally when iron is low or superoxide radicals are present in high concentrations (Park and Gunsalus, 1995). Also *fumC* and *fumA* were reported to be most abundant under aerobic conditions (Tseng, 1997). The up-regulation of a glyceraldehyde-3-phosphate dehydrogenase A was seen as in *S. sonnei*.

Furthermore, an up-regulation of an aldehyde dehydrogenase (ALDH) was observed. ALDHs are linked to oxidative stress, because by lipid peroxidation toxic aldehydes are formed (Esterbauer et al., 1991), and cells use ALDHs to oxidize the aldehydes to corresponding acids (Yoshida et al., 1998). By an overxpression of ALDHs in *Aradopsis thaliana* a better stress tolerance was observed (Sunkar et al., 2003).

As well were an up-regulation of an outer membrane protein and a putative periplasmatic protein observed, which again might be part of the up regulation of virulence parameters during desiccation as discussed above.

Trehalose. The accumulation of trehalose during desiccation was shown for translation into enzymes and the actual accumulation of the disaccharide. For *S. sonnei* an up-regulation of the protein spot intensity of trehalose-6-phosphate synthase could be observed. For *S. sonnei* and *S.* Enteriditis a HPLC measurable up-regulation of intracellular trehalose during desiccation was observed. The seen up-regulation is an average for all cells, dead and alive, at the point of extraction. Most of the dead cells will probably not have been able to accumulate trehalose, because as seen in Chapter Five, more than 90% percent of the

population have died after 20 min. Due to this fact the accumulation of trehalose in viable cells is likely to be more than 10-fold higher than values reported. Therefore, trehalose apparently plays an important role in the drying behavior of *S. sonnei* and *S.* Enteriditis. More research has to be done to prove for *S. sonnei* and *S.* Enteriditis that trehalose influences the survival during desiccation positively.

Chapter Nine

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Staining of gram-negative bacteria to determine the survival rate and changes of the bacterial membrane during desiccation

INTRODUCTION

The integrity of the bacterial membrane may play a crucial role in the survival of bacteria during drying stress. There are several staining methods that can be used to determine the integrity of the membrane.

Propidium iodine (PI) is a stain that is able to penetrate only the ruptured cell membrane because of its relatively large size of 668 Da. The stain can not permeate the membrane of intact cells and no fluorescent dye is formed. The stain reacts with the DNA and forms a flourescent molecule (http://www.probes.com/media/pis/mp07007.pdf). Therefore, staining with PI can determine if cells have an intact (no fluorescence) or ruptured (fluorescence) membrane, implying that the cells are viable or dead, respectively.

Styo 9 is able to stain both viable and dead cells green fluorescent, because of its relatively small size of ~10 Da. When used in combination with PI, only viable cells are stained green, whereas dead cells are stained red. This combination of stains was used to track physiological changes of *Salmonella typhimurium* under osmotic and starvation stress (Caro et al., 1999) and to enumerate injured *E. coli* cells (Braux et al., 1997).

1-*N*-Phenyl-naphtylamine (NPN) is a hydrophobic molecule, which is not able to permeate an intact cell membrane of a gram-negative bacterium. Once a cell membrane is damaged, NPN can enter the phospholipid layer of the membrane of a gram-negative bacterium. When in phospholipid environments, strong flourescence occurs (Träeuble and Overath, 1973). With the help of NPN, the permeabilization of gram-negative bacteria was assessed (Helander and Mattila-Sandholm, 2000) and the integrity of the outer cell membranes of *E. coli* during pressure treatment was determined (Gänzle and Vogel, 2001).

The flourocrome stain 33258 H (Hoechst) (2-[2-(4-hydroxy-phenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazole 3 HCl) stains the DNA of viable cells (Singh and Stephens, 1986). Hoechst was used for the detection of viable *Toxoplasma*

gondii (Borel et al., 1998) as well as for the detection of *Chlamydia trachomatis* (Grossgebauer and Rolly, 1982).

As mentioned in Chapter One and Seven, bacteria must maintain their membranes in a liquid-crystalline state (Chapter One, Figure 1.2) to ensure biological activity (Denich et al., 2002). Temperature plays an important role for the fluidity of the membrane. Bacteria grown in a cold environment have a relatively higher amount of unsaturated, short-chain fatty acids in the membrane, whereas more saturated and long-chain fatty acids are incorporated into the membrane at or above optimum growth temperatures (Denich et al., 2002).

The objectives of this study were to determine membrane integrity as a function of desiccation. Therefore, stains that would accurately follow the death rate during desiccation in combination with membrane damage were investigated. Furthermore, the change in fatty acid composition when cells are grown at different temperatures, and the impact of the drying process on the fatty acid composition of the cell membranes of *S. sonnei* and *S.* Enteriditis were investigated.

MATERIAL AND METHODS

Test strains and media preparation. A human isolate of *Shigella sonnei* was obtained from Dr. Trevor Suslow, UC Davis. *Salmonella enterica* serovar Enteriditis Phage Type 30 was isolated from raw almonds associated with an outbreak of salmonellosis (Isaacs et al., 2005).

Preparation of inoculum. Inoculum was prepared as described in Chapter Three. Briefly, before each experiment, a sample from a frozen stock culture was grown on TSA and an isolated colony was subcultured in tryptose soy broth (Difco) (37°C for 24 h). Single loop transfers were prepared at two consecutive 18 to 24 h intervals. Overnight cultures were collected by centrifugation and suspended MilliQ water. Cells were washed twice in MilliQ water to ensure complete removal of the growth medium. For fatty acid analysis, the resulting

pellet was used. Appropriate dilutions were made in MilliQ water and transferred to the microtiter plate.

To produce non-viable populations, cells were washed twice in MilliQ water and pasteurized for 5 min in a water bath at 80ºC. After heating, cells were washed an additional time in MilliQ water. Pasteurized cells were plated on TSA to confirm that viable cells were below the detection limit (2.1 log CFU).

When the influence of a lower or higher growth temperature on the fatty acid composition of the cell membrane was determined, a culture from a TSA plate of *S. sonnei* and *S.* Enteriditis cells was transferred to TSB and incubated in a waterbath at 13°C and 45°C. Cells required 4 days to reach stationary phase for 13°C and 8 h for 45°C (Chapter Seven).

Cultures were collected by centrifugation (12,000 rpm for 2 min) and suspended in an equal volume of MilliQ water. Cells were washed two times in MilliQ water to ensure complete removal of the growth medium and cells were adjusted to the same optical density (OD = 0.8) to achieve a similar initial inoculum. The resulting pellet was also used for fatty acid analysis.

Inoculation of microtiterplates. Approximately 10^8 CFU of inoculum (10 µl) was spot-inoculated into a 96 well microtiter plate well (Sarstedt Inc., Newton, NC). An inoculum of 10^6 CFU did not provide enough fourescence to be detected by the fluorimeter. Microtiterplates were stored under a biohazard hood with the fan running at room temperature (28 ± 2°C).

Propidium Iodine and Styo 9. Propidium idodine (PI) and Styo 9 (LIVE/DEAD® BacLight™, Molecular Probes, Eugene, Oregon) were used at a final concentration of 30 µM and 6 µM, respectively, dissolved in MilliQ water. After 200 µl of staining solution was mixed with cells, the mixture was allowed to react for 15 min in the dark. Fluorescence

readings were done with a HP (Hewlett Packard Company, Huston, TX) flourimeter at 485 nm excitation and 635 nm emission.

***N*-Phenyl-1-naphtylamine (NPN).** NPN (Sigma, St. Louis, MO) was used according to Helander and Mattila-Sandholm (2000). Briefly, a 10 mmol/l stock solution in ethanol was diluted to a concentration of 20 µmol/l with imidazol buffer (pH = 7.4) (Merck, Darmstadt, Germany). Cells were mixed with 200 µl of staining solution and fluorescence readings were immediately taken with a spectra fluor (TECAN, Crailsheim, Germany) flourimeter at 340 nm excitation and 420 nm emission.

Hoechst 33342. Hoechst 33342 (Molecular Probes, Leiden, Holland) was used at a concentration of 10 mg/ml dissolved in Li Chrosolv water (Merck, Darmstadt, Germany). Cells were mixed with 200 µl of staining solution and fluorescence readings were immediately taken with a spectra fluor (TECAN, Crailsheim, Germany) flourimeter at 340 nm excitation and 420 nm emission.

Fatty acid analysis. Fatty acid extraction and analysis was performed accordingly to Watkins et al. (2001). *S. sonnei* and *S.* Enteriditis cells were washed in MilliQ water. To determine the fatty acid composition of a non-dried droplet, fatty acids were extracted from approximately 10^{10} CFU. To determine the fatty acid composition of a dried droplet, 10 µl of washed bacteria solution (10^8 CFU) were inoculated onto a glass slip and 100 single droplets were dried for 3 h. After drying, slips were put directly into Folch reagent (chloroform – methanol mixture (1:1, vol:vol)) to prevent any further changes in the fatty acid composition and the fatty acids were extracted.

Fatty acids were extracted by the following procedure: 6.0 ml Folch reagent was added to washed or harvested cells in 50 ml Falcon-tubes (Benton Dickinson and Company, Franklin Lakes, NJ). The mixture was vortexed for 30 s at high speed and was allowed to sit for 15 min. KCl (1.8 ml, 0.01 M) was added and mixture was vortexed. The tube was centrifuged in a Allegra 6 Centrifuge (Beckman Coulter, Fullerton, CA) at 3,000 rpm for 5 min. Using a 5

ml pipette (Fisher Scientific, Pittsburgh, PA), the bottom organic phase of the sample was removed and placed in 4 ml amber storage vials (Fisher Scientific, Pittsburgh, PA). A 300 µl sample was transferred to a 1.5 ml GC vial.

Silica Gel 60 thin-layer chromatography (TLC) plate (10 x 20 cm) (Merck, Darmstadt, Germany) impregnated with 1 ml EDTA, pH 5.5 (Sigma, St. Louis, MO), and washed by ascending development. Just before use, plates were activated by heating at 110°C for 10 min and allowed to cool to room temperature. Sample extracts were dried under nitrogen and spotted onto EDTA-impregnated TLC plates. Two TLC standard lanes consisting of authentic PC and PE were spotted on the outside lanes of the TLC plate as reference samples. The mobile phase employed for the separation of PL classes consisted of chloroform/methanol/acetic acid/water (100:67:7:4, by vol.). All chromatography solvents were obtained from Fisher (Pittsburgh, PA).

Individual PL classes were identified by comparison with the authentic standards chromatographed in the reference lanes. Reference samples were visualized by cutting the reference lanes from the plate, dipping them in 10% cupric sulfate/8% phosphoric acid, and charring them at 200°C. The reference lanes were then compared to the uncharred plate to determine the location of individual lipid classes in the sample.

Each lipid fraction was scraped from the plate and methylated by incubating the silica in 3 N methanolic-HCL in sealed vials under a nitrogen atmosphere at 100°C for 45 min, followed by a neutralization step with K_2CO_3.

Fatty acid methyl esters were extracted with hexane containing 0.05% butylated hydroxytoluene (Sigma) and prepared for gas chromatography by sealing the hexane extracts under nitrogen. Fatty acid methyl ester standards were obtained from Nu-Chek-Prep (Elysian, MN). Fatty acid methyl esters were separated and quantified by capillary gas chromatography using a Hewlett-Packard (Wilmington, DE) gas chromatograph (model 6890) equipped with a 30 m 225 ms capillary column (Agilent, Folsom, CA), a flame-ionization detector, and

Hewlett-Packard ChemStation software. Fatty acid methyl ester standards were obtained from Nu-Chek-Prep (Elysian, MN).

RESULTS

***S. sonnei* cells grown at 13 and 37°C stained with Styo 9 during desiccation.** The number of viable cells was followed quantitatively at 13 and 37˚C during desiccation by staining with Styo 9. Unexpectedly the RFU for 100% viable cells at 37˚C was double that at 13˚C, whereas the RFU for 100% dead cells at 37˚C was only half that of at 13˚C. Also unexpected was that the RFU for 100% viable cells was lower than the RFU for the initial inoculum at 37˚C and the RFU for 100% dead cells was higher than the RFU for cells at the end of the drying period (Figure 9.1). When *S. sonnei* cells were grown at 13˚C initial RFU were the same as for 100% viable cells and dropped to the RFU for 100% dead cells after 40 min of drying (Figure 9.2).

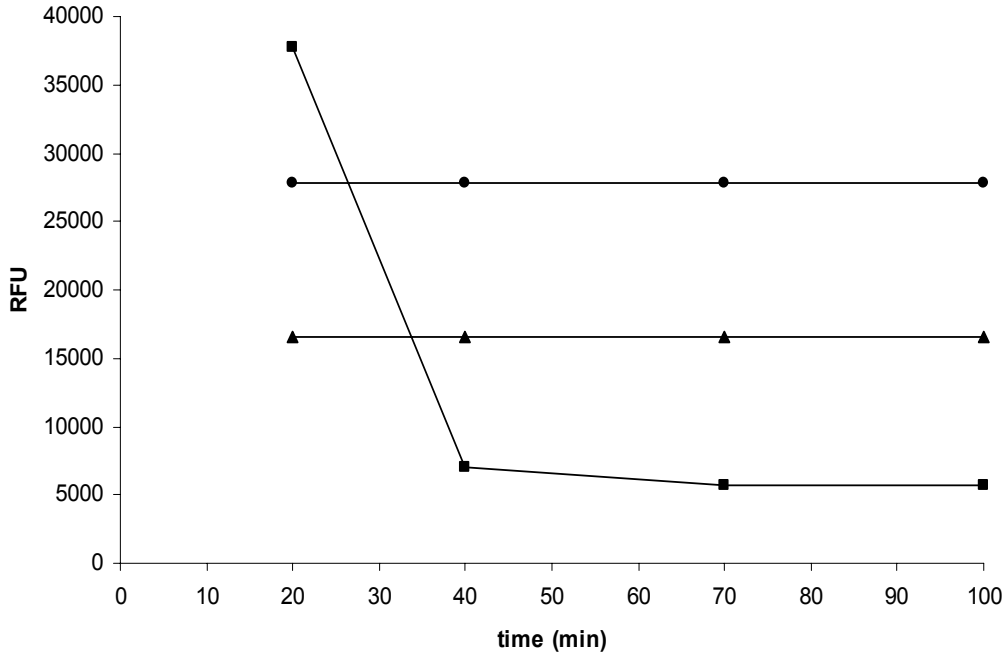

Figure 9.1: *Relative fluorescence units (RFU) measured during drying of* S. sonnei*, stained with Styo 9. Pasteurized cells (100% dead, triangles), fresh undried culture grown at 37˚C (100% viable, circles), and culture grown at 37˚C and dried in microtiter plate wells (squares).*

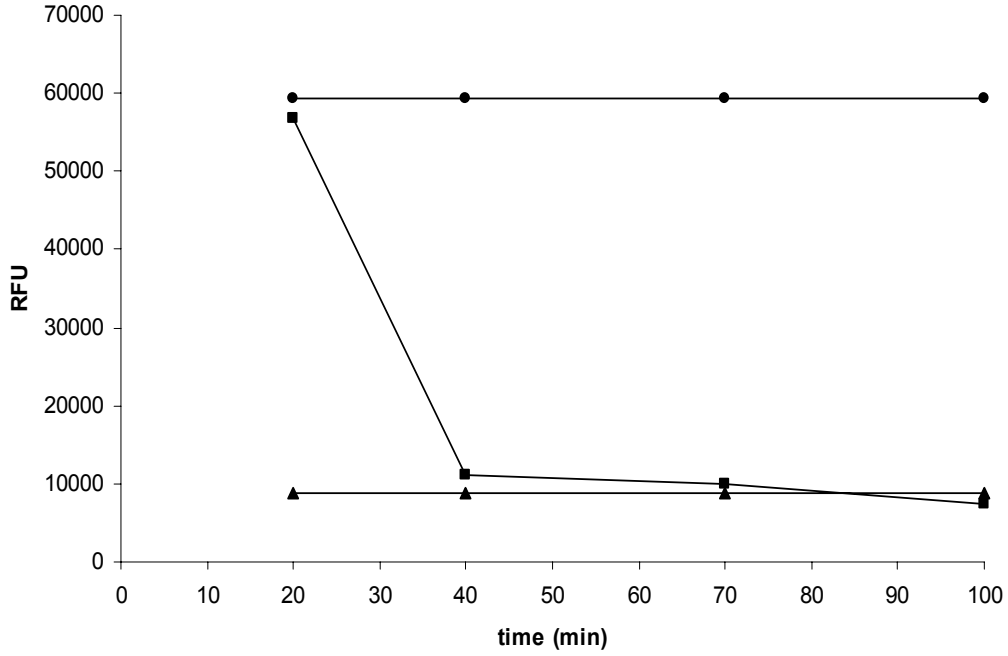

Figure 9.2: *Relative fluorescence units (RFU) measured during drying of* S. sonnei*, stained with Styo 9. Pasteurized cells (100% dead, triangles), fresh undried culture grown at 37˚C (100% viable, circles), and culture grown at 13˚C and dried in microtiter plate wells (squares).*

***S. sonnei* cells grown at 13 and 37°C stained with PI during desiccation.** When the number of dead cells was followed quantitatively during desiccation by staining with PI, the RFU for 100% viable cells was the same as the RFU for the initial inoculum when cells were grown at 37˚C. At the end of the drying period the RFU of dead cells was below the RFU for 100% dead cells (Figure 9.3). When *S. sonnei* cells were grown at 13˚C initial RFU were similar to those for 100% viable cells, but did not increase to indicate to presence of dead cells (Figure 9.4).

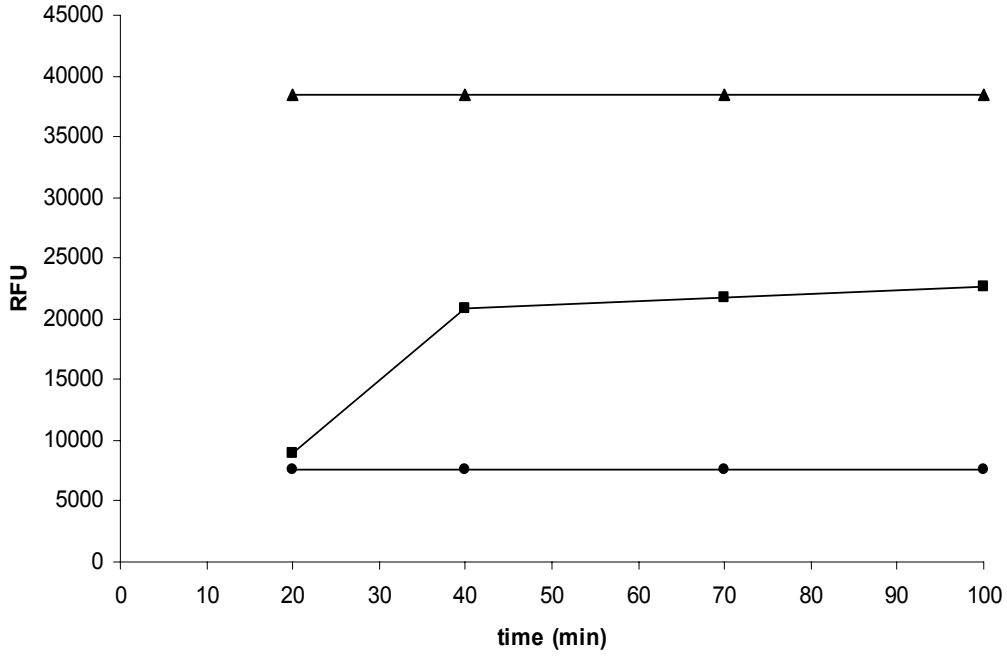

Figure 9.3: *Relative fluorescence units (RFU) measured during drying of* S. sonnei*, stained with PI. Pasteurized cells (100% dead, triangles), fresh undried culture grown at 37˚C (100% viable, circles), and culture grown at 37˚C and dried in microtiter plate wells (squares).*

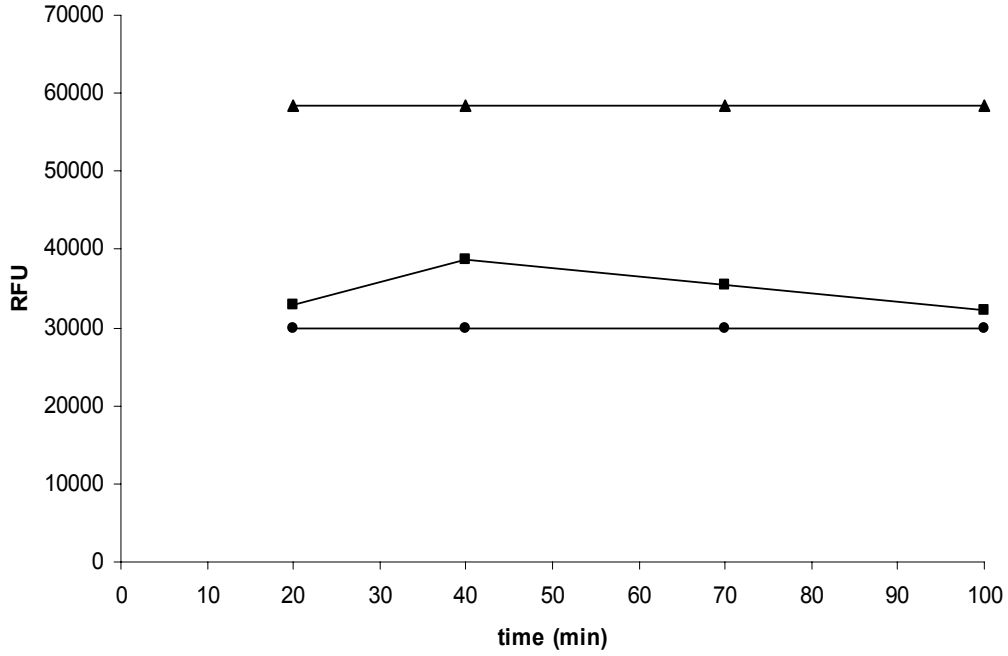

Figure 9.4: *Relative fluorescence units (RFU) measured during drying of* S. sonnei*, stained with PI. Pasteurized cells (100% dead, triangles), fresh undried culture grown at 37˚C (100% viable, circles), and culture grown at 13˚C and dried in microtiter plate wells (squares).*

***S.* Enteriditis cells grown at 13 and 37°C stained with Styo 9 and PI during desiccation.** The results for *S.* Enteriditis cells grown at 13 and 37˚C could be included in one graph, because the RFU values for 100 % viable and 100% dead cells were the same at both temperatures (Figure 9.5).

When the number of viable cells was followed quantitatively for both growth temperatures during desiccation by staining with Styo 9, the RFU for 100% viable cells was lower than the RFU for the initial inoculum and the RFU for 100% dead cells was higher than RFU for cells at the end of the drying period (Figure 9.5).

When cells grown at 37˚C were stained with PI, the initial RFU was the same as 100% viable cells. For cells grown at 13˚C, however, the RFU was well above 100% viable cells indicating the presence of dead cells (Figure 9.6).

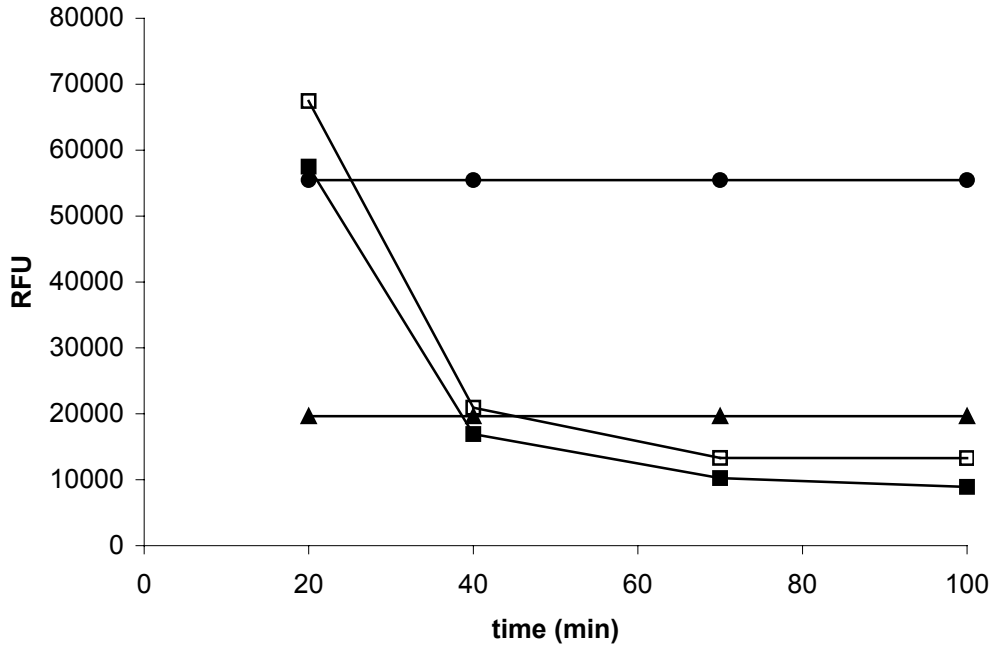

Figure 9.5: *Relative fluorescence units (RFU) measured during drying of* S. *Enteriditis, stained with Styo 9. Pasteurized cells (100% dead, triangles), fresh undried culture grown at 37˚C (100% viable, circles), and culture grown at 37˚C (closed squares) or 13˚C (open squares) and dried in microtiter plate wells.*

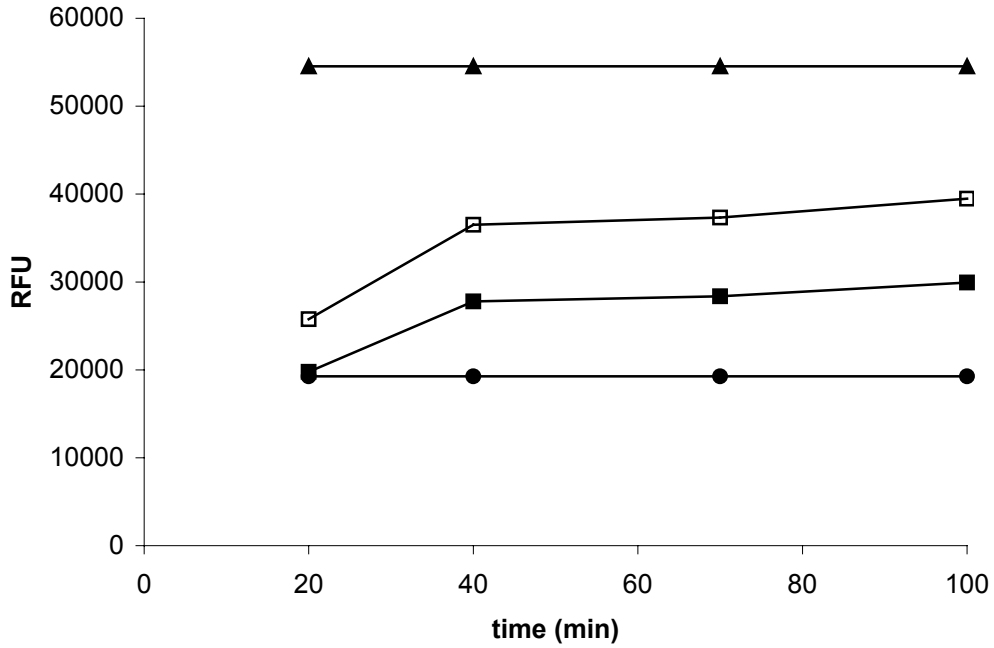

Figure 9.6: *Relative fluorescence units (RFU) measured during drying of* S. *Enteriditis, stained with PI. Pasteurized cells (100% dead, triangles), fresh undried culture grown at 37˚C (100% viable, circles), and culture grown at 37˚C (closed squares) or 13˚C (open squares) and dried in microtiter plate wells.*

***S. sonnei* cells grown at 37°C stained with NPN during desiccation.** When cells were grown at 37˚C and stained with NPN the initial RFU reading was the same as 100% viable cells, but no increase in RFU was observed after 20 to 30 min, which would indicate a damaged and permeable membrane (Figure 9.7).

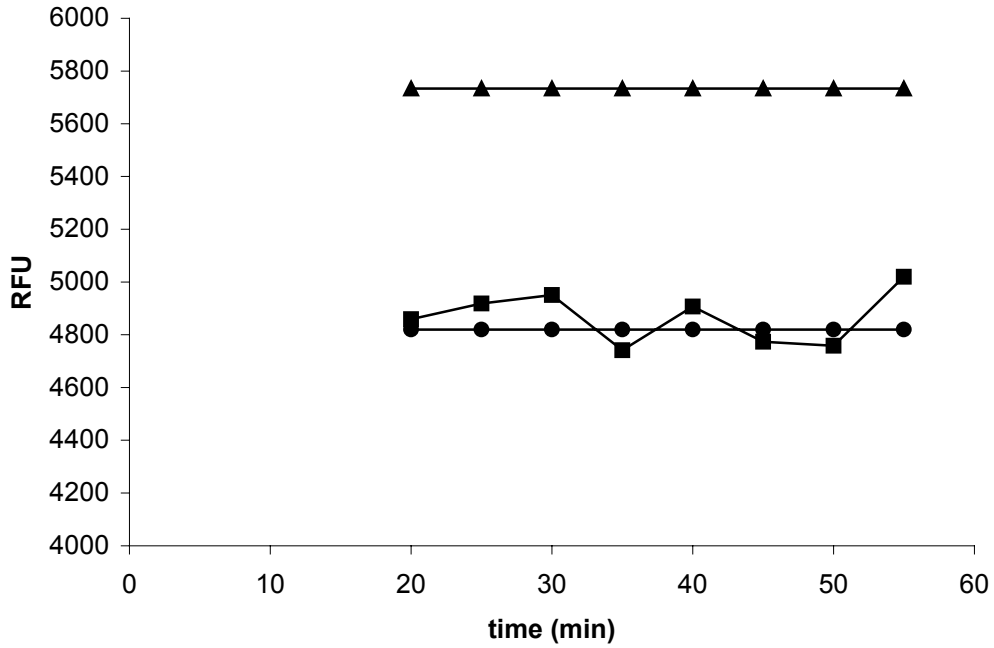

Figure 9.7: *Relative fluorescence units (RFU) measured during drying of* S. sonnei*, stained with NPN. Pasteurized cells (100% dead, triangles), fresh undried culture grown at 37˚C (100% viable, circles), and culture grown at 37˚C and dried in microtiter plate wells (squares).*

***S. sonnei* cells grown at 37°C stained with Hoechst during desiccation.** When cells were grown at 37˚C and stained with Hoechst, the initial RFU was the same as 100% viable cells, rising as soon as the droplet dried, indicating the presence of dead cells (Figure 9.8).

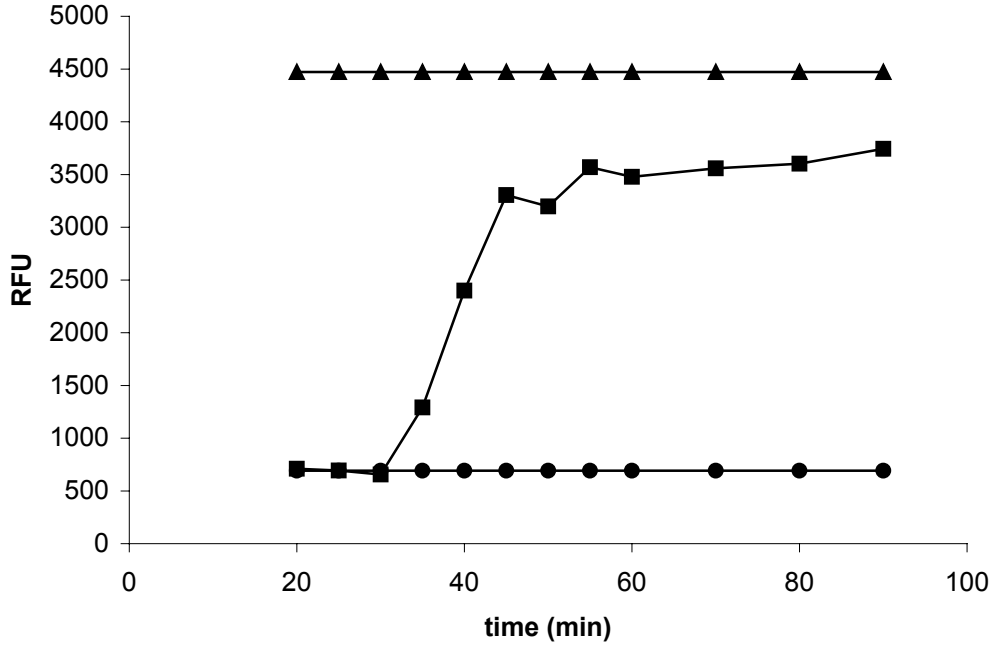

Figure 9.8: *Relative fluorescence units (RFU) measured during drying of* S. sonnei*, stained with Hoechst. Pasteurized cells (100% dead, triangles), fresh undried culture grown at 37˚C (100% viable, circles), and culture grown at 37˚C and dried in microtiter plate wells (squares).*

Membrane fatty acid composition. When the fatty acid composition of stressed and non-stressed *S. sonnei* and *S.* Enteriditis cells was compared no major change of the evaluated fatty acids could be observed. However, a slight increase of the unsaturated 15:1 fatty acid going along with a slight decrease of the 18:0 fatty acid was observed for both bacteria.

When the fatty acids of *Salmonella* grown at 13, 37, and 45°C was compared, no difference in the composition was observed (Table 9.1).

When the fatty acids of *Shigella* grown at 37 and 45°C was compared, a shift from the short chained unsaturated fatty acid 15:1 towards the saturated fatty acids 16:0 and 18:0 was observed (Table 9.1). This is opposite of the behavior observed during drying stress.

Table 9.1: *Fatty acid composition in percent of non-stressed and stressed* S. sonnei *and* S. *Enteriditis grown at 13, 37, and 45°C. The total amount of all shown fatty acids was taken as 100% and single fatty acids were calculated accordingly.*

	non-stressed 37°C	stressed 37°C	non-stressed 13°C	non-stressed 45°C
Salmonella				
14:0	3	3	3	3
15:1	21	25	21	22
16:0	24	24	25	24
18:0	52	48	51	51
Shigella				
14:0	3	3	nd[a]	4
15:1	22	24	nd	16
16:0	24	24	nd	27
18:0	51	49	nd	53

[a]: not done

When investigating the GC chromatograms of the fatty acid samples two peaks with a retention time of approximately 18.9 and 21.4 min increased when *S. sonnei* and *S.* Enteriditis were drying-stressed (Fig 9.9 and Fig. 9.10). With the standards used the peaks could not be identified.

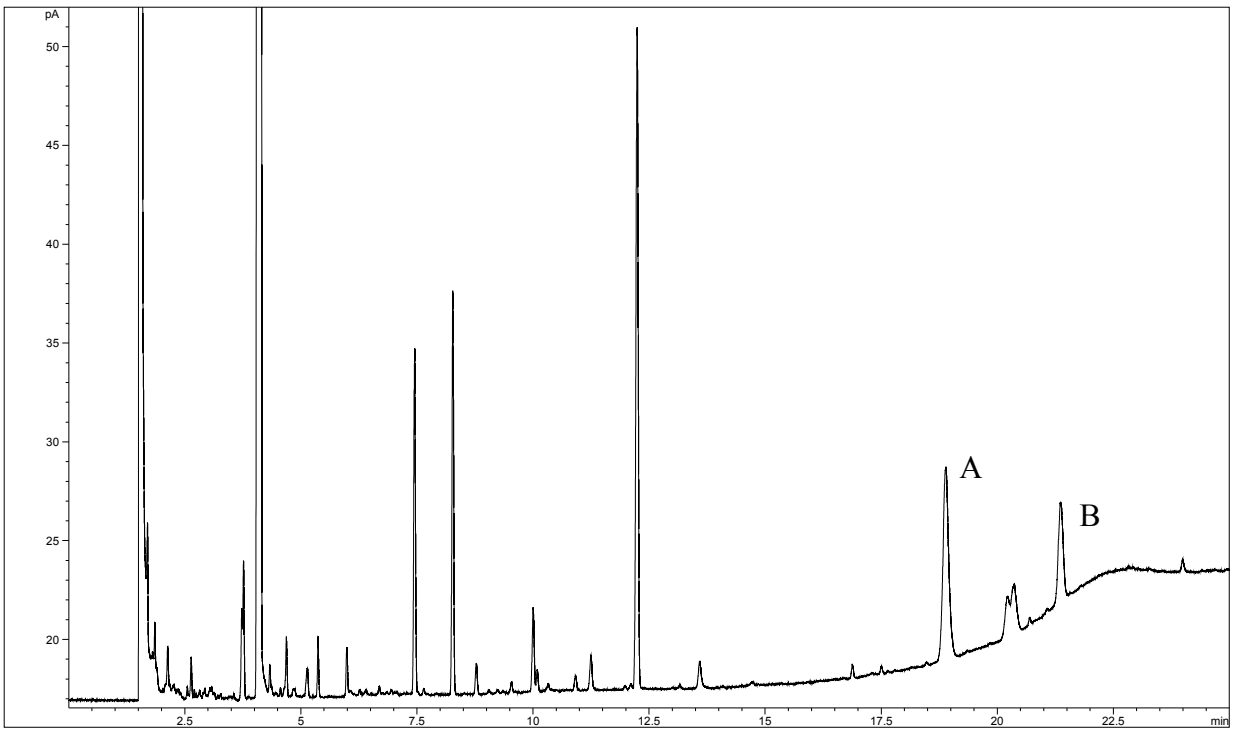

Figure 9.9: *GC chromatogram of a non-stressed* S. *Enteriditis sample. The two unidentified peaks are marked with A and B.*

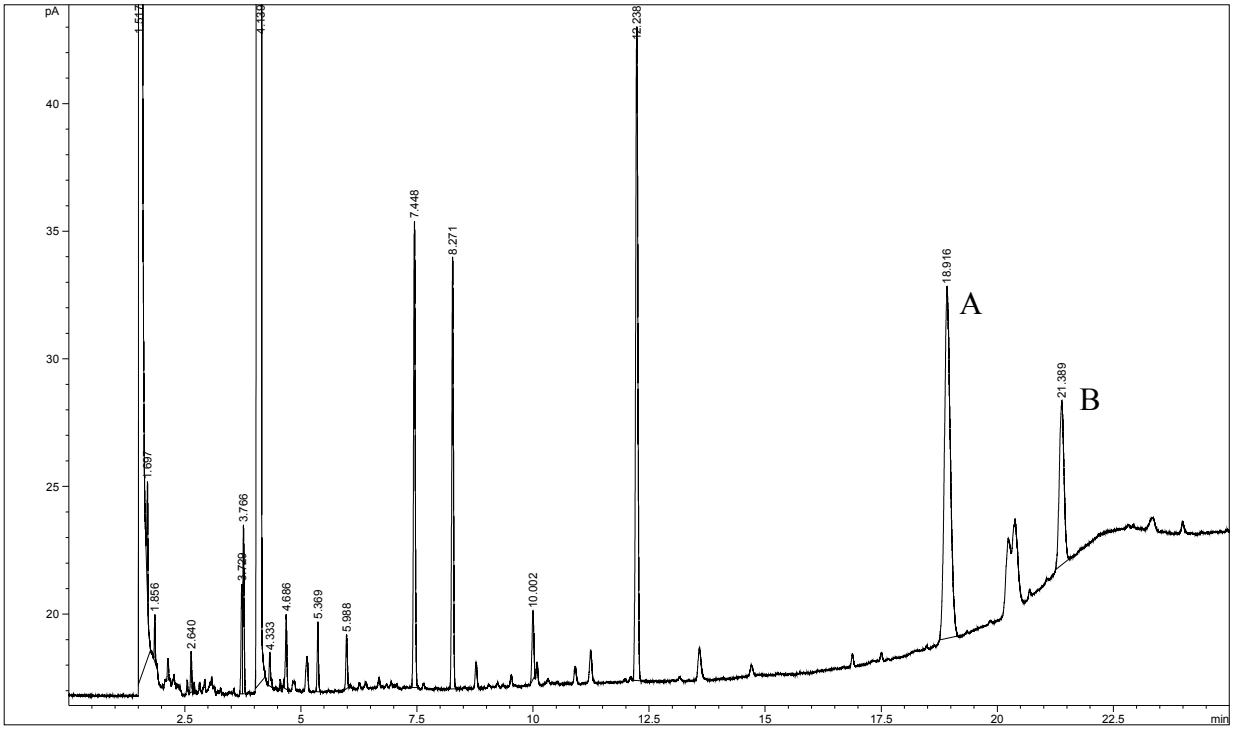

Figure 9.10: *GC chromatogram of a drying-stressed* S. *Enteriditis sample. The two unidentified peaks are marked with A and B.*

DISCUSSION

Cell staining. When *S. sonnei* and *S.* Enteriditis cells grown at 13 and 37˚C were stained with Styo 9 and the decrease of viable cells during the drying process was followed, results did not correlate with plating determinations from Chapter Five (Table 9.3). The RFU values at 40, 70, and 90 min indicated either an almost complete elimination of viable cells, or even indicated the presence of more than 100% dead cells. This stain did not provide data that complemented plating for *S. sonnei* and *S.* Enteriditis during desiccation.

When cells were stained with PI, the percentage of dead cells calculated for *S. sonnei* was 92 and 50% for cells grown at 13 and 37˚C, respectively, at the end of the drying period. The percentage of dead cells for *S.* Enteriditis was 43 and 69% for 13 and 37˚C, respectively, at the end of the drying period. The data generated by plating (Chapter Five) provided survival rates of 15 and 13% for *S. sonnei* and *S.* Enteriditis, respectively, at 37˚C at the end of the drying period (see Table 9.3).

With the use of NPN, the decrease of viable cells during desiccation could not be followed. The reason might be that the stain is not able to lock into the membrane once it is dried.

When the Hoechst stain was used, the results were very similar to the plating method. When stained and fluorescence was measured, a survival rate of 18% after the drying period for *S. sonnei* was calculated, whereas the calculated survival rate of the plating method was 15% (Table 9.3).

Table 9.3: *Percentage of dead and viable* Shigella *and* Salmonella *at 20 min and 90 min of the drying period, for bacteria grown at 37˚C.Values of >100% were calculated when values of samples were higher than samples values for 100% dead or viable cells.*

		Start of drying period		End of drying period	
		Viable	Dead	Viable	Dead
Salmonella					
	Plating	100	0	13	87
	PI	100	0	69	31
	Styo 9	106	-	-	132
Shigella					
	Plating	100	0	15	85
	Hoechst	100	0	18	82
	NPN	98	2	80	20
	PI	95	5	50	50
	Styo 9	189	-	-	194

Membrane fatty acid composition. For *Shigella* and *Salmonella* very similar fatty acid profiles were observed. However, Tomlins et al. (1982) reported a deviant fatty acid composition for *S. typhimuriium*. In that study a much lower proportion of 18:0 fatty acid and a higher proportion of 12:0, 16:1, and 18:1 fatty acids was observed.

When *S. sonnei* was grown at supra-optimal temperatures longer and saturated fatty acid (18:0) amounts increased, whereas short-chained and unsaturated fatty acids (15:1) decreased. Adams and Russel (1992) observed that the higher the temperature *V. costicola* was grown in, the higher the amount of long-chained and saturated fatty acids incorporated into the membrane.

As shown in these experiments, *S. sonnei* and *S.* Enteriditis can slightly change their cellular fatty acid composition during a drying period of 3 h. As discussed for trehalose in chapter eight, the absolute number of the increase is probably higher than that measured due to the death of bacteria at the beginning of the drying period. These dead cells could not change their fatty acid composition. The amount of the unsaturated 15:1 fatty acid increased while 18:0 fatty acid decreased by a similar amount.

Chapter Ten

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Conclusion and possible future work

Conclusion

In this study a detailed picture of various aspects involved in the drying of bacteria suspended in a droplet were shown.

The Hoechst stain was useful for following the decrease of viable cells during desiccation as results were comparable with plate counts. It is possible that this method could be used instead of the more time and material consuming plating method, where appropriate.

Shigella, *Salmonella*, *Escherichia coli* O157:H7, and lactobacilli were very similar in their behavior when population effects during desiccation were studied. It was observed that cells generally survive significantly better at higher densities (Table 10.1). However, a significant difference was observed between the three gram-negative bacteria and gram-positive lactobacilli. The lactobacilli showed a significant better survival rate than *Salmonella*, *Shigella*, and *E. coli* O157:H7 when inoculated at densities of approximately 10^6 and 10^8 CFU per slip.

Table 10.1: *Approximate declines for* Shigella*,* Salmonella*,* E. coli *O157:H7, and lactobacilli in log (CFU) inoculated onto slips with MilliQ water as carrier (n=6)*

Inoculum		Decrease after 90 min
10^8 CFU		
	S. sonnei	1.0
	S. Enteriditis	1.0
	E. coli O157:H7	1.6
	L. sanfranciscensis	0.1
10^6 CFU		
	S. sonnei	3.2
	S. Enteriditis	2.9
	E. coli O157:H7	3.4
	L. sanfranciscensis	0.3
	L. reuteri	0.5
10^5 CFU		
	L. sanfranciscensis	1.9
	L. reuteri	1.7

The carrier medium had a significant influence on the survival during drying. The higher the organic load, the better the observed survival. While survival-enhancing mechanisms are desirable for starter cultures as lactobacilli, they are undesirable with pathogenic bacteria. Conditions that favor high cell density and high organic load should be avoided with pathogenic bacteria.

The growth temperature of *Shigella* and *Salmonella* also had a significant impact on survival during desiccation. When cells were grown at their optimal growth temperature of 37°C survival was better than when grown at sub- and supra-optimal growth temperatures. That implies that cold or heat pre-stress does not lead to a better desiccation resistance. Growth at non-optimal temperatures might have imposed further stress on the cells so that they could not react to the drying stress with all their resources. For the food industry attempting to keep minimum temperatures during storage, handling, and production could minimize the risk of long term survival when cells become desiccated.

Shigella survived better when inoculated onto the surface of a strawberry when compared to glass, steel, and parafilm surfaces. Availability of nutrients, a higher humidity and possibly quorum sensing effects may be an explanation for this observation. Stainless steel, commonly used in food processing environments and glass surfaces supported equallly the survival of gram-negative bacteria. Considering the widespread use of plastic surfaces in the food industry, survival thereon should also be investigated. Also an altered hydrophobicity (parafilm) did not have an influence on the survival rate. Altogether contamination on organic surfaces as strawberries is of greater concern compared to contamination on non-organic surfaces, because of the better survival. However, other organic surfaces can show different survival patterns due to the variability from smooth and waxy to highly complex and rough, and from dry to relatively moist (Chapter Five).

Differences at the proteom level were observed between antibiotic resistant and non-resistant strains, as well as between desiccation-stressed and non-stressed cells. The proteins

up-regulated during drying were identified as general stress proteins, membrane proteins, and proteins of energy pathways. The up-regulation of membrane proteins are indicative of an up-regulation of virulence parameters during drying stress, which might explain why as few as 10 cells of *Shigella* and *Salmonella* are thought to be sufficent to cause illness.

The composition of the membrane fatty acids is changing during desiccation. The change is comparable to that of cold-stressed bacteria, when more unsaturated fatty acids are incorporated into the membrane.

Trehalose accumulated within drying stressed *Shigella* and *Salmonella* cells in a short time period of 3 h. As discussed earlier, the protection effect on the membrane and proteins might be a reason for this increased production. Further experiments should be done to demonstrate that trehalose enhances survival during desiccation, e.g., with the help of a trehalose-negative mutant.

Less than 10% of a *Shigella* or *Salmonella* population survive the first 1.5 h of drying stress. Therefore, only few cells manage to produce trehalose in an adequate amount to ensure survival. Cells are known to produce trehalose as they enter the stationary phase in the absence of other stress factors (Strom and Kaasen, 1993). In this study cells were always harvested from the stationary phase, therefore, a part of the population might have been able to initiate the production of trehalose in an amount that ensures the ability to further up-regulate the trehalose production and thus enhance survival.

Changes of the intracellular trehalose level and the change of the fatty acids within the membrane, which was seen for a part of the dried cell population, presumeably the surving part, might help cells to reach a dormitory state, enabling the cells to survive without nutrition and the presence of moisture. This could explain why *Salmonella* Enteriditis has been isolated in orchard soil for over 4 years (Harris, personal communication).

All described tools and methods improve the understanding and influences of the survival of bacteria - pathogenic bacteria as well as starter cultures - in a suspension during

drying. But many more aspects are involved in the drying process which could not be investigated within this work. Therefore, the following topics for future research are proposed.

Possible future work

Based on the results of this study a mathematical model of the survival during desiccation could be calculated, including different parameters such as cell density and carrier medium.

The study showed an influence of an antibiotic resistance on the survival on *Shigella* and *Salmonella*, but the ways and methods of this influence still have to be determined.

Survival of pathogenic bacteria on plastic surfaces, e.g. polypropylene, should be investigated in comparison to inert surfaces to be able to make a risk assessment for surfaces most often used in the food industry.

Of additional interest is whether pre-stressed cells will be more resistant to drying, since growth in cold and hot environments decreased the survival rate during drying. Further possible pre-stresses that could be tested are: osmotic, acid, pressure, and cold and heat shock.

Identification the up- and down-regulated protein spots when comparing the rifampicin resistant and non-resistant strain of *S. sonnei*.

Work with RT-PCR could yield valuable results for the up- and down-regulation of proteins and genes during desiccation, possibly confirming the proposed path-ways.

Further work is under way to determine if virulence parameters are expressed during desiccation and if a particular temperature during drying is necessary to express those parameters.

It is also important to ensure the identity of the fatty acid peaks and identify the peaks via mass spectrometry which were seen on the GC chromatograms in Chapter Nine and increased in size during desiccation.

Phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) are the main components of the cell membrane of *E. coli* with 20 – 25% and 70 – 80%, respectively

(Dowhan, 1997). An influence of different growth temperatures on the ratio of PE to PG was shown in membranes of *Vibrio costicola* (Adams and Russel, 1992). Therefore, a possible change of this ratio during desiccation should be investigated including the interpretation of such data.

Chapter Eleven

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Chapter Twelve

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Appendix

Appendix 1 - Media recipes

Tryptic soy agar (TSA)	
Pancreatic digest of casein	17.0 g
Enzymatic digest of soybean meal	3.0 g
Dextrose	2.5 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
To produce tryptic soy agar, agar was added	15.0 g

1 l of DI water was added and autoclaved at 121°C for 15 min.
Final pH 7.2

MacConkey agar (MAC)	
Peptone	17.0 g
Proteose peptone	3.0 g
Lactose	2.5 g
Bile salts No. 3	5.0 g
Sodium cloride	2.5 g
Agar	15.0 g
Neutral red	0.03 g
Crystal red	0.001 g

1 l of DI water was added and autoclaved at 121°C for 15min.
Final pH 7.1.

Bismuth sulfite agar (BSA)	
Beef extract	5.0 g
Peptone	10.0 g
Dextrose	5.0 g
Disodium phosphate	4.0 g
Ferrous sulfate	0.3 g
Bismuth sulfite indicator	8.0 g
Brilliant green	0.025 g
Agar	20.0 g

1 l of DI water was added and boiled for 1min.
Final pH 7.7.

Double strength *Shigella* broth (DSSB)	
Tryptone	40.0 g
Potassium phosphate dibasic	4.0 g
Potassium phosphate monobasic	4.0 g
Sodium chloride	10.0 g
Glucose	2.0 g
Tween 80	3.0 ml

1 l of DI water was added and autoclaved at 121°C for 15 min. Steril filtrated novobiocin (0.5 mg/l) was added after autoclaving.
Final pH 7.0.

MRS4	
Peptone digest from casein	10.0 g
Yeast extract	5.0 g
Beef extract	5.0 g
Glucose	5.0 g
Maltose	10.0 g
Fructose	5.0 g
Potassium phosphate dibasic	4.0 g
Potassium phosphate monobasic	2.6 g
Amonium chloride	3.0 g
Cystein-HCl	0.5 g
Tween 80	1.0 g
To produce MRS4 agar, agar was added	15.0 g

Chemicals, except sugars, magnesium-mangan-vitamin solution (next page) were dissolved in 800 ml of DI water, sugars were separately dissolved in 200 ml DI water and chemicals and sugars were autoclaved separately at 121°C for 15 min and mixed afterwards. Sterile filtered magnesium-mangan-vitamin solution was added after media cooled down after autoclaving.
Final pH 6.8.

Magnesium, mangan, vitamin solution	
Biotin	0.2 g
Cobalamin	0.2 g
Folic acid	0.2 g
Nicotic acid	0.2 g
Panthotheic acid	0.2 g
Pyridoxal	0.2 g
Riboflavin	0.2 g
Thiamin	0.2 g
$MgSO_4$	0.2 g
$MnSO_4$	0.05 g

Appendix 2 – Primer sequences

Primers used

Alkyl-hydroperoxide-reductase C22 subunit (*Ahr*); house keeping gene for *Shigella sonnei*:

Ahr-forward: 5’ gac gtt gct gac cac tac 3’,

Ahr-reverse: 5’ tcg cac ggt cag cca gac 3’

Trehalose-6-phosphate synthase (*OtsA*):

OtsA-forward: 5’ tga cag aac cgt taa ccg 3’,

OtsA-reverse: 5’ tca agc tcc acc att gag 3’

Virulence B (*InvE*):

InvE-forward: 5’ tct gga tgg cac tcg tag 3’,

InvE-reverse: 5’ cgc gcg aga cag att cac 3’

Virulence F (*VirF*):

VirF-forward: 5’ caa cac tcc tat tct gag g 3’,

VirF-reverse: 5’ gac gcc atc tct tct cga 3’

Invasive plasmid gene (*IpaH*):

*Ipa*H (forward): 5’gtt cct tga ccg cct ttc cga tac cgt c3’,

*Ipa*H (reverse): 5’gcc ggt cag cca ccc tct gag agt ac3’.

Appendix 3 - Gradient used for HPLC

Pressure lower limit: 50 bar

Pressure upper limit: 270 bar

A: Bi-distilled water

B: 1 M sodium acetate

C: 250 mM sodium hydroxide

Gradient

Time (min)	eludent	percentage
-40	A	0
	B	50
	C	50
-29.9	A	100
	B	0
	C	0
-21	A	88
	B	0
	C	12
0 = Injection		
2	A	90
	B	0
	C	10
11	A	80
	B	0
	C	20
18	A	70
	B	0
	C	30
28	A	44
	B	32
	C	24
39	A	36
	B	40
	C	24
49	A	50
	B	50
	C	0

Appendix 4 - Buffers for protein extraction

Buffers for protein extraction for protein gels

Tris buffer	
TrisHCl	20 mM
DTT	120 mM
10 ml of bidestiled water was added and the pH adjusted to 8.0 with 4 N NaOH	

SDS buffer	
SDS	0.9%
Trisbase	0.1 M
10 ml of bidestiled water was added and the pH adjusted to 8.6 with 4 N NaOH	

Swelling buffer		
Urea	6.1 M	21.00 g
Thiourea	1.79 M	7.10 g
DDM	65.06 mM	1.66 g
DTT	1%	0.50 g
Pharmylate 3-10	0.50%	0.75 ml
5 ml of bidestiled water was added		

Buffers for protein extraction for trehalose amount

TE-buffer	
Tris-HCL	25 mM
EDTA	1 mM
Final pH 8.8	

Digest buffer	
kH2PO4	50 mM
NaCl	100 mM
Glycerin	10%
n-Dodecyl-β-D-Maltoside	1%
Final pH 8.0	

Appendix 5 - Buffers for isoelectrical focusing

Swelling buffer	
Urea	6.0 M
Thiourea	2.0 M
DDM	1.0%
DTT	0.4%
Pharmalyte 3-10	0.5%

T buffer	
Trisbase	18.2 g
SDS	0.4 g

100 ml of bidestiled water was added and the pH adjusted to 8.8 with 4 N HCl

Equillibration buffer	
Urea	36.0 g
Glycerin	30.0 g
SDS	2.0 g
T buffer	3.3 ml

45.7 ml of bidestiled water was added

Appendix 6 - Media for 2D gels

D buffer	
Trizma base	36 g
SDS	30 g

300 ml of bi-distilled water was added, solution filtered, bi-destiled water was added to a final volume of 500 ml, pH was adjusted to 8.6 with 4 N HCl

Acrylamide gels (12%)	
Acrylamide solution (30.8%)	389.5 ml
D buffer	250.0 ml
Glycerin	50.0 g
TEMED	55.0 µl
APS	7.0 ml

Tank buffer	
SDS	19.9 g
Glycin	299.6 g
Trizmabase	58.0 g

19.9 l of bi-distilled water was added

Appendix 7 – Programs for isoelectrical focusing and gel runs

Isoelectrical focusing program	
150 V	2.0 h
300 V	3.5 h
600 V	2.0 h
1500 V	2.0 h
3000 V	14.0 h

Gel run program	
40 V	1.5 h
60 V	1.5 h
40 V	1.5 h
60 V	1.5 h
150 V	1.5 h
300 V	1.5 h
600 V	1.5 h
1500 V	1.5 h
3500 V	16.0 h

Appendix 8 - Silver staining solutions

Fixing solution	
Ethanol (v/v)	80%
Acetic acid (v/v)	10%
Bi-distilled water	10%

Sensibilisation solution	
Sodiumthiosulfate (w/v)	0.02%
Bi-distilled water	99.98%

Silver staining solution	
Silver nitrate (w/v)	0.20%
Formaldehyde (37%) (v/v)	0.02%
Bi-distilled water	97.98%

Developer	
Sodium carbonate (w/v)	3.00%
Formaldehyde (37%) (v/v)	0.05%
Sodiumthiosulfate (w/v)	0.0005%
Bi-distilled water	96.50%

Stop solution	
Glycin (w/v)	0.5%
Bi-distilled water	99.5%

Shrinking solution	
Ethanol	30%
Glycerin	12%
Bi-distilled water	58%

Silver staining method	
Fixing of gels	3 h
Washing	3 x 20 min
Sensibilisation	1 min
Washing	3 x 20 s
Silver staining	20 min
Washing	2 x 20 s
Developing	5 min
Washing	1 x 20 s
Stop	5 min
Washing	3 x 10 min
Shrinking	5 min

Appendix 9 - Cooomassie blue staining solutions

Fixing solution	
o-phosphoric acid (85%)	1 ml
Methanol	20 ml
Bi-distilled water	79 ml

Coomassie blue staining solution	
Roti-blue concentrate (5x)	20 ml
Methanol	20 ml
Bi-distilled water	60 ml

Washing solution	
Methanol	25 ml
Bi-distilled water	75 ml

Coomassie blue staining method	
Fixing of gels	1 h
Coomassie blue staining	2 days
Washing	3 x 20 min

Sources

In this study data, which were produced by Holger Teichert within a Semester and a Diplom thesis at the Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, were used in Capters Five and Eight.

The fatty acids (Chapter Nine) were analyzed after the extraction by Bob Ward in Bruce Germans Labor at the University of California Davis, Department of Food Science & Technology.

All pictures of the bacteria in Capters Two and Four were taken by Stephan Flessa at the University of California Davis, except the picture of *Lactobacillus sanfranciscensis* in Chapter Two, which was taken by Holger Teichert at the Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt.

Publications

Excerpts from Chapters Three, Five, and Eight were presented as posters with the following titles at the listed conferences:

Flessa, S., R. F. Vogel, and L.J. Harris. 2004. Drying Stress dependent expression of proteins of *Shigella sonnei* and *Salmonella enterica* serovar Enteriditis. Annual Meeting, International Association of Food Protection, Phoenix, AZ. (Abstract P055)

Flessa, S., R. F. Vogel, and L.J. Harris. 2003. Survival of *Shigella sonnei* during desiccation on surfaces is dependent upon density of inoculum and inoculum carrier. Annual Meeting, International Association of Food Protection, New Orleans, LA. (Abstract P234)

Flessa, S. and L.J. Harris. 2002. Survival of *S. flexneri* on strawberries stored at –20, 4 and 24°C. Annual Meeting, International Association of Food Protection, San Diego, CA. (Abstract P164)

Curriculum vitae

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Education

2002-present Doctor of Philosophy (Dr.-Ing.), Technische Universität München, Wissenschaftszentrum Weihenstephan and Department of Food Science and Technology, University of California, Davis. Dissertation title: Drying stress and survival of *Shigella* and *Salmonella* in food derived model systems.

1995-2001 Master of Sciences (Dipl.-Ing.), Technische Universität München, Wissenschaftszentrum Weihenstephan. Study subject: Technology and Biotechnology of Food.

1998-1999 Diploma thesis, study at the United States Department of Agriculture and at the Department of Viticulture and Enology at the University of California, Davis. Topic: Processing effects on antioxidant amount and activity of tomatoes.

1986-1995 Rudolf-Diesel-Gymnasium Augsburg

1982-1986 Grundschule Kissing

Industry Experience

1996 Internship, Scheuerer brewery, Moosbach, Germany. Working in the whole production process of beer

1995 Internship, Riegele Brewery, Augsburg, Germany. Quality assurance of beer, non-alcoholic drinks, and intermediate products with microbiological and chemical methods and working in all steps of the production process of beer and non-alcoholic drinks

Research Experience

2001-2002 Postgraduate Research Associate, University of California, Davis, Department of Food Science & Technology. Research: Bacterial contamination of pistachios from harvest to roasting. Determination of a possible *Salmonella* contamination of almonds during processing. Developement of a detection method for *Shigella* on strawberries. Examination of industrial washing methods of strawberries.

Professional Activities

2000-present Member, International Association of Food Protection

1996- present Member, Verein der Deutschen Ingenieure (Society of German engineers)

1996 and 2000 President of the student body (Fachschaft für Milchwissenschaft, Brauwesen und Lebensmitteltechnologie e.V.) at the Technische Universität München

1997 Member of the department council of the faculty for brewing and food technology at the Technische Universität München

1990-1995 President of the graduating students for 2 years at the Gymnasium
Student's president for 1 year at the Gymnasium
Editor in Chief of high school newspaper for 2 years at the Gymnasium