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Pooling of ground meat samples during analysis of *Salmonella* for compliance with regulations on microbiological criteria for foodstuffs

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Contents

Abstract

Most *Salmonella* outbreaks, that has occurred in Sweden so far, have been associated with consumption of beef and pork meat. During routine testing for *Salmonella* in meat, the merging of several samples to one analysed sample, so called pooling, occurs. It is allowed by regulations given that it has no negative effect on the detection of one *Salmonella* in 25g meat. This study aims to investigate whether pooling affects the method's sensitivity by comparing *Salmonella* detection in pooled ground beef and ground pork samples and in individual samples.

Five samples of 25g each were pooled: one sample spiked with *Salmonella* with four blank samples. Four different freeze dried strains were used in the study. Two pooling methods were tested separately: wet- or dry-pooling. Moreover, level of background flora was determined in each meat package. The ability of freeze-dried bacteria to grow on selective media was investigated to estimate the stress level of the tested *Salmonella* strains.

Most findings showed equal sensitivity in pooled and individual spiked samples. Sensitivity was lower in pooled samples compared to individual samples below 3,0 cfu per 25g for dry-pooling, and below 2,5 cfu per 25g for wet-pooling. The two pooling methods performed equally well above 3 cfu per 25g. Levels of *Enterobacteriaceae* were similar in packages of ground beef and ground pork, while there were more aerobic bacteria in the latter. The amount of background flora did not affect *Salmonella* detection in pooled samples. Also, freeze-drying was sufficient to mimic stress during meat manufacturing.

1. Introduction

1.1 Background

What is Salmonella?

Salmonella is a genus of enteric bacteria that belongs to the family *Enterobacteriaceae* (1). It comprises of two species: *Salmonella enterica* and *Salmonella bongori* (1). The vast majority of *Salmonella* bacteria belongs to the species *Salmonella enterica* (1). It is subsequently divided into six subspecies (2,3), where the subspecies *enterica* is most frequently identified in human and animal infections (1). There are over 2500 *Salmonella* serotypes (3), classified based on the molecular structure of the surface lipopolysaccharide (O-antigen) and the flagella (H-antigen) (1).

Most salmonella are zoonotic, *i.e.* they can infect both humans and animals (4), while others are specially adapted to one host (5). For example, the serotypes Typhi and Paratyphi are strictly human pathogens and usually do not affect animals (3). The serotype Dublin is specific to cattle, but transmission to humans may occur (5).

Salmonellosis

Salmonella infection, also called, salmonellosis, is known to cause gastroenteritis (6). Characteristic symptoms start usually within 12-36 hours following infection and include acute fever, diarrhea, abdominal pain and sometimes also vomiting (6,7). Depending on the causative *Salmonella* serotype as well as the age and immune-competence of the host, the severity of disease may vary (8,9). Most commonly, symptoms are mild and the infection is self-limiting (6). However, in some cases patients may suffer from severe diarrhea, or septicemia may arise (6,7). Salmonellosis has also been associated with chronic conditions such as reactive arthritis (7). Human infection with *S.* Dublin has been shown to cause more severe disease compared to other serotypes (9).

Food types and serotypes involved in human cases

A wide range of domestic and wild animals carry *Salmonella* in their intestinal tract, often without symptoms (6,10). Upon contact with fecal material from infected animals, water as well as foodstuffs of both animal and plant origin may be contaminated (6,10). In the European Union (EU), eggs and pig meat are the most common source of human salmonellosis (6). Most *Salmonella* outbreaks, that has occurred in Sweden so far, have been caused by consumption of beef and pork meat, closely followed by salad and sprouts (11). Poultry, eggs and dairy products come on third place (11).

Salmonella Enteritidis, closely followed by *S.* Typhimurium, are the most commonly isolated serotypes in human infections in the EU (7). In Sweden, *S.* Typhimurium dominates human infections as well as broiler hens and pigs (10). In cattle, *S.* Typhimurium and *S.* Dublin are the most common serotypes (10).

Favourable salmonella status in Sweden

Salmonellosis is one of the most common zoonotic infections in the EU (12). However, in Sweden, around 80 percent of the *Salmonella* infections are travel-related (12), while domestically procured infections dominate in most of the other member states (6). Finland is the only other EU-country with nearly as high percentage of cases with foreign origin as Sweden (6). A *Salmonella* serological study of eight countries identified Sweden and Finland as the two countries with lowest seroincidence (13), *i.e.* the number of people that have been exposed to *Salmonella,* per inhabitants.

The low *Salmonella* prevalence in Swedish food producing animals contributes greatly to the low number of domestic cases. According to the trend between 1995 and 2012, below 0,3 percent of adult pigs, cattle and poultry were tested positive for Salmonella (10). The highest prevalence was in pigs and lowest in poultry (10).

Stringent national control

Based on the low *Salmonella* prevalence, Sweden and Finland were granted the so called *Salmonella* guarantees when entering the EU in 1995 (12). The conditions are described in Commission regulation (EC) No 1688/2005. The regulation states that these two countries can require certificates of *Salmonella* free fresh meat and eggs from *Salmonella* free poultry flocks imported from other EU countries into Sweden and Finland (12). Cattle, pigs and poultry, and their meat (including ground meat) imported from countries with higher *Salmonella* prevalence than in Sweden are covered by this regulation (12). Absence of *Salmonella* attained by approved analytical methods is attested by documentation provided by the sender (12). The Swedish border control and importers checks documentation and verifies laboratory results by random sampling of consignments (14).

The application of preventive measures to ensure the absence of *Salmonella* in the food-chain is a prerequisite for maintaining the good *Salmonella* status required for the *Salmonella* guarantees and for preservation of good public health (12). This is monitored and controlled by the Swedish national *Salmonella* strategy program. Around 30,000 samples from cattle, pigs and poultry at abattoirs and cutting plants are sampled and analysed every year (10). The goal is to eliminate the pathogen in all stages of food production: 1) feed, 2) carcasses of livestock, 3) and processed meat products (15).

Food safety legislation

All food business operators (FBOs) must ensure the safety of their products in different ways by both EU and national legislation (16,17). For example, they must run their business in accordance with current regulations on the subjects of: 1) Food safety (16), 2) Food hygiene (18), 3) Special hygiene rules regarding animal good products (19), 4) *Salmonella* guarantees (14), and 5) Microbiological criteria for foodstuffs (20).

The regulation of microbiological criteria includes sampling plans for a number of microorganisms including *Salmonella* (20). For all meat products, no *Salmonella* should be found in a minimum of five tested samples of 25grams each (20). The regulation on microbiological criteria states ISO 6579/2002 as reference analytical method for *Salmonella* detection, but alternative equivalent methods may also be used (20).

One such method is NMKL method number 71, published by the Nordic Committee on Food Analysis (21). In addition to ISO 6579/2002, it is also referred to in the regulation on *Salmonella* guarantees and, further used for *Salmonella* analysis within the Swedish national control program (10,14). The methods are considered technically equivalent despite minor differences (21,22).

Pooling of samples and the practice of commercial laboratories

The tests included in the food businesses' internal control for food safety are done by commercial laboratories. The food legislation allows FBOs to choose their own procedures to fulfil the legal requirements (23). Pooling of samples is an approach that has increasingly been considered for compliance with the microbiological criteria (24). Pooling reduces both the work load and costs when testing many samples (24). There are two ways of sample compositing (pooling) described: (1) dry-pooling, which involves the composition of dry sample units, and (2) wet-pooling, which refers to the pooling of pre-enriched samples (25).

The use of pooling during analyses of *Salmonella* in meat at Swedish commercial laboratories was confirmed by employees of three laboratories (26–28). Dry-pooling is the preferred method (26–28). The representatives from two of the laboratories reported pooling of five samples (26,28). At the third lab, up ten samples may be pooled, but five samples is the most common pooling routine (27). Furthermore, two laboratories analysed only in the context of the internal control of food businesses (26,28), while the remaining laboratory analysed also for the fulfilment of the requirements stated in the regulation of *Salmonella* guarantees (27).

According to the analytical method ISO 6579/2002, pooling of up to ten samples is allowed as long as there is evidence of no negative effect on sensitivity (22). However, the validity of pooling has not yet been established and that infers uncertainty of whether pooling can be used for compliance with the regulation of microbiological criteria. Since pooling may impair detection of low numbers of microorganisms, the International Commission on microbiological specification of foodstuffs (ICMSF) has urged that pooling should be approached with caution and should be validated for each specific combination of food and pathogen (24).

So far, two pooling studies of *Salmonella* have been done. Sörensen et al (2007) compared prevalence based on five pooled swine carcass swab samples with results from individual samples (20), and Mooijman at the European Union Reference Laboratory (EU-RL) for *Salmonella*, tested both dry- and wet pooling in poultry meat (30). Up to now, information is lacking for how pooling of other types meat samples affects the sensitivity of *Salmonella* detection.

1.2 The goal of the project

The aim of this study is to compare the analytical sensitivity of Salmonella analysis in ground meat of beef and pork when pooling and with the sensitivity obtained when testing individual samples. Results from the study will lead to enhanced knowledge about the effect of pooling and also serve as a basis for food authorities when deciding if pooling of five samples of ground meat is an approved method for compliance with the regulation of microbiological criteria (20).

Ground beef and pork are particularly interesting for three reasons: (1) their contribution to salmonellosis cases, (2) their import is regulated in accordance with the *Salmonella* guarantees, and (3) they both make an excellent substrate for bacterial growth. Mincing meat allows the distribution of bacteria from the surface to spread throughout the meat (31). Further, more meat surface is exposed to bacteria in minced meat compared to whole meat cuts (31). Our investigation focuses on the zoonotic *Salmonella* serotypes *S.* Enteritidis, *S.* Typhimurium, *S.* Dublin and *S.* Stockholm.

2. Material and methods

2.1 Food matrix and strains

Fresh ground beef and ground pork prepared in the food store shortly before purchase were used as food matrix. For each meat type, all packages contained meat with the same fat content. The purchase was made on the same day as sample preparation and spiking. Thereafter, the meat was stored at refrigeration temperature (4 °C ± 2 °C) ca 24 hours until weighing.

Vials of freeze-dried strains of *Salmonella enterica* serotypes Typhimurium, Enteritidis, Dublin and Stockholm were provided from the strain collection of the Microbiology Division at the National Food Agency. One vial contained only one strain. Information about the vials such as SLV-identification number, bacterial concentration (log cfu per vial), year of preparation and storage conditions were provided by Laurence Nachin, Microbiology Division (Table 1). All vials were moved to -20°C before project start and were stored there during the project.

Table 1. Information about freeze-dried vials containing *Salmonella* strains. For each strain, SLV identification number, as well as year of preparation and storage conditions until project start are listed.

Strain	Year of preparation	Storage conditions
S. Dublin SLV-242	2013	-55°C
S. Enteritidis SLV-436	2013	-55°C
S. Typhimurium SLV-248	2012	-20°C
S. Stockholm SLV-390	2014	-20°C

2.2 Estimation of stress level caused by freeze-drying

One vial of each of the four strains was resuspended in an appropriate volume of peptone water (PW; Oxoid, Basingstoke, UK, catalogue number LP00034) and 0,1 ml of each bacterial suspension was inoculated in triplicate using a sterile plastic spreader on plate of Nutrient Agar (NA; Oxoid, Basingstoke, UK, cat. no. CM0003), Xylose-Lysine-Desoxycholate Agar (XLD; Oxoid, Basingstoke, UK, cat. no. CM0469), and Brilliance™ Salmonella agar (BriS; Oxoid, Basingstoke, UK, cat. no. CM1092). NA and XLD plates were incubated at 37°C for 24±3 h and BriS plates for 48 h at the same temperature. As a measure of the stress effects of selective pressure on bacterial growth, the difference between NA and XLD (both Oxoid) as well as between NA and BriS (both Oxoid) was calculated based on mean cfu per ml of the triplicates.

2.3 Study design of pooling experiments

Each *Salmonella* strain was tested in separate experiments in either ground meat of beef or pork. Further, for each experiment, one of the four strains was inoculated in the meat and subjected to either wet- or dry-pooling experiments. As seen in Figure 1, five parallel poolings were tested at each experiment occassion and each pooling consisted of five samples: 1 x 25g inoculated sample and 4 x 25g blanks. Sample preparation and *Salmonella* detection was done according to NMKL method number 71 (21). For dry- and wet pooling, procedures described by Jarvis and Mooijman were applied (30,32,33).

For confirmation, as first choice one typical colony from XLD was isolated. If it was not possible to isolate from XLD, colonies were chosen from either Mannitol Lysine Crystal Violet Brilliant Green Agar (MLCB) or BriS. If suspected *Salmonella* colonies were found on negative control plates, one colony per sample was serologically tested.

2.4 Dry-pooling experiments

Sample preparation

For each pooling, a representable sample of ground meat from different parts of the meat package was taken to yield one test portion of 100 g (4 x 25g) and one test portion of 25g. In parallel, two 25g portions were sampled for positive and negative controls respectively. A deviation of ±0.15 g was accepted. The samples were stored in 4 ±2°C until spiking later the same day.

Spiking of samples

The contents of one vial was resuspended and serially diluted 10-fold in PW (Oxoid) to yield a theoretical concentration of 1-10 cfu/ml. Then, 1 ml of the suitable dilution was used for spiking of one 25g meat portion and positive control. Both spiked and blank samples were incubated at 4 ±2℃ overnight to allow the bacteria to attach to the food matrix.
The actual number of bacteria in the inoculum was determined as follows: 100 µl of each dilution including the undiluted bacterial suspension was inoculated in duplicate on NA plates (Oxoid) using a sterile plastic spreader. Plates were incubated at 37 ºC for 24±3 h. The colonies were counted and the number of colony forming units (cfu) per 1 ml used for inoculation was calculated based on the data from all the plates.

The dry-pooling procedure
Following the overnight incubation of the meat samples, 5 x 25g samples were pooled by adding 100g (4x25g) of un-spiked meat to 25g spiked sample in a stomacher® filter bag (Seward, Worthing, UK, prod. no. BA6041/STR) to make one pooling (Figure 1)**.**

Pre-enrichment

As evident in Figure 2, the pooling was added to 225 ml of buffered peptone water (BPW; Oxoid, Basingstoke, UK, cat. nr. CM0509) and homogenized in a laboratory blender

apparatus (Laboratory Blender Stomacher 400, type BA 7021; Seward, Worthing, UK, serial no. 32666) at normal speed for 30 s. Thereafter, 900 ml of BPW (Oxoid) was added to a final volume of 1125 ml. To each of the positive and negative controls, 255 ml of BPW was added and samples were homogenized as described above. All samples were pre-enriched by incubation at 37±1 ºC for 18±2 h.

Enrichment

The pre-enriched BPWs were mixed by circular movement, first 20 times clockwise, then 20 times counter-clockwise. From each pre-enriched sample, 100 µl was transferred to 10 ml of Rappaport-Vassiliadis Soya Peptone Broth (RVS, Oxoid, Basingstoke, UK, cat. nr. CM0866), pre-warmed at 42 ±0,2 ℃ in a water bath (Figure 1). All samples were incubated at 42 ±0,2 ℃ in water bath for 24 h±2 h.

Isolation

The RVS tube was vortexed thoroughly and 10µl of the enriched sample was streaked onto selective plates using a plastic loop. For each sample, three plates: XLD (Oxoid), BriS (Oxoid), and Mannitol Lysine Crystal Violet Brilliant Green Agar (MLCB; Oxoid, Basingstone, UK, cat. nr. CM0783) were inoculated. The enriched samples were vortexed before each inoculation.

Serological confirmation

Typical colonies from XLD, MLCB or BriS were streaked onto NA-plates and incubated for 24±3 hours. Examples of such colonies are shown in Figure 1. Agglutination tests with polyvalent H and polyvalent O antisera (both Reagensia, Solna, Sweden, prod. nr. 100, 200) were prepared using Saline (0,9 NaCl) as negative control. The presence of O and H antigens result in agglutination of the antibodies in the antiserum (21).

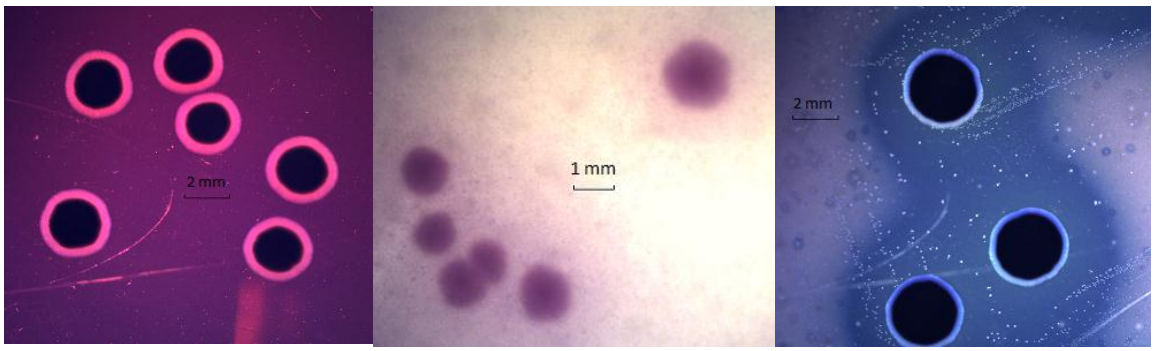

Figure 1. Typical Salmonella colonies on Xylose-Lysine-Desoxycholate Agar (left), Brilliance™ Salmonella agar (middle), and Mannitol Lysine Crystal Violet Brilliant Green Agar (right).

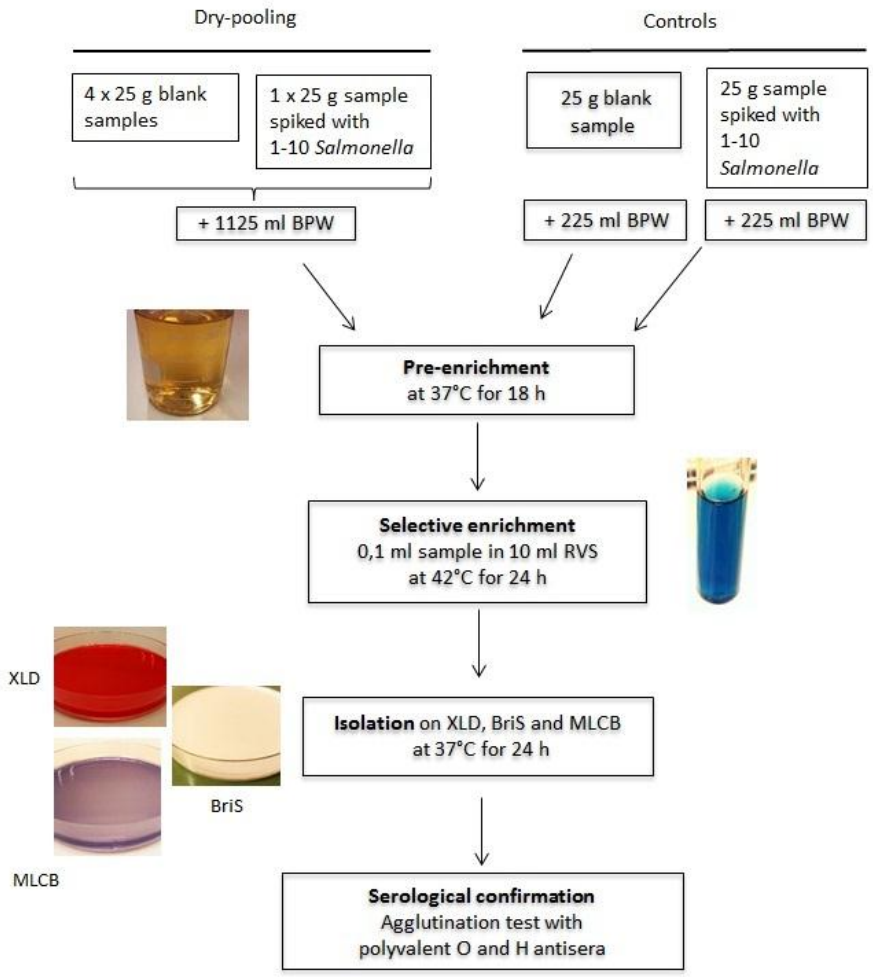

Figure 2. **Principles of analysis of dry-pooled and control samples**. First, meat samples (one spiked + four blank) were added together, *i.e*. dry-pooled. The pooled sample, parallel to a positive and negative control was analysed for *Salmonella* by the NMKL method number 71. BPW = buffered peptone water; RVS = Rappaport-Vassiliadis Soya Peptone Broth; XLD = Xylose-Lysine-Desoxycholate Agar; BriS = Brilliance™ Salmonella agar; MLCB = Mannitol Lysine Crystal Violet Brilliant Green Agar

2.5 Deviations in the wet-pooling experiments

Samples were spiked as described in section 2.3 with the exception that only one 25g food sample was spiked per pooling. As shown in Figure 3, these sample was both included in pooling and used positive control. For pooling, one of the blank samples was a negative control. All samples were homogenized separately in 225 ml BPW each.

The wet-pooling procedure was done as follows: For each pooling, pre-enrichments from five samples (one spiked sample and four blank samples) were pooled together by transferring 5 ml from each sample to a sterile glass flask, yielding a final volume of 25 ml. For the enrichment in RVS, 0,5 ml was transferred from each wet pooling to 50 ml pre-warmed RVS.

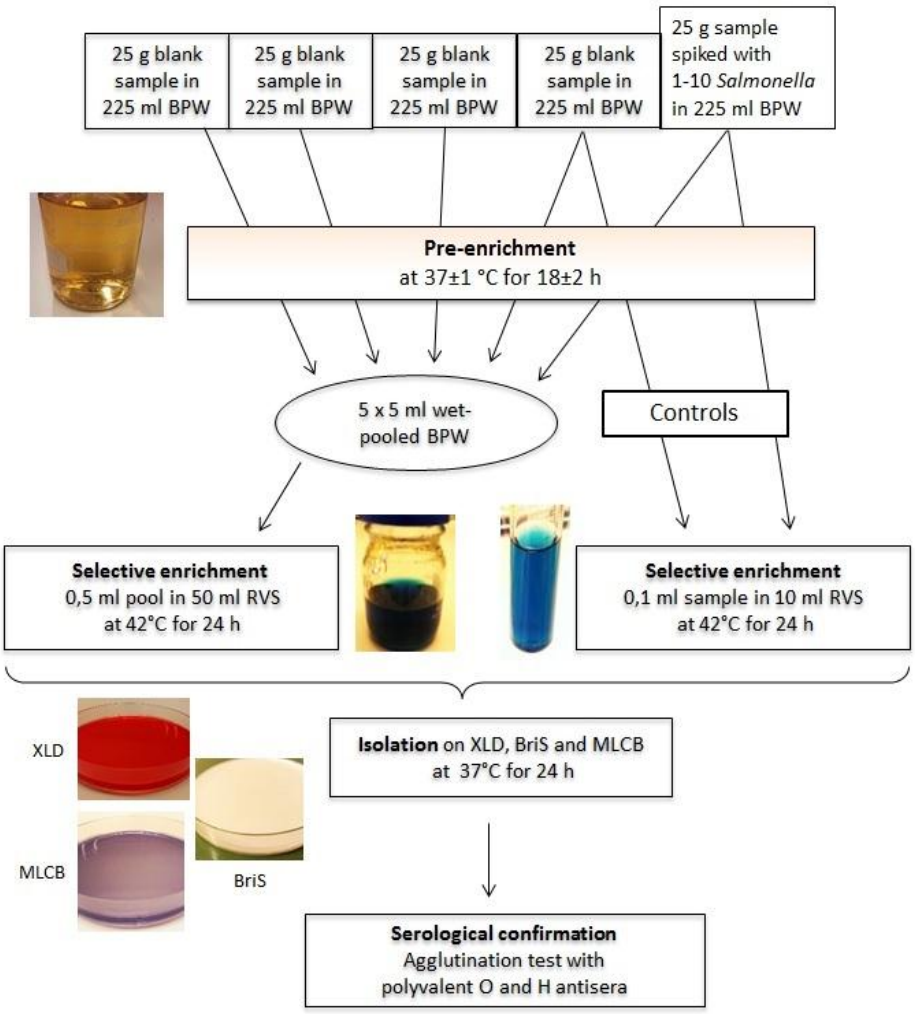

Figure 3. **Principles of analysis of wet-pooled and control samples**. One spiked and four blank meat samples were pre-enriched separately. Wet-pooling means the mixing of equal volumes from each pre-enriched sample. Besides being included in the wet-pool, the spiked sample and one of the blank samples served as positive and negative control, respectively. Both pooled and control samples were analysed for *Salmonella* using the NMKL method number 71. BPW = buffered peptone water; RVS = Rappaport-Vassiliadis Soya Peptone Broth; XLD = Xylose-Lysine-Desoxycholate Agar; BriS = Brilliance™ Salmonella agar; MLCB = Mannitol Lysine Crystal Violet Brilliant Green Agar

2.6 Enumeration of background flora

The number of *Enterobacteriaceae* and aerobic bacteria in the meat used for pooling were determined according to NMKL method number 144 (34) and number 86 (35) respectively. Since adding 225 ml BPW to 25g sample yields a 250 ml total volume, the bacteria are diluted 10-fold.

Prior to incubation of the pre-enrichment broth, one 10-fold dilution series per ca 700 g meat was prepared from the negative control. 1 ml from the homogenized sample (1:10 dilution) and 1 ml from each dilution tube was inoculated by pour-plating of Violet-red-Bile-Glucose Agar (VRBG; Acumedia, Lansing, USA, cat. nr. 7425) and of Plate Count Agar (PCA; Acumedia, Lansing, USA, cat. nr. 7157) respectively. For VRBG (Acumedia), a dilution series from 10^{-1} to 10^{-4} and for PCA (Acumedia), a dilution series from 10^{-3} to 10^{-6} was used. Plates of VRBG (Acumedia) were incubated at 37 ºC for 24±3 h and plates of PCA (Acumedia) at 30 ºC for 72±6 h.

Five typical colonies were picked out from plates of VRBG (Acumedia), re-streaked onto a plate of NA (Oxoid) and incubated for 18±2 h. Then, each colony were tested for the presence of cytochrome oxidase using oxidase strip (Bactident® Oxidase; Merck Millipore, product no. 1133000001). *Enterobacteriaceae* are oxidase negative (36).

2.7 Statistical tests

For a statistical estimation of the probable number of bacteria being transferred in spiking, Poisson distribution was used. The range of values was calculated by Jonas Toljander, risk benefit assessor at the National Food Agency, using the software @RISK (Palisade Corporation) and kindly provided to the author. Also, an Excel spreadsheet, generously shared by Magnus Simonsson, microbiologist at the National Food Agency, was used by the author to calculate the probability of transferring 0 cfu, or >1 cfu bacteria, respectively.

3. Results

3.1 Quantification of background flora in different meat packages

Aerobic bacteria and bacteria belonging to the family *Enterobacteriaceae* are naturally occurring in beef and pork meat (37,38). In order to map the level of this background flora in the meat used for this project, aerobic plate count (APC) and level of *Enterobacteriaceae* (EBAC) were determined in each meat package using NMKL method number 144 (34) and number 86 (35) correspondingly. These methods were chosen because they are suitable specifically for the determination of the number of aerobic bacteria and of *Enterobacteriaceae*, respectively, in food stuff by the colony count procedure (34,35). To account for the heterogeneity in bacterial distribution within the packages, the number of measurements made from each package was proportional to its weight. For packages over 700 g, mean value and corresponding standard deviation of several measurements was calculated.

Data from ground beef are presented in Figure 4, and results from ground pork are presented in Figure 5. In ground beef, APC varied from approximately 4 log cfu/g to just nearly 6,5 log cfu/g and EBAC was in the range of about 2,5-5 log cfu/g. In ground pork, EBAC was between approximately 3 log and 4,5 log cfu/g. Levels of APC was around 6 log cfu/g on average, with high standard deviation in one of the packages, where APC reached up to above 7,0 log cfu/g.

Results reveal that level of background flora may vary between different packages of the same meat type. In addition, the level of *Enterobacteriaceae* seems to be quite similar in both meat types. On the other hand, there was more aerobic bacteria observed in ground pork than in ground beef. Data representing several measurements shows that, to a varying extent, there were differences in levels of background flora within some packages (Figures 4-5).

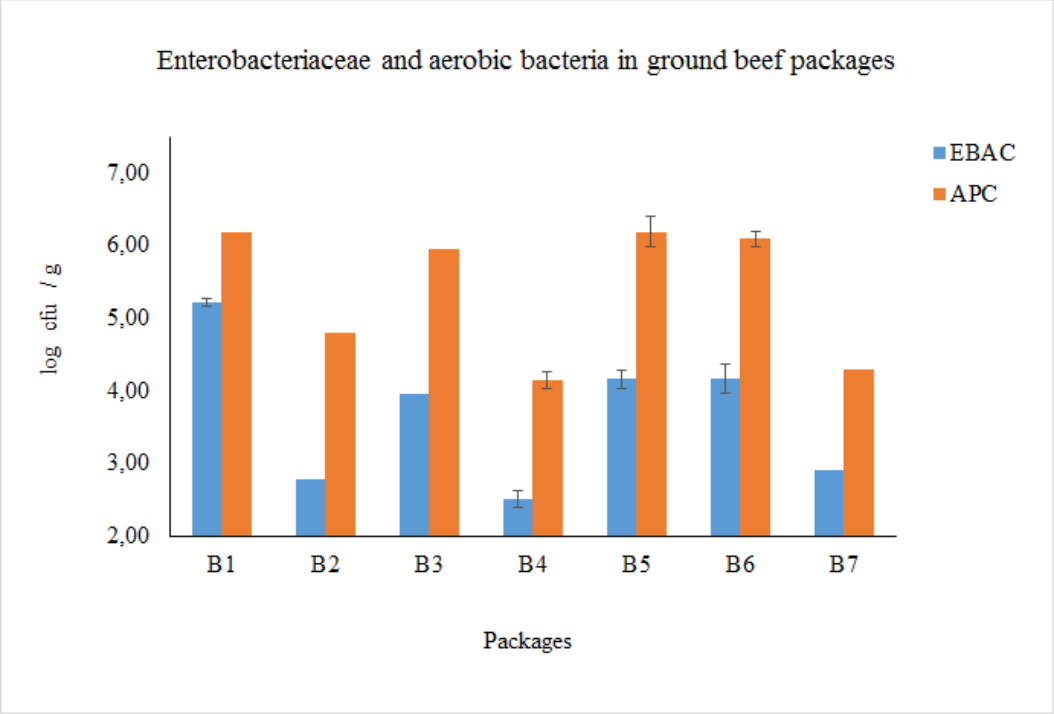

Figure 4. Variation in levels of *Enterobacteriaceae* (EBAC) and aerobic plate count (APC) in different packages of ground beef. On the x-axis, package number is shown. One measurement was taken per ca 700 g meat, thus mean value of several measurements are shown for packages B1, B5-B7, except that only one APC measurement was attained for B2. Standard deviation for each mean value is shown by error bars. *Results vary both between different packages and between measurements taken from the same package (see error bars.)*

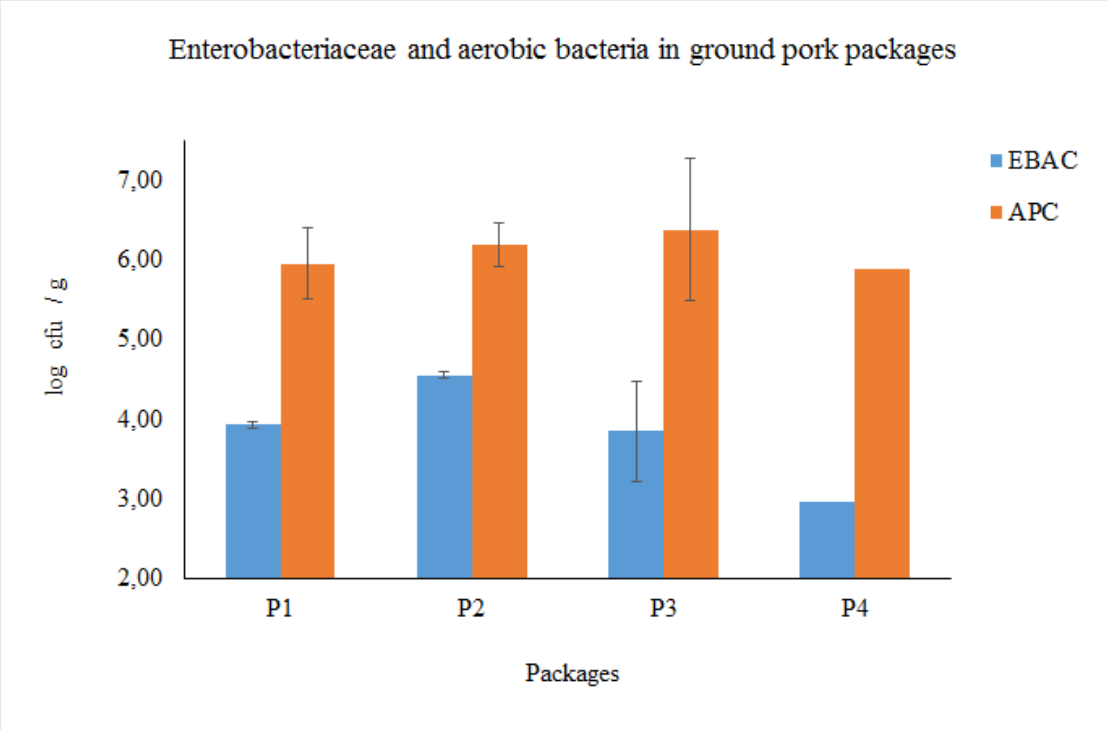

Figure 5. Variation in aerobic plate count (APC) and Enterobacteriaceae (EBAC) in different packages of ground pork. On the x-axis, package number is shown. One measurement was taken per ca 700 g meat, thus mean value of several measurements are shown for packages P1-P3, which weighed over 700 g. Standard deviation for each mean value is shown by error bars. *Results are variable, and especially interesting is the pronounced difference within package P3.*

3.2 Detection of four Salmonella serotypes in pooled and in individual samples

In the pooling experiments, both dry - and wet -pooling were tested to elucidate any differences between the two approaches. Three medically relevant *Salmonella* serotypes (Enteritidis, Typhimurium, and Dublin), in addition to *S.* Stockholm, were tested in each combination of pooling type and meat type. *S.* Stockholm was included to increase the variation in investigated strains.

Salmonella in food stuff may be injured, and is typically found in low numbers and in the presence of a much higher number of other *Enterobacteriaceae* (21). The NMKL method number 71 (21) was used as it includes procedures necessary for recovery and isolation of *Salmonella* from food stuff: 1) pre-enrichment in media that facilitates growth of all bacteria , 2) enrichment in media that favours the growth of *Salmonella* and 3) cultivation on agar that is suitable especially for *Salmonella* isolation (21). According to NMKL-71, the use of Xylose-lysine-desoxycholate Agar (XLD) and a second agar of choice is obligatory (21). XLD allows the isolation of H_2S-positive *Salmonella* that do not ferment lactose and saccharose (39, 40). For the detection of *Salmonella* with different biochemical profiles, both Brilliance™ Salmonella Agar and Mannitol Lysine Crystal Violet Brilliant Green Agar were included in addition to XLD (41,42).

In each experiment, five identical batches of pooling were analysed to account for any natural variation. Individual samples spiked with *Salmonella* (positive control) and unspiked samples (negative control) were analysed parallel to the pooled samples.

In the following sections, for dry-pooling and wet-pooling separately, the results of serological confirmation are described, and compared to estimated inoculation level and the amount background flora specific for the particular meat package in question.

Dry-pooling

The result from all dry-pooling experiments are shown in Table 2. In analyses with *S.* Enteritidis, ground beef and ground pork was spiked with 8,9 and 8,7 cfu per 25g, respectively. All pooled samples yielded positive results, corresponding to 100 percent detection rate. Same results were obtained when 6,7 cfu per 25g of *S.* Typhimurium was added to ground beef and ground pork. One suspicious colony was observed in the negative control for beef but could not be serologically confirmed. Further results of 100 percent detection rate was found following the analysis of *S.* Stockholm in both meat types with an inoculation level of 3,3 and 4,6 cfu per 25g. Data obtained from analyses with *S.* Dublin in ground pork showed detection level of 100 percent at 3 cfu per 25g. On the other hand, spiking of ground beef with 2,5 cfu per 25g gave four out of five (80 percent) confirmed positive pooled samples.

All in all, data point toward that 3 cfu per 25g was the lowest inoculation level resulting in a 100 percent detection rate in pooled samples.

Observations regarding background flora are presented in Table 2. As for *S.* Dublin, the level of *Enterobacteriaceae* (EBAC) was 2,91 log cfu/g and aerobic plate count (APC) 4,30 log cfu/g respectively, while corresponding numbers in pork were 3,92 and 6,01 log cfu/g. Also, for *S.* enteritidis, EBAC and APC were higher in pork (3,92 and 6,01 log cfu/g, respectively) than in beef (2,78 and 4,80 log cfu/g, respectively). An opposite pattern was observed with *S.* Typhimurium. The detection rate was 100 percent for all tests irrespective of meat type, suggesting that the background flora had no apparent effect on the analytic sensitivity at the observed inoculation levels.

Wet-pooling

Results from wet-pooling experiments are presented in Table 3. 8,8 and 8,6 cfu of *S.* Enteritidis was added per spiked sample in ground pork and ground beef, correspondingly. In the ground pork experiment, all five pooled samples were positive. When testing the negative control for beef, there were three suspicious colonies, of which two could be confirmed as *Salmonella* negative.

When ground pork was spiked with *S.* Typhimurium with 3,2 cfu per 25g, five out of five (100 percent) pooled samples were positive In an initial experiment with ground beef, with the inoculation level of 1,3 cfu per 25g, only one out of five pooled samples (20 percent) was positive. This was consistent with the detection rate in positive controls. The low detection rate could have been a consequence of the low inoculation level. In a subsequent repeat of this experiment, 6,7 cfu per 25g was used. This gave positive results in all five pooled samples and all five positive controls.

Following the analysis of ground pork spiked with 2,5 cfu *S.* Stockholm per 25g , all pooled samples were confirmed positive. For ground beef, an inoculation level of 3,1 cfu per 25g was used. Four out of five pooled samples and four of the five positive controls were found to be positive.

Table 2. A summary of results from experiments where dry-pooling was used, divided by serotypes and meat type. Inoculation level and level of Enterobacteriaceae (EBAC) and aerobic bacteria (APC) are displayed to allow comparison with result from serological confirmation.

Serotype	Sample ¥	Meat type	Inoculation level (cfu / 25 g)	Samples with suspected colonies	Serological confirmation		Background flora (log cfu / ml)	
					+	-	EBAC	APC
Dublin	Pooled (n = 5)	Beef	2,5	5	4	1	2,91	4,30
	Positive control (n = 1)	Beef	2,5	1	1	0	2,91	4,30
	Negative control (n = 1)	Beef	0	1	0	1	2,91	4,30
Dublin	Pooled (n = 5)	Pork	3	5	5	0	3,93	6,07
	Positive control (n = 1)	Pork	3	1	1	0	3,93	6,07
	Negative control (n = 1)	Pork	0	0	Nd	Nd	3,93	6,07
Enteritidis	Pooled (n = 5)	Beef	8,9	5	5	0	2,78	4,80
	Positive control (n = 1)	Beef	8,9	1	1	0	2,78	4,80
	Negative control (n = 1)	Beef	8,9	0	Nd	Nd	2,78	4,80
Enteritidis	Pooled (n = 5)	Pork	8,7	5	5	0	3,93	6,07
	Positive control (n = 1)	Pork	8,7	1	1	0	3,93	6,07
	Negative control (n = 1)	Pork	0	0	Nd	Nd	3,93	6,07
Typhimurium	Pooled (n = 5)	Beef	6,7	5	5	0	4,18	6,10
	Positive control (n = 1)	Beef	6,7	1	1	0	4,18	6,10
	Negative control (n = 1)	Beef	0	1	Nd*	Nd*	4,18	6,10
Typhimurium	Pooled (n = 5)	Pork	6,7	5	5	0	2,95	5,88
	Positive control (n = 1)	Pork	6,7	1	1	0	2,95	5,88
	Negative control (n = 1)	Pork	0	1	0	1	2,95	5,88
Stockholm	Pooled (n = 5)	Beef	3,3	5	5	0	3,96	5,96
	Positive control (n = 1)	Beef	3,3	1	Nd*	Nd*	3,96	5,96
	Negative control (n = 1)	Beef	0	1	0	1	3,96	5,96
Stockholm	Pooled (n = 5)	Pork	4,6	5	5	0	3,93	6,07
	Positive control (n = 1)	Pork	4,6	1	1	0	3,93	6,07
	Negative control (n = 1)	Pork	0	0	Nd	Nd	3,93	6,07

Nd = note done

* suspicious colonies were not possible to isolate

¥ pooled sample = 1 x 25g inoculated + 4x25g blank; positive control = 1x25g inoculated; negative control = 1 x 25g blank

When the detection of *S.* Dublin in ground pork was analysed, the inoculation level was 2,2 cfu per 25g (Table 3). Four out of five (80 percent) pooled samples and all positive controls were confirmed positive. There were suspected colonies observed for negative controls, but only one out of four could be isolated and confirmed negative. In pooled ground beef, samples were spiked with 3,6 cfu per 25g and Salmonella was detected in four out of five samples (80 percent). However, due to confirmed *Salmonella* presence in two out of five negative controls, only two pooled samples were considered positive. In summary, results from wet-pooling showed 100 percent detection rate in pooled samples at 2,5 cfu per 25g at the lowest.

As for background flora, a similar pattern as in dry-pooling experiments was also observed in wet-pooling. The same meat package was used in the experiments with *S.* Dublin and *S.* Enteritidis, and a considerable difference can be observed between ground pork (EBAC log 4,56 cfu/g and APC log 6,24 cfu/g) and ground beef (EBAC log 2,52 cfu/g and APC log 4,16 cfu/g). In the case of *S.* Typhimurium and *S.* Stockholm, only the level of aerobic bacteria differed between the two meat types. There was no difference in pooling results between ground pork ground beef in any of these experiments.

Statistical uncertainty during spiking of samples

Poisson distribution is applicable to rarely occurring random events, such as the distribution of low number of bacteria in large quantity of food or other material (32). Thus, it is suitable for our study. To describe the possible outcomes of the transfer of bacteria, the Poisson distribution for each suspension was calculated with 90 percent confidence interval. Table 4 shows, that when pipetting a bacteria solution, the number of bacteria that are transferred may vary statistically between different pipetting occasions. For the concentrations 1,3, 2,2 and 2,5 cfu/ml, the confidence intervals include 0 (zero) transferred bacteria. Concentrations of 3,0 cfu/ml and above include values that statistically indicate 1 bacteria or more.

Also, from the Poisson distribution the probability of each possible outcome, expressed in percent can be estimated (32). In Table 4, the probability of transferring 0 cfu (*i.e.* absence of Salmonella) and the probability of transferring 1 or more cfu (*i.e.* presence of Salmonella) is shown. In general, the probability of 0 cfu decreases with increasing concentration. For the level 1,3 cfu ml, the chance of transferring 0 cells is as high as 27,3 percent, for 2,2 cfu/ml it decreases to 11,1 percent and for 2,5 ml it is 8,2 percent.

Table 3. A summary of results from experiments where wet-pooling was used, divided by serotypes and meat type. Inoculation level and level of Enterobacteriaceae (EBAC) and aerobic bacteria (APC) are displayed to allow comparison with result from serological confirmation.

Serotype	Sample ¥	Meat type	Inoculation level	Samples with suspected colonies	Serological confirmation		Background flora log cfu / ml	
					+	-	EBAC	APC
Dublin	Pooled (n = 5)	Beef	3,6	5	4 *	0	2,52	4,16
	Positive control (n = 5)	Beef	3,6	5	5	0	2,52	4,16
	Negative control (n = 5)	Beef	0	3	2	1	2,52	4,16
Dublin	Pooled (n = 5)	Pork	2,2	5	4	1	4,56	6,24
	Positive control (n = 5)	Pork	2,2	5	5	0	4,56	6,24
	Negative control (n = 5)	Pork	0	4	0	1 *	4,56	6,24
Enteritidis	Pooled (n = 5)	Beef	8,8	5	5	0	2,52	4,16
	Positive control (n = 5)	Beef	8,8	5	5	0	2,52	4,16
	Negative control (n = 5)	Beef	0	3	0	2 *	2,52	4,16
Enteritidis	Pooled (n = 5)	Pork	8,6	5	5	0	4,56	6,24
	Positive control (n = 5)	Pork	8,6	5	5	0	4,56	6,24
	Negative control (n = 5)	Pork	0	2	0	2	4,56	6,24
Typhimurium	Pooled (n = 5)	Beef	1,3	2	1	1	4,17	6,22
	Positive control (n = 5)	Beef	1,3	3	1	2	4,17	6,22
	Negative control (n = 5)	Beef	0	1	0	1	4,17	6,22
Typhimurium (rerun)	Pooled (n = 5)	Beef	6,7	5	5	0	4,18	6,10
	Positive control (n = 5)	Beef	6,7	5	5	0	4,18	6,10
	Negative control (n = 5)	Beef	0	2	0	2	4,18	6,10
Typhimurium	Pooled (n = 5)	Pork	3,2	5	5	0	4,04	6,73
	Positive control (n = 5)	Pork	3,2	5	5	0	4,04	6,73
	Negative control (n = 5)	Pork	0	0	Nd	Nd	4,04	6,73
Stockholm	Pooled (n = 5)	Beef	3,1	4 **	4	0	4,17	6,22
	Positive control (n = 5)	Beef	3,1	4 **	4	0	4,17	6,22
	Negative control (n = 5)	Beef	0	3	2	1	4,17	6,22
Stockholm	Pooled (n = 5)	Pork	2,5	5	5	0	4,04	6,73
	Positive control (n = 5)	Pork	2,5	5	5	0	4,04	6,73
	Negative control (n = 1)	Pork	0	0	0	1	4,04	6,73

Nd = note done

* not all suspicious colonies were possible to isolate
** pooled and individual samples correspond to each other
¥ pooled sample = 1 x 25g inoculated + 4x25g blank; positive control = 1x25g inoculated; negative control = 1 x 25g blank

Table 4. The statistical number of bacteria cells that may be transferred (*column 4*) from the bacteria suspensions used for spiking (*column 3*), and probability (*P*) of the absence of *Salmonella* (0 cfu) and corresponding probability of presence of *Salmonella* (>1 cfu) in the transferred volume (*column 5 and 6, respectively*). *Salmonella* detection rate for individual samples in the pooling experiments are listed for comparison (*column 7*). The statistical data in columns 4-6 were obtained using Poisson distribution, in a statistical simulation of infinite independent trials. *The highest chance of transferring 0 cfu were associated with lowest concentrations; and the probability of <1 cfu rose with increasing concentration*.

Serotype	Pool. type / meat	Conc. (cfu/ml)	Trans. cell (90% CI)	P: 0 cfu (%)	P: >1 cfu (%)	Detect. (%)
Dublin	Dry / beef	2,5	0-5	8,2	91,8	100
	Dry / pork	3,0	1-6	5,0	95,0	100
	Wet / beef	3,6	1-7	2,7	96,3	100
	Wet / pork	2,2	0-5	11,1	88,9	100
Enteritidis	Dry / beef	8,9	4-14	0,0	100,0	100
	Dry / pork	8,7	4-14	0,0	100,0	100
	Wet / beef	8,8	4-14	0,0	100,0	100
	Wet / pork	8,6	4-14	0,0	100,0	100
Typhimurium	Dry / beef	6,7	3-11	0,1	99,9	100
	Dry / pork	6,7	3-11	0,1	99,9	100
	Wet / beef	1,3	0-2	27,3	72,7	20
	Wet / beef *R*	6,7	3-11	0,1	99,9	100
	Wet / pork	3,2	1-6	4,0	96,0	100
Stockholm	Dry / beef	3,3	1-7	3,7	96,3	100
	Dry / pork	4,6	1-8	1,0	99,0	100
	Wet / beef	3,1	1-6	4,5	95,5	80
	Wet / pork	2,5	0-5	8,2	91,8	100

CI = confidence interval
R = rerun

3.3 Stress study to estimate the effects of freeze-drying on selective growth

Vials containing freeze-dried pure cultures of *S.* Dublin, *S.* Enteritidis, *S.* Typhimurium and *S.* Stockholm were available at the Microbiology Department of the National Food Agency. To experimentally assess the stress level caused by freeze-drying, the ability of each strain to grow on the selective agars Xylose-lysine-desoxycholate Agar (XLD) and Brilliance™ Salmonella Agar (BriS) was compared to growth on the non-selective Nutrient Agar (NA). The selective agars were chosen because they facilitate the growth of *Salmonella* while inhibiting the growth of other bacteria (39,41). NA, on the other hand, is used for general bacteria culturing (43).

In Figure 6, results of the stress evaluation are shown as percentage of growth (cfu/ml) on NA. Below 100 percent of the bacteria growing on the non-selective NA, also grew on the selective agars but at a lower level. The lowest recovery was observed in for XLD, the levels were lowest for *S.* Dublin (63%), followed by *S.* Enteritidis (75%), *S.* Typhimurium (87%), and *S.* Stockholm (94%) (Fig.6). There is less variation in results in terms of growth on BriS. However, there were only a few comparisons and the results should be considered only as general observations. An overall conclusion was that not all bacteria that is able to grow on NA could always be expected to grow on XLD and BriS.

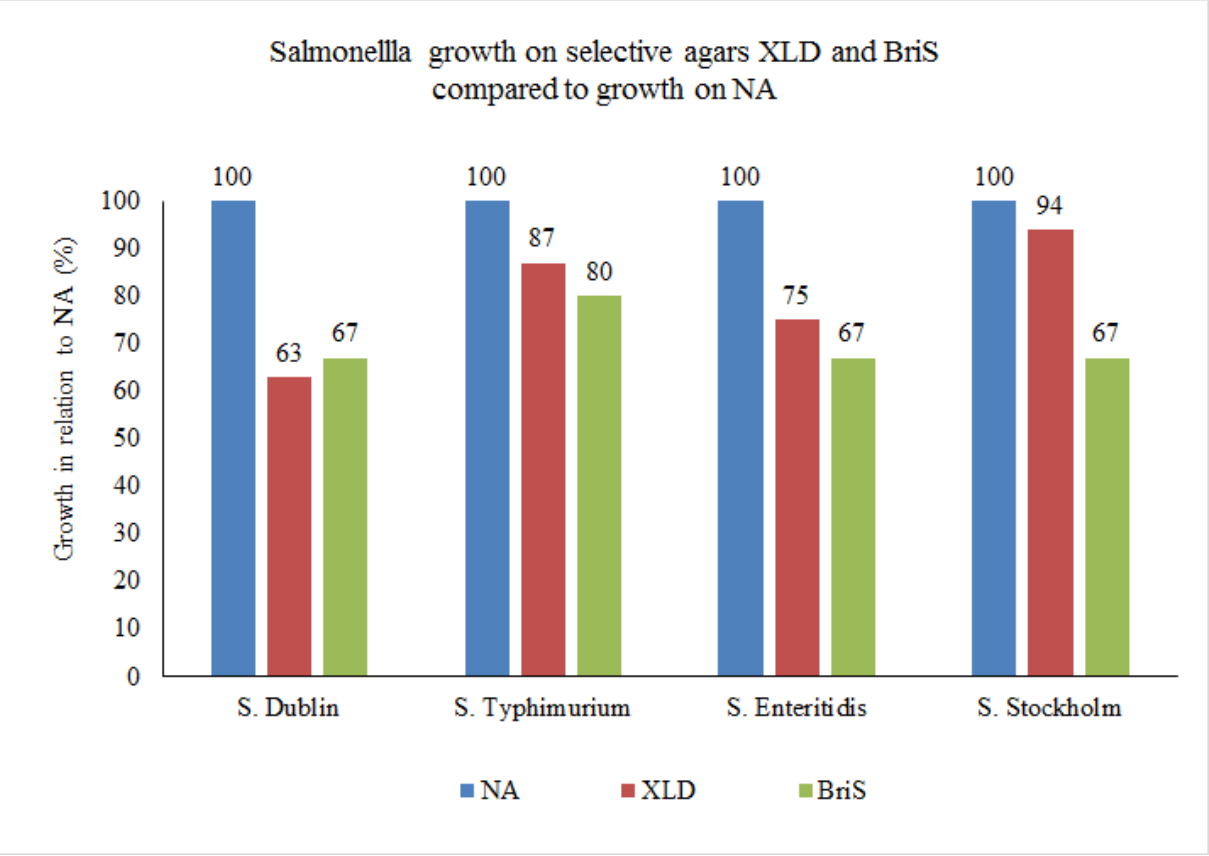

Figure 6. Growth of four Salmonella strains previously exposed to stress due to freeze-drying. The level of stress was investigated by cultivation on plates of Nutrient Agar (NA), Xylose-Lysine-Desoxycholate Agar (XLD) and Brilliance™ Salmonella agar (BriS) and subsequent comparison of growth on the non-selective NA and growth on the selective XLD and BriS agar. Statistical significance of results was not possible to determine. *Less bacteria grew on XLD and BriS in comparison to NA. Except for S. Dublin, a larger proportion of bacteria that grew on NA, also grew on XLD than in the case of BriS.*

4. Discussion

4.1 Sensitivity in pooled versus in individual samples

The aim of the project was to compare the sensitivity of the NMKL-71 method when pooling five samples compared to analysis of individual samples. The regulation on microbiological criteria requires the ability to detect a single *Salmonella* in 25g meat (20). For food producers, pooling must be used without compromising compliance with regulations, *i.e.* the same sensitivity should be achieved when analysing pooled samples.

Most data showed the same detection rate in pooled and in individual samples, indicating that pooling usually does not affect the analytical sensitivity. The lowest inoculation level resulting in 100 percent detection in pooled and individual samples was 3,0 and 2,5 cfu per 25g for dry- and wet-pooling respectively.

A question is whether 100 percent sensitivity is required for an acceptable test. According to the NordVal (part of the Nordic Committee on Food analysis) protocol for validation certification of alternative microbiological methods, 95 percent is an accepted sensitivity limit (44). This could be used as guidance for satisfactory sensitivity also in pooling of samples.

In the present study, sensitivity in pooling was below 95 percent at inoculation levels of 2,5 cfu per 25g in dry-pooling and of 2,2 cfu per 25g in wet-pooling, while the sensitivity was higher in corresponding individually spiked samples. Interestingly, both pooling of five swab samples from pig carcasses, and pooling of five samples from poultry indicated decreased sensitivity (29,33). In the scientific assessment of pooling, Jarvis states that pooling may put increased demands on the method's sensitivity because bacteria are present in a larger sample volume and pre-enrichment broth during pooling (24). Statistical modelling dictates a minimum bacterial concentration to ensure the transfer of one single bacteria (24). To obtain this concentration, the number of bacteria in a solution must increase proportionally with volume. The statistical uncertainty is also present when samples are transferred from the enrichment buffer to the selective plates (24).

The statistical modelling our results showed that there is a possibility that zero *Salmonella* cells may be transferred at concentrations below 3 cfu per ml. For results where there was difference between pooled and individual samples. If the loss in sensitivity would have been caused by statistical uncertainty during spiking, it would have affected both pooled and individual samples. The difference in sensitivity between pooled and invidual samples is more likely to be a consequence of statistical uncertainty introduced by the large volumes used during pooling rather than the possibility of spiking zero *Salmonella* cells.

4.2. Comparison between dry-pooling and wet-pooling

According to Jarvis, wet-pooling is preferable to dry-pooling, since samples are pre-enriched separately in this approach (24). Thus, pre-enrichment in a large volume is avoided, which eliminates the main source of limitation to sensitivity (24). In addition, the handling of large volumes of *Salmonella* contaminated material is practically difficult and involves contamination risks for the involved laboratory personnel.

In the present study, the detection of *Salmonella* was equally successful in both pooling methods when comparing dry- and wet-pooling at similar inoculation levels. However, this

observation is limited to 3 cfu per 25g and higher. At 2,5 cfu per 25g, detection rate was higher in wet-pooled samples than dry-pooled samples. For wet-pooling the lowest tested inoculation levels were 2,2 cfu per 25g and 1,3 cfu per 25g. A lower level than 2,5 cfu per 25g was never investigated for dry-pooling. This study suggests that the two pooling methods perform equally well, but further studies are needed to verify this at lower contamination levels than 3 cfu per 25g.

4.3. Methodological considerations

Inoculation with 1,3 cfu per 25g resulted in 20 percent positive samples both in pooled and individually spiked samples. The pooled samples and the positive controls, that were shown not to contain *Salmonella*, are from the same spiked sample. Therefore, the negative results could have a common explanation. The risk of transferring zero *Salmonella* during several independent pipettings from a suspension with the concentration of 1,3 cfu/ml is as high as 27,3 percent. This greatly exceeds the risks involved with higher concentrations., These results suggest uncertainty in the success of spiking with 1 cfu per 25g, and could explain the 20 percent detection rate when spiking with 1,3 cfu per 25g

There are also other relevant methodological considerations. In dry-pooling, there was only one positive control per five poolings. Consequently, the control's value of one fifth corresponded to each pooling. A better approach would be to include one positive and one negative control per each pooling batch, just as in wet-pooling.

Furthermore, colony isolation is important. XLD agar selects for H_2S-positive *Salmonella* that do not ferment lactose and saccharose (39,40). BriS and MLCB were chosen as supplementary agars to allow detection of variants with a broader biochemical profile including lactose and saccharose fermenters (40,41). Even so, isolation of typical colonies was sometimes not possible, and most frequently on XLD agar. Excessive growth of background flora on plates may result in lack of single *Salmonella* colonies well isolated from the background, if at all.

The RVS enrichment broth is composed to fit the growth conditions of *Salmonella* more than other *Enterobacteriaceae* e.g. *Escherichia coli* and *Klebsiella* (45), but does not completely exclude the growth of the latter. Here, yellow colonies that could belong to *Enterobacteriaceae* (40), were frequently observed on XLD agar. Background flora was also noticed on BriS and MLCB. Overall, these observations of high background flora were more often made in experiments with high levels of *Enterobacteriaceae* in the meat. Thus, it is likely that the level of *Enterobacteriaceae* can affect the ability to isolate *Salmonella* colonies.

4.4 Significance of background flora

Our results showed that the aerobic plate count (APC) was 7 log cfu/g, and level of *Enterobacteriaceae* was around 5 log cfu/ml at most. These measurements are comparable to Mooijman's findings in poultry (33).

In previous reports, *Enterobacteriaceae* and aerobic bacteria were found in higher numbers on beef with high fat content compared to lean beef (46). Hence, more of background flora should be present on pork carcasses than on beef, as pork contains more fat (47,48). Here, the aerobic flora was consistently somewhat higher in pork than in beef. Surprisingly, the level of *Enterobacteriaceae* was similar in pork and beef.

The pre-enrichment step is essential for the recovery of sub-lethally injured *Salmonella* (21). The efficiency of recovery and the overall increase in growth might be affected by broth growth support of both the target organism and the competing background flora. This might influence the detection of low numbers of *Salmonella* in the contaminated food. The results of this study show that in experiments where meat had different level of background flora, but were inoculated with similar numbers of *S.* Enteritidis, there was no difference in the success of Salmonella detection. This could be because the inoculation level was relatively high, 8-9 cfu per 25g. However, *Salmonella* was confirmed in five pooled samples in experiments with *S.* Dublin and *S.* Typhimurium, even at relatively low inoculation level of around 3 cfu per 25g in combination with relatively high background flora. These observations, suggest that background flora level does not have an apparent influence on *Salmonella* detection in ground meat.

4.5 Were the *Salmonella* strains sufficiently stressed?

Deviation from the conditions a microorganism is specially adapted to can inflict stress on it (49). This commonly leads to compensatory responses, which promote survival rather than growth (49). Carcasses are tenderized by hanging in 2-4℃ for minimum 7 days (50,51), and the cold and low water content during this process could cause stress to the bacteria on the meat.

Freeze-dried cultures of *Salmonella* were chosen since they may have experienced stress levels similar to natural conditions. In the present study, the effects of stress were assessed based on the bacteria's ability to grow in selective media compared to non-selective media. The selectivity of XLD relies on *Salmonella*'s resilience to the inhibitor sodium desoxycholate (40). *Salmonella* is differentiated from other bacteria on XLD and BriS through degradation of chromogens by specific enzymes in the bacteria (40,41). In some microorganisms, stress response to cold alter the genetic expression that influence biochemical processes amongst others (49). Therefore, freeze-drying might have changed the ability of *Salmonella* to biochemically interact with the components of the agar. Supporting this idea, lower numbers of *Salmonella* grew on the selective agars compared to the non-selective.

During freeze-drying, the concentrated bacterial suspension is first frozen, then the water is removed from the frozen matrix by sublimation of ice (52). Depending on the freezing temperature, ice crystals of different sizes may form (52), and could injure the bacteria. All considered, this process seems to be sufficient to mimic meat storage conditions, and even exceeds them in severity. This idea is reminiscent of Mooijman's (34) report of storage at a lower temperature (-20℃) resulting in a higher amount of stress than storage at 2-4℃.

4.6 Conclusions

- The majority of findings showed equal sensitivity in pooled and individually spiked samples.

- *Salmonella* detection in pooled samples is limited. Loss of sensitivity in pooled samples compared to individual samples was observed below 3,0 cfu per 25g for dry-pooling, and below 2,5 cfu per 25g for wet-pooling.

- The two pooling methods performed equally well above 3 cfu per 25g. At 2,5 cfu per 25g, wet-pooling was better than dry-pooling.

- Statistical modelling revealed that bacteria may be absent when transferring bacteria from a suspension with concentration less than 3 cfu/ml.

- Level of *Enterobacteriaceae* was similar in packages of ground pork and ground beef, while there were more aerobic bacteria in the pork. The background flora did not affect *Salmonella* detection in pooled samples, indicating that contamination level is the more important.

- Freeze-drying of *Salmonella* strains is suitable to mimic stress caused by conditions in the manufacturing process of meat.

4.7 Future prospects

- Only the pooling of five samples was investigated whereas ISO 6579/2002 states that pooling up to ten samples is allowed if the sensitivity is equal to testing individual samples. Pooling of ten samples was excluded because it was associated with many practical restrictions. For instance, handling of large volumes (> 2,5 litres BPW x 5) of *Salmonella* contaminated material could not be done within the framework of our study. With the necessary laboratory resources available, a new study could be designed where pooling of ten samples could be investigated instead of five.

- There is high uncertainty of transferring one bacteria during inoculation with less than 3,0 cfu per 25g. Still, a possible idea would be to explore the lowest limits of inoculation further than the scope of this study. Then, perhaps the lowest possible inoculation level could be included in new pooling experiment and tested in a higher number of samples to account for statistical variables.

- Another suggestion for the future is further comparison of dry- and wet-pooling where experimental conditions are identical for both approaches.

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References

1. International Commission on Microbial Specifications for Foods (ICMSF). Microorganisms in Foods 5: Characteristics of microbial pathogens. 1st edition. London: Blackie Academic & Professional; 1996.

2. Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. Salmonella Nomenclature. J Clin Microbiol. 2000;38(7):2465–7.

3. Chen H-M, Wang Y, Su L-H, Chiu C-H. Nontyphoid salmonella infection: microbiology, clinical features, and antimicrobial therapy. Pediatr Neonatol. 2013;54(3):147–52.

4. National Veterinary Institute (SVA). Allmänt om salmonella - SVA [homepage on the Internet]. No date [cited 2014 April 22]. Available from: http://www.sva.se/sv/Mer-om-SVA1/Salmonellaportal/Allmant-om-salmonella/

5. Uzzau S, Brown DJ, Wallis T, Rubino S, Leori G, Bernard S, et al. Host adapted serotypes of Salmonella enterica. Epidemiol Infect. 2000;125(2):229–55.

6. European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. EFSA Journal. 2014;12(2):3547.

7. European Centre for Disease Prevention and Control (ECDC). Annual epidemiological report 2013. Reporting on 2011 surveillance data and 2012 epidemic intelligence data [document on the Internet]. ECDC; 2013 [cited 2014 April 23]. Available from: http://www.ecdc.europa.eu/en/publications/Publications/annual-epidemiological-report-2013.pdf

8. Fierer J, Guiney DG. Diverse virulence traits underlying different clinical outcomes of Salmonella infection. J Clin Invest. 2001;107(7):775–80.

9. Jones TF, Ingram LA, Cieslak PR, Vugia DJ, Tobin-D'Angelo M, Hurd S, et al. Salmonellosis Outcomes Differ Substantially by Serotype. J Infect Dis. 2008;198(1):109–14.

10. National Veterinary Institute (SVA). Surveillance of infectious diseases in animals and humans in Sweden 2012 . Uppsala, Sweden; SVA report series no 26 ISSN 1654-709811.

11. National Veterinary Institute (SVA). Surveillance of zoonotic and other animal disease agents in Sweden 2010 . Uppsala, Sweden; SVA report series no 22 ISSN 1654-7098.

12. The National Board of Health and Welfare. Salmonella – ett nationellt strategidokument [Document on the Internet] The National Board of Health and Welfare; 2013 [Cited 2015 April 1]. Available from www.socialstyrelsen.se

13. Falkenhorst G, Simonsen J, Ceper TH, van Pelt W, de Valk H, Sadkowska-Todys M, et al. Serological cross-sectional studies on salmonella incidence in eight European countries: no correlation with incidence of reported cases. BMC Public Health. 2012;12(1):523.

14. Comission regulation (EC) No 1688/2005 of 14 October 2005 implementing Regulation (EC) No 853/2004 of the European Parliament and the Council as regards special guarantees concerning salmonella for consigment to Finland and Sweden of certain meat and eggs.

15. National Food Agency (SLV). Kontrollhandbok Provtagning - Del 6 Provtagning för kontroll av salmonella [document on the Internet]. National Food Agency (SLV); 2014 [cited 2014 May 05]. Available from: http://www.slv.se/upload/dokument/livsmedelsforetag/vagledningar/kontrollhandbok-provtagning-del-6.pdf

16. Regulation (EC) No 178/2002 of the European Parliament and the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety

17. Livsmedelslag, SFS 2006:804 [statute on the Internet]. Sveriges Riksdag; 2006. [Cited 2014 April 21]. Available from: http://rkrattsdb.gov.se/SFSdoc/06/060804.PDF

18. Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs.

19. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs.

20. Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuff.

21. Nordic Committee on Food Analysis. Nordic Committee on Food Analysis - Method No 71, 5th edition [document on the Internet]. Nordic Committee on Food Analysis; 1999 [cited 2014 April 0101]. Available from: http://www.nmkl.org/ABONNEMENT/METPDF/71sk.PDF

22. International Organization for Standardization (ISO). Standard ISO 6579/2002. Microbiology of food and animal feeding stuff -- Horizontal method for detection of Salmonella spp. Geneva, Switzerland: ISO;2002

23. National Food Agency (SLV). Så kontrolleras din verksamhet - Livsmedelsverket [homepage on the Internet]. No date [cited 2014 May 05]. Available from: http://www.slv.se/sv/grupp1/livsmedelsforetag/sa_kontrolleras_din_verksamhet/

24. Jarvis B. On the compositing of samples for qualitative microbiological testing. Lett Appl Microbiol. 2007;45(6):592–8.

25. Basil Jarvis. Proposal to revise ISO 7218 by inclusion of a statement and procedure for compositing of food samples for qualitative tests. Working Group 2 Statistics of ISO/TC 34/SC 9, International Organization for Standardization (ISO), 2012 (Unpublished)

26. Monica Sundelin. Laboratorierutiner vid poolning av prover i samband med mikrobiologisk analys av livsmedel [personal communication]. 2014. (Unpublished)

27. Forsell J. Frågor kring poolning av prover vid mikrobiologisk analys av livsmedel [e-mail correspondence]. 2014. (Unpublished)

28. Lidborg C. Poolning, Salmonella [e-mail correspondence]. 2014. (Unpublished)

29. Sørensen LL, Wachmann H, Alban L. Estimation of Salmonella prevalence on individual-level based upon pooled swab samples from swine carcasses. Vet Microbiol. 2007;119(2-4):213–20.

30. Mooijman K. The 17th EURL-Salmonella workshop - 14 and 15 May 2012, Chalkida, Greece, RIVM report 330604026/2012 [document on the Internet]. National Institute for Public Health and the Environment (RIVM); 2012. [Cited 2014 March 13]. Available from: http://www.rivm.nl/dsresource?objectid=rivmp:193187&type=org&disposition=inline&ns_nc=1

31. Food Safety and Inspection Service, United Sates Department of Agriculture. Ground Beef and Food Safety [homepage on the Internet]. 2014 [cited 2014 May]. Available from: http://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/meat-preparation/ground-beef-and-food-safety/ct_index

32. Jarvis B. Statistical Aspects of the Microbiological Examination of Foods. Academic Press; 2008.

33. Mooijman K. ISO and CEN activities for Salmonella [presentation on the Internet]. 2012 [cited 2014 March 20]. Available from: http://www.eurlsalmonella.eu/Workshops/Archive_Workshops/Workshop_2012

34. Nordic Committee on Food Analysis. Nordic Committee on Food Analysis - Method No 144; 3rd edition [document on the Internet]. Nordic Committee on Food Analysis; 2005 [2014 cited April 18]. Available from: http://www.nmkl.org/ABONNEMENT/METPDF/144sk3utg.pdf

35. Nordic Committee on Food Analysis. Nordic Committee on Food Analysis - Method No 86, 5th edition [document on the Internet]. Nordic Committee on Food Analysis; 2013 [cited 2014 April 18]. Available from: http://www.nmkl.org/ABONNEMENT/METPDF/86sk5utg2013.pdf

36. Murray PR, Rosenthal KS, Pfaller MA. Medical Microbiology. 6th edition. Philadelphia: Elsevier Mosby; 2009

37. Lindblad M. Mikroprofil Nötkreatur - Kartläggning av mikroorganismer i slaktkroppar, SLV Report No. 1/2008 [document on the Internet]. National Food Agency (SLV), 2008 [cited 2014 April 03]. Available from: http://www.slv.se/upload/dokument/rapporter/bakterier_virus_mogel/2008_livsmedelsverket_1_mikroprofil_notkreatur.pdf

38. Lindblad M. Mikroprofil Gris - Kartläggning av mikroorganismer på slaktkroppar, SLV Rapport No. 1/2006. [document on the Internet]. National Food Agency (SLV), 2006 [Cited 2014 May 05]. Available from: http://www.slv.se/upload/dokument/rapporter/bakterier_virus_mogel/Mikroprofil%20Gris%20SLV%20Rapport%201%202006.pdf

39. Danielsson-Tham M-L. Några vanliga biokemiska tester inom bakteriologin. 3rd revised edition. Uppsala: Sveriges lantbruksuniversitet; 1993.

40. Oxoid Ltd. CM0469, X.L.D. Agar | Oxoid - Product Detail [homepage on the Internet]. 2014 [cited 2014 April 22]. Available from: http://www.oxoid.com/uk/blue/prod_detail/prod_detail.asp?pr=CM0469&org=124&c=uk&lang=en

41. Oxoid Ltd. CM1092, Brilliance Salmonella Agar | Oxoid - Product Detail [homepage on the Internet]. 2014 [cited 2014 May 06]. Available from: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM1092&sec=&org=124&c=UK&lang=EN

42. Oxoid Ltd. CM1092, MLCB Agar | Oxoid - Product Detail [homepage on the Internet]. 2014 [cited 2014 May 07]. Available from: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0783&org=124&c=UK&lang=EN

43. Oxoid Ltd. CM0003 Nutrient Agar | Oxoid - Product Detail [homepage on the Internet]. 2014 [cited 2014 May 07]. Available from: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0003

44. NordVal. Protocol for the validation of alternative microbiological methods [document on the Internet]. Nordic Committee on Food Analysis (NMKL), 2009 [Cited 2014 June 05]. Available from: http://www.nmkl.org/dokumenter/nordval/NordValProtocol.pdf

45. Oxoid Ltd. CM0866, Rappaport-Vassiliadis Soya Peptone Broth (RVS Broth) | Oxoid - Product Detail [homepahe on the Internet]. 2014 [cited 2014 May 05]. Available from: http://www.oxoid.com/uk/blue/prod_detail/prod_detail.asp?pr=CM0866&org=124&c=uk&lang=en

46. Borch E, Kant-Muermans M-L, Blixt Y. Bacterial spoilage of meat and cured meat products. Int J Food Microbiol. 1996;33(1):103–20.

47. Svenskt kött i Sverige AB. Nötfärs [homepage on the Internet]. 2014 [cited 2014 May 06]. Available from: http://www.svensktkott.se/om-kott/styckdetaljer/not/fars/

48. Svenskt kött i Sverige AB. Färs [homepage on the Internet].2014 [cited 2014 May 06]. Available from: http://www.svensktkott.se/om-kott/styckdetaljer/gris/fars/

49. Beales N. Adaptation of Microorganisms to Cold Temperatures, Weak Acid Preservatives, Low pH, and Osmotic Stress: A Review. Compr Rev Food Sci Food Saf. 2004;3(1):1–20.

50. Lundkovallkött Society. Hängmörning - [homepage on the Internet]. 2014 [cited 2014 May 22]. Available from: http://www.lundkovallkott.se/text1_18.html

51. Svenskt Kött i Sverige AB. Nöt [homepage on the Internet]. 2014 [cited 2014 April 16]. Available from: http://www.svensktkott.se/om-kott/styckdetaljer/not/

52. Labconco. A Guide to Freeze Drying for the Laboratory [homepage on the Internet]. 2014 [cited 2014 May 02]. Available from: http://chiron.no/pdf/Labconco_guide_to_freeze_drying.pdf