

Growth and survival of exponential and stationary phase *Salmonella* during sausage fermentation

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Abstract

When raw meat is contaminated with enteropathogens, the growth state may appear in a mixture of phases. Survival for exponential and stationary phase cells differs, with stationary phase cells being generally more resistant. Our aim of this study was to investigate the survival of exponential and stationary phase *Salmonella* during freezing and to follow the survival/growth of these cells during subsequent sausage fermentation. Minced meat was inoculated with exponential and stationary phase *Salmonella* Typhimurium, respectively, and frozen at -20°C for up to 35 days. The meat was thawed overnight at 5°C prior to sausage production. The sausages were fermented at 25°C and growth/survival was observed for 3 days. In the freezing period before sausage production, no reduction of stationary phase cells was observed after more than 35 days whereas exponential phase cells were reduced more than 1.5 log₁₀ units. Despite this reduction, exponential phase cells were able to grow to the same level as the stationary phase cells during fermentation of sausages simulating failure of the starter culture. However, the pH drop caused by the starter culture prevented growth of both exponential as well as stationary phase *Salmonella*. These results show that failure of a starter culture to lower pH may lead to growth of *Salmonella*, independent of the phases.

Introduction

During slaughter raw meat will occasionally be contaminated with enteropathogens such as *Salmonella* and *Yersinia*. This can be a food safety problem in raw fermented sausages. Several *Salmonella* outbreaks have been linked to fermented sausages where epidemiological and molecular subtyping studies confirmed the etiology (Bremer et al., 2004, Nygård et al., 2007, Bone et al., 2010).

The growth state of the bacteria during contamination of meat is unknown, but they may appear in a mixture of phases. The ability to respond to environmental changes is fundamental for bacterial survival. The stress sensitivity between exponential and stationary phase cells may be different. An acid challenge experiment in juice at different temperatures showed e.g. that the stationary phase cells were considerably more resistant than exponential growing cells and that the low temperature protection was only observed for the stationary cells (Gawande 2002).

Freezing of meat for sausage production arrest bacterial growth but it is questionable whether it can be used as a decontamination step as only 0.5 log unit reduction of stationary phase *Salmonella* Typhimurium was observed after freezing for 10 weeks at -20°C (Barrel, 1988).

In this study the aim was to investigate: 1) the survival of exponential and stationary phase *Salmonella* during freezing in minced meat and 2) survival/growth of exponential and stationary cells during sausage fermentation in sausages made of meat that had been frozen for different periods.

Material and Methods

Bacterial strains and preparation of inocula. *Salmonella enterica* serotype Typhimurium DT12 and *Salmonella enterica* serotype Typhimurium U292 isolated from an outbreak 2009 in Denmark were used. From stock culture, one loop full of culture was streaked onto blood agar plates and incubated over night at 37°C and then sub-cultivated by transferring one loop full of single colonies to 20 ml Luria Broth (LB). After 19 hours of incubation at 37 °C, the cells had reached stationary phase and was used directly for sausage production after dilution. To adjust the number of bacteria, the stationary culture was first diluted to OD₆₀₀ = 0.1, which corresponds to a cell level of approximately 8.4 log₁₀ cfu/ml. This culture was diluted to 7 log₁₀ cfu/ml and was used as inoculum. For preparation of exponential cells, the 19 hours culture was diluted to 4 log₁₀ cfu/ml. Subsequently the culture was grown for about 4.5 hours until the cells had reached 7 log₁₀

cfu/ml (OD600 = 0.05) and immediately after used as inoculums for sausage production.

Sausage production. For sausage production, minced meat, with a fat content of 16-20%, was purchased from a local shop. The procedure for sausage production for exponential and stationary cells was identical. Initially, 1200 g minced meat was added an inoculum of 24 ml of exponential and stationary cells, respectively, resulting in a start level of approximately $4.5 \log_{10}$ cfu. Then the inoculated meat was divided into bags with 300 g each. One bag was immediately used for sausage production; the other three bags were frozen at -18°C for 0, 7, and < 35 days, respectively, before sausage production.

For the sausage production the final batter consisted the Salmonella inoculated meat with the following ingredients added: NaCl (3 wt/wt %), dextrose (0.7%) dextrose and Na-nitrite (0 or 100 ppm) varied. Then the batter was divided into two portions: 150 g batter added starter culture (F-1 Bactoform containing a mixed culture of *Staphylococcus xylosum* and *Pediococcus pentosaceus*, Chr. Hansen) and 150 g batter was without starter culture. The sausage batter was then stuffed into sterile syringes with removed tip and incubated at $24-25^{\circ}\text{C}$ (standard fermentation temperature) ad modum Heir et al. (2010). The pH decline was measured during the fermentation.

Microbial analysis and sampling. To analyse the effect of freezing of the raw meat, 5 g meat was macerated by stomaching in 15 ml 0.9% NaCl for 2 min. Tenfold dilutions were prepared in 0.9% NaCl and colony forming units were determined on Salmonella selective Xylose Lysine Deoxycholate (XLD) agar plates by spotting 3 times $10 \mu\text{l}$ from appropriate dilutions.

Enumeration of Salmonella during sausage fermentation was performed at day 0 and two times a day the following three days. The plungers push a piece of sausage out of the syringe for each sampling. The first 0.5 cm sausage was discarded. A sample of 5 g of sausage was cut off and transferred into a stomacher bag with 15 ml 0.9% NaCl and treated as in the freezing experiment. All plates were incubated at 37°C for approximately 24 hours.

Results

The effect of freezing (-18°C) is illustrated in figure 1 for the two phage types of *S. Typhimurium*, DT12 and U292. Exponential cells were noticeable more sensitive than stationary cells and also a difference between the phage types was observed after 7 days. Exponential DT12 cells were reduced approximately $0.6 \log_{10}$ units whereas exponential U292 cells were reduced approximately $0.9 \log_{10}$ units. However, after more than 35 days of freezing the no difference could be measured. The stationary cells were marginally affected by the freezing with only approximately $0.1 \log_{10}$ unit reduction.

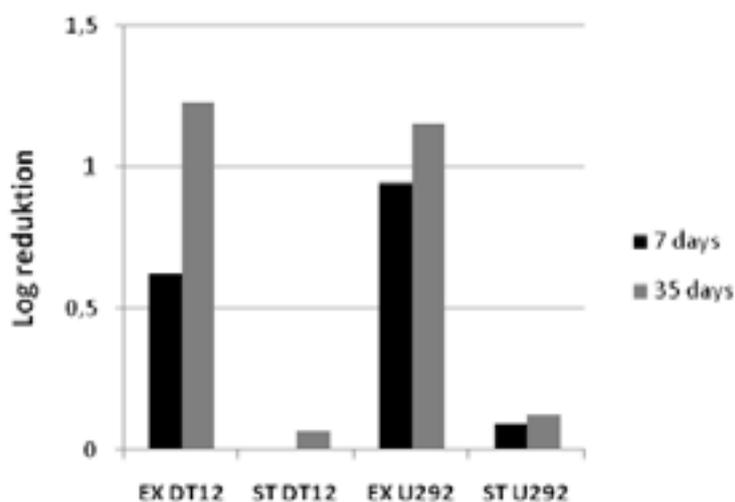


Figure 1. The effect of freezing on stationary and exponential *Salmonella Typhimurium* cells in minced meat. EX: exponential cells, ST: stationary cells. Black columns: 7 days of freezing, grey columns: more than 35 days of freezing.

The growth/survival of *Salmonella Typhimurium* DT12 during sausage fermentation at 25°C is outlined in figure 2. Minced meat that was inoculated with *Salmonella* and then frozen for 7 and < 35 days, respectively, was used for sausage production. Comparison of parameters such as with/without starter culture and exponential/stationary cells on growth/survival of *Salmonella* were analysed. Sausage batter added starter culture, prevented growth or slightly reduced (max. $1 \log_{10}$ unit) both exponential and stationary cells during fermentation, independently of the freezing period of the meat.

Freezing the meat for more than 35 days reduced the number of exponential cells with at least 1 log₁₀ unit whereas the number of stationary cells was unchanged. When fermenting batter with no starter culture, the number of exponential cells started more than 1 log₁₀ units lower than the stationary cells, but they grew to the same level during three days of fermentation. The same growth/survival pattern was observed for U292 during sausage fermentation. Addition of NaNitrite did not affect Salmonella (data not shown).

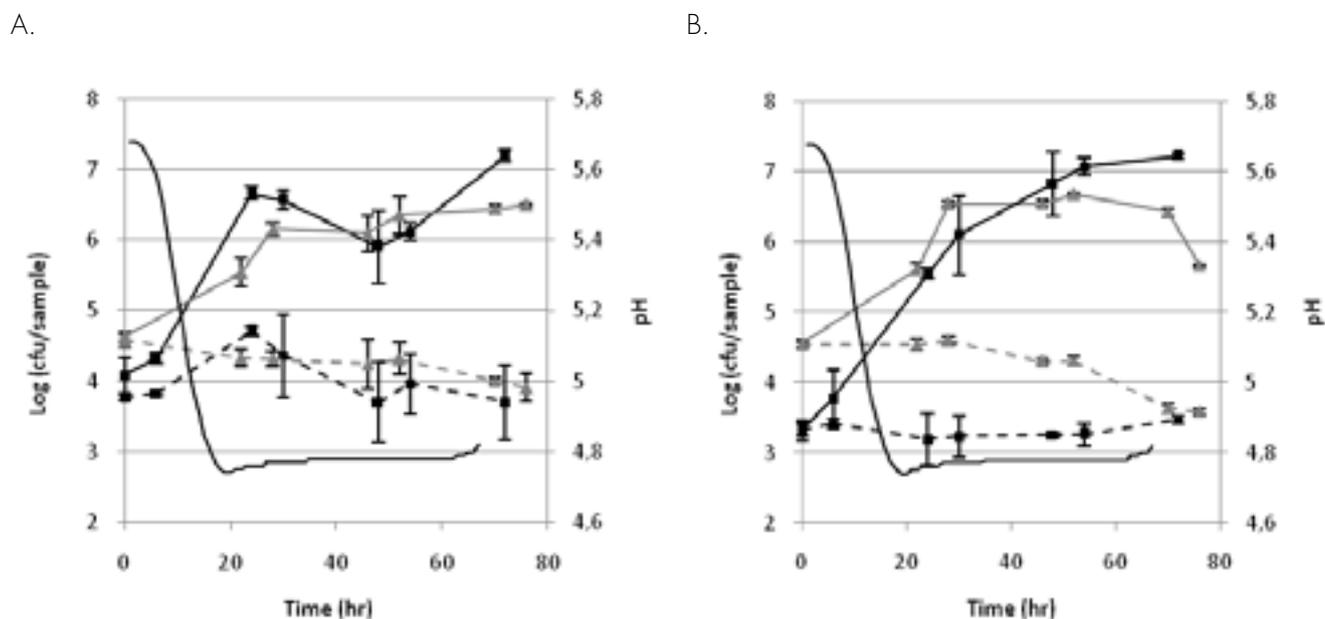


Figure 2. Growth/survival of *Salmonella Typhimurium* DT12 during sausage fermentation at 25°C without NaNitrite. Unbroken lines: sausages with no starter culture, dotted lines: sausage with starter culture, black square: exponential cells, grey triangle: stationary cells, black line: pH measurement. A) and B) sausages made of meat that has been frozen for 7 and more than 35, respectively.

Discussion

Exponential cells are more sensitive to freezing than the stationary cells in minced meat. This observation was also found in a previously freeze-thaw treatment of *Salmonella* in chicken exudates (Obafemi et al, 1986). Freezing minced meat before sausage production has a relatively minimal effect if the bacteria primarily are in the stationary phase. Thus, the freezing of raw material routine used in production of fermented sausage seems ineffective as an intervention.

Fermenting sausages with no starter culture should mimic failure of fermentation. Under these condition *Salmonella* are able to multiply and grow to a high level during the fermentation at 24-25°C. However, the subsequent drying process of the sausage has shown to reduce the number of *Salmonella*. A modeling study, calculated that *Salmonella* can be reduced by 0.3 to 2.4 log₁₀ during the sausage drying process.

Conclusion

It can be concluded that:

- Exponential cells are more sensitive to freezing than stationary cells in a food matrix.
- Growth of starter culture (sausage fermentation) arrest growth of both exponential and stationary cells.
- Failure fermentation can lead to growth of both exponential and stationary cells.

References

- BARRELL, R. A. (1988). The survival and recovery of salmonella typhimurium phage type U285 in frozen meats and tryptone soya yeast extract broth. *International Journal of Food Microbiology* 6, 309-16.
- BONE, A., H. NOEL, S. LE HELLO, N. PIHIER, C. DANAN, M. E. RAUENAUD, S. SALAH, et al. (2010). Nationwide outbreak of salmonella enterica serotype 4,12:l:- infections in france, linked to dried pork sausage, march-may 2010. *Euro Surveillace : Bulletin European Sur Les Maladies Transmissibles = European Communicable Disease Bulletin*, 15, 19592.
- BREMER, V., K. LEITMEYER, E. JENSEN, U. METZEL, H. MECZULAT, E. WEISE, D. WERBER, et al. (2004). Outbreak of salmonella goldcoast infections linked to consumption of fermented sausage, germany 2001. *Epidemiology and*

Infection, 132, 881-7.

GAWANDE, P. V., and A. A. BHAGWAT. (2002). Protective effects of cold temperature and surface-contact on acid tolerance of salmonella spp. *Journal of Applied Microbiology*, 93, 689-96.

HEIR, E., A. L. HOLCK, M. K. OMER, O. ALVSEIKE, M. HOY, I. MAGE, and L. AXELSSON. (2010). Reduction of verotoxigenic escherichia coli by process and recipe optimisation in dry-fermented sausages. *International Journal of Food Microbiology*, 141, 195-202.

NYGARD, K., B. A. LINDSTEDT, W. WAHL, L. JENSVOLL, C. KJELSO, K. MOLBAK, M. TORPDAHL, and G. KAPPERUD. (2007). Outbreak of salmonella typhimurium infection traced to imported cured sausage using MLVA-subtyping. *Euro Surveillance : Bulletin Europeen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin*, 12, E070315.5.

OBAFEMI, A., and R. DAVIES. (1986). The destruction of salmonella typhimurium in chicken exudate by different freeze-thaw treatments. *The Journal of Applied Bacteriology*, 60, 381-7.