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Inactivation of *Escherichia coli*, *Listeria monocytogenes* and *Yersinia enterocolitica* in fermented sausages during maturation/storage

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ABSTRACT

The purpose of this study was to evaluate maturation and storage conditions as a way to increase the safety of non-heat treated fermented sausages. The specific objectives were to investigate the effects of storage time and temperature on the levels of *Escherichia coli*, *Listeria monocytogenes* and *Yersinia enterocolitica* in fermented sausages and in broth, and to validate how well the broth experiments and some published models can predict inactivation in sausage. One strain each of *E. coli*, *L. monocytogenes* and *Y. enterocolitica* with induced acid tolerance was inoculated into sausage batters representing a typical Swedish recipe for cold-smoked sausages. The sausages were fermented at 27 °C for 39 or 48 h and then stored at different temperatures (8, 15, or 20–22 °C) for up to 44 days. The levels of the experimental strains, lactic acid bacteria, and pH, a_w, and lactic acid was measured during the maturation/storage period. Inactivation in BHI broths adjusted to pH 4.4 or 4.6, water activity of 0.93, and with 1, 1.3 or 2% lactic acid added was also studied. For all strains inactivation rates increased with temperature in both broths and sausages. At 8 °C the storage time required for a one-log reduction in sausage ranged from 21 days for *E. coli*, >16 days for *L. monocytogenes*, to 18 days for *Y. enterocolitica*. At temperatures of 20 °C or more, the storage time needed for a one log reduction was shorter: between 7 to 11 days for *E. coli*, 4 to 7 days for *L. monocytogenes*, and 1 to 4 days for *Y. enterocolitica*. A published model based on temperature only yielded a good prediction of *E. coli* inactivation in sausage. A linear model based on the rate estimated in broth yielded a fair prediction of *L. monocytogenes* inactivation. The performance of other inactivation models validated was unsatisfactory. Significant *E. coli* growth which occurred in batters without salt during initial phases of fermentation resulted in a subsequent increased inactivation rate, possibly due to increased susceptibility to stress of exponential phase bacteria. The results indicate that the practice of utilising a short maturation period and storage at refrigeration temperatures may result in unsatisfactory reductions of pathogens if present. Thus, inclusion of a maturation period above refrigeration temperatures before distribution may increase the safety of these products.
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1. Introduction

Fermentation of raw materials to improve the safety, shelf life and acceptability of foods has a long tradition. In fermented sausages produced from raw meat without any heat treatment, conditions resulting from fermentation generally inhibit the growth of most pathogens due to a combination of several factors, e.g. pH, lactic acid, water activity. However, studies have indicated that several bacteria including *Escherichia coli*, *Salmonella* spp. and *Listeria* spp. (Glass and Doyle, 1989; Glass et al., 1992; Whiting, 1993; Calicioglu et al., 2002), can survive in these products and, thus, may not be completely eliminated during the production process. Shigatoxin producing *E. coli* (STEC) O157:H7 exhibiting high acid tolerance and high virulence, e.g. a low ID_{50} dose (infectious dose to 50% of exposed individuals), have been considered especially important in this respect and can pose a significant problem. Although fermented sausages generally enjoy a well-founded reputation for safety, notable outbreaks of foodborne illness associated with fermented sausages have occurred. Examples include salmonella and raw fermented sausage (Bremer et al., 2004), STEC O157:H7 and dry-cured salami (Anonymous, 1995), STEC O111:H- and semi-dry fermented sausage (mettwurst)(Henning et al., 1998), and STEC O103 and mörrpölse (Schimmer et al., 2008).

During fermentation and drying of fermented sausages, levels of *E. coli* O157:H7 have been shown to decrease about 1 to 2 log units from initial inoculated levels (Hinkens et al., 1996; Faith et al., 1997; Faith et al., 1998; Riordan et al., 1998; Muthukumarasamy and Holley, 2007; Naim et al., 2003). The inactivation increases with decreasing pH levels and increasing salt and nitrite levels (Riordan et al., 1998; Casey and Condon, 2000). Subsequent storage at low temperatures results in a low inactivation rate, whereas storage at ambient temperatures

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results in faster inactivation (Sharma et al., 2004; Faith et al., 1997; Faith et al., 1998; Calicioglu et al., 2001). For example, Faith et al. (1997) showed that a 5 log reduction of *E. coli* O157:H7 levels was obtained after storage of pepperoni slices under air at 21 °C for four weeks, whereas only a 2 log reduction was observed after storage at 4 °C for three months. Inactivation of *E. coli* O157:H7 over time during fermentation and drying of sausages has been modeled as a function of water activity and pH (Pond et al., 2001), or water activity, pH and allyl isothiocyanate added as a preservative (Palanichamy et al., 2008). There are also models describing the inactivation rate of *E. coli* O157:H7 in uncooked fermented sausages during fermentation, drying and storage as a function of temperature (Ross and Shadbolt, 2001), or temperature and water activity (Ross et al., 2004). In the Pathogen Modeling Program inactivation of *E. coli* O157:H7 can be predicted based on temperature, water activity, pH, concentration of lactic acid and nitrite level (PMP, 2007).

Inactivation of *Listeria monocytogenes* in various types of sausages is dependent on the same parameters as is inactivation of *E. coli* (Junttila et al., 1989; Farber et al., 1993; Nissen and Holck, 1998; Hew et al., 2005; Thevenot et al., 2005; Bonnet and Montville, 2005). In addition, much research has addressed the effects of interactions with starter cultures (Farber et al., 1993; Drosinos et al., 2006; Tyopponen et al., 2003; Benkerroum et al., 2005). Nissen and Holck (1998) reported better survival of *L. monocytogenes* than *E. coli* O157:H7 in Norwegian fermented dry sausage. Depending on the type of sausage inactivation may be most rapid during fermentation (Farber, et al., 1993) or during maturation/storage (Thevenot et al., 2005), and is slower for adapted, i.e. isolated from processing environment, than for non-adapted strains (Thevenot et al., 2005). Available predictive models describing inactivation of *L. monocytogens/innocua* under conditions typical of fermented sausages include factors such as pH, water activity, total lactic acid or undissociated lactic acid concentration (e.g. Buchanan et al., 1997; Hajmeer et al., 2005; Janssen et al. 2007). Drosinos et al. (2006) reported that in fermented sausages typical of four European countries inactivation during fermentation and ripening increased in the presence of a bacteriocin producing *Lactobacillus sakei* strain and was best described by the model of Baranyi and Roberts (1994). Inactivation models for *L. monocytogenes* are also included in PMP and the ComBase Modelling Toolbox (Combase Modelling Toolbox, 2008).

Few studies have investigated inactivation of *Yersinia enterocolitica* in fermented sausages but the importance of nitrite, starter cultures, pH and acidulants on inactivation has been reported. In Turkish dry fermented sausage (Sucuk) produced using starter culture and inoculated with about 5 log *Y. enterocolitica* per g, levels were below detection after 3 days of fermentation during which pH was reduced from 6.3 to 4.7. In contrast, in sausages without starter culture (final pH 5.6) levels of *Y. enterocolitica* were still detectable after 4 days of fermentation and 12 days of drying (Ceylan and Fung, 2000). To eliminate similar levels of *Y. enterocolitica* O:3 in fermented sausages within 28 days, addition of 80–120 mg nitrite/kg was required (Asplund et al., 1993). *Y. enterocolitica* persisted during the 35 days trial if less than 50 mg nitrite/kg was added (Asplund et al., 1993). A log-logistic model previously used to describe thermal inactivation of micro-organisms was used to predict survival of *Y. enterocolitica* (0:3) under conditions of sub-optimal growth temperature (0–23 °C) and growth inhibiting pH values achieved with different acidulants, including acetic, lactic, citric, and sulphuric acids (Little et al., 1994). Predictions for survival in mayonnaise agreed well with observations whereas survival was overestimated in natural yoghurt both at 20 and 4 °C (Little et al., 1994). Models taking not only pH and temperature into consideration but also the amount of lactic acid concentration (Virto et al., 2005) as well as the undissociated lactic concentration and microbiological interactions with starter cultures have also been presented (Vereecken et al., 2003; Janssen et al., 2006).

In 2002, an outbreak of STEC O157:H7 infection caused by consumption of cold-smoked fermented sausage occurred in Sweden (Sartz et al., 2008). This outbreak prompted work to improve our understanding of factors affecting inactivation of STEC and other pathogens during production of cold-smoked fermented sausages. The present study addresses the maturation/storage stage with the purpose to evaluate the potential of temperature and time as a way to improve the safety of these products. The objectives were to 1) investigate the effect of storage time and temperature on the inactivation of *E. coli, L. monocytogenes* and *Y. enterocolitica* in broth, 2) investigate the effect of storage temperature and time on the inactivation of these bacteria in fermented sausages, 3) evaluate how well broth experiments can predict inactivation in sausage, and 4) validate available inactivation models for these bacteria with data from inactivation in fermented sausages during maturation/storage.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Strain 683, a generic strain of *E. coli*, strain L8, a rifampicin resistant (rif^+) strain of *L. monocytogenes*, and strain Y5A, a rif^+ strain of *Y. enterocolitica*, were used in the experiments. The strains were selected (see below) from a collection of strains originating from various surveys carried out by the Swedish National Food Administration.

Strains were stored at −70 °C in TSB (Trypticase Soy Broth, Oxoid, England) with 20% glycerol. Prior to an experiment strains were inoculated on nutrient agar (NA) plates (Oxoid) and incubated for 24 h at 37 °C. Working cultures of strains with induced acid tolerance used to inoculate broths or sausages were prepared by the following procedure. A small amount of bacteria from a pure colony on the NA plate was inoculated into 50 ml of TSB+G (TSB supplemented with 1% glucose, filter sterilized) in an E-flask which was incubated overnight (20–24 h) at 37 °C. Addition of glucose to TSB broth has been reported to induce the acid tolerance response in *E. coli* and *L. monocytogenes* (Buchanan and Edelson, 1996; Samelis et al., 2003; Berry et al., 2004). A volume of 0.1 ml of the culture was added to 50 ml TSB+G, and the incubation was repeated. Finally, from this culture, 0.05 ml was added to 50 ml TSB+G (or 1% culture to the desired volume), and the incubation was repeated. The rifampicin resistant strains of *Listeria monocytogenes* and *Yersinia enterocolitica* were cultured using the same procedure but with rifampicin added to the TSB+G medium. Appropriate dilutions of the resulting culture suspensions were used in the experiments.

To facilitate the procedure for detecting *Listeria monocytogenes* and *Yersinia enterocolitica* in sausage, rifampicin-resistant strains were selected as described by Foegeding et al. (1992) and used in the experiments. A stock solution containing 250 mg Rifampicin (R3501, Sigma, Aldrich) in 20 ml methanol was prepared and stored in the dark. Four ml of this solution was added to 1 l autoclaved Tryptic Soy agar (TSA, Oxoid) after the temperature had decreased to less than 60 °C. The concentration of rifampicin in the resulting agar plates was 50 $\mu g\ l^{-1}$ and the plates were used to isolate resistant strains as well as to quantify survival of strains during storage of sausages. Colony morphology of the rif^+ strains was identical to that of the wild-type strains. Log reductions of rif^+ strains were the same as for the parent strains when incubated 20 h in BHI (Difco) medium (with 3% lactic acid, pH 4.3) and quantified on TSA-rif or TSA only. Rif-resistance in the strains was maintained following growth under non-selective conditions, i.e. sub-culturing in TSB with no rifampicin.

2.2. Microbial analyses

Ten g samples of sausage were homogenized for 2 min in 90 ml Peptone water (Oxoid) in Stomacher 400 filter bags (Seward, London, UK). Cell counts were determined using a spiral plater (Eddy Jet, IUL Instruments, Germany), or by manually spreading appropriate volumes of decimal dilutions of the homogenate or inoculated broths

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