International Journal of Food Microbiology 285 (2018) 81–91

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Staphtox predictor - A dynamic mathematical model to predict formation of Staphylococcus enterotoxin during heating and fermentation of meat products

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ARTICLE INFO

Keywords:
S. aureus
Predictive modelling
Growth
Meat
Heat treatment
Fermentation

ABSTRACT

Existing growth models for *S. aureus* predict growth in relation to temperature, a_w/NaCl and pH, and the assessment of probable Staphylococcus enterotoxin (SE) formation is based solely on the number of *S. aureus*. However, during the production of meat products such as fermented sausages and semi-processed hams, growth of *S. aureus* is a critical control point in HACCP plans. There is a need to develop a model that evaluates the safety of the product regarding SE formation in relation to the product composition, changes in pH or temperature during the processing and the number of *S. aureus* in the final product. The objective of the present work is to develop a mathematical model that predicts both the increase in the number of *S. aureus* and whether SE formation is possible in different meat product processes.

A total of 78 experiments were carried out in a meat model system. The experiments covered a range of different temperatures (10–40 °C), pH (4.6–6.0), water phase salt (WPS) (2.2–5.6%) and Sodium nitrite concentrations (0–150 ppm). The meat model system was inoculated with approximately 10^3 CFU/g of a multi-strain cocktail and incubated at the different temperatures. The cocktail consisted of three strains of *S. aureus* producing the Staphylococcus enterotoxins A to D (SEA to SED) and a methicillin-resistant strain producing SEG, SEI, SEM, SEN, SEO and SEU. Enumeration of *S. aureus* was performed several times during the incubation, SE was extracted from samples with > 5 log CFU/g, and the SEA-E content was analysed by an ELISA method. Maximum growth rates and lag times calculated from microbiological data, together with temperature, pH, WPS and Sodium nitrite, were used to develop a SE and a growth model. The growth model was developed by training a neural network and the SE model based on logistic regression.

The SE and growth models were validated on separate data sets (N = 200 SE model, N = 63 growth model) including both dynamic and static conditions. The SE model predicted all occurrences of toxin formation in the validation data sets. The growth model is a fail-safe model and the prediction errors are comparable to laboratory reproducibility. In conclusion, the models are applicable for predicting the increase in *S. aureus* and for evaluating if SE formation is likely during processing of meat products. The models are available to producers and other interested parties at www.dmripredict.dk.

1. Introduction

Staphylococcal food poisoning (SFP) occurs after intake of food containing heat stable Staphylococcus enterotoxins (SE) formed during growth of *Staphylococcus aureus*. Under the right conditions, SEs are primarily formed and secreted during the late exponential growth phase (Balaban and Rasooly, 2000; Derzelle et al., 2009). SEs are heat stable, and as little as 20–100 ng of SE per g food can result in food poisoning (Bang et al., 2008; Schelin et al., 2011). SFP typically results in nausea, vomiting, abdominal cramps and sometimes diarrhoea.

A total of 21 SEs is known and designated SEA to SEIV (Schelin et al., 2011), and they are all short extracellular water-soluble proteins. The reported outbreaks are commonly associated with the classical toxins SEA to SEE. These toxins are very stable and are resistant to both heat and degrading enzymes (Denny et al., 1966; Genigeorgis, 1989; Le Loir et al., 2003). In development of mathematical models, it is relevant to apply strains that produce the same SE's as associated with the reported outbreaks.

In 2012, bacterial toxins caused 127 outbreaks in the EU corresponding to 16.6% of all outbreaks with strong evidence. In 35 of these

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https://doi.org/10.1016/j.ijfoodmicro.2018.07.030
Received 27 October 2017; Received in revised form 14 June 2018; Accepted 22 July 2018
Available online 26 July 2018
0168-1605/ © 2018 Published by Elsevier B.V.

outbreaks, the cause was related to SFP due to time-temperature abuse, slow cooling, or inadequate heat treatment. The food vehicles involved in the SFP outbreaks were pig meat and pork products (5.7%), broiler meat (8.4%), cheese (20%), mixed foods (31.4%) and other foods (34.3%) (EFSA, 2014).

SFP is mainly caused by food contaminated during handling followed by improper storage e.g. on a buffet at room temperature (20–25 °C). In the industry, growth of *S. aureus* and SE formation constitute a hazard in the following cases: 1) mild heat treatment of salted meat, e.g. boneless ham, followed by a long cooling time (Rajkovic, 2012) and 2) if the time exceeds 1200 degree hours (time above 15 °C until pH < 5.3) during the production of fermented sausages at 24 °C (AMI, 1997).

Existing predictive models for growth of *S. aureus*, such as Combase Predictor (1996) (http://browser.combase.cc/ComBase_Predictor.aspx?model=1), Pathogen Modelling Program (https://www.ars.usda.gov) and Therm 2.0 (https://meathaccp.wisc.edu/pathogen_modeling/therm.html), were developed mainly based on data from liquid media resulting in growth of *S. aureus* to, for example, 8 log CFU/g after 40 h at 24 °C, pH 5.3, 4% water phase salt (WPS). Several studies have shown that there is a significant increase in the growth rate for bacteria in the planktonic state compared to immobilised bacteria on surfaces (Becker et al., 2001; Beenken et al., 2004; Resch et al., 2005; Stoodly et al., 2002). A predictive model for growth of *S. aureus* based on data from experiments conducted in foods will provide risk assessors and quality managers with a more reliable model. None of the existing models predict if SE formation is likely to occur. Normally, a high number of enterotoxigenic *S. aureus* is a good indicator of the formation of sufficiently high level of SE to cause intoxication (Bennet, 2005). Therefore, the number of bacteria is usually counted to determine whether a food product is safe for human consumption. Several authors have detected SE with *S. aureus* concentrations in the range of 10^5 to 10^8 *S. aureus*/g. The time to detection and concentration of SE depend on the composition of the products (Notermans and van Otterdijk, 1985; Rajkovic, 2012; Wallin-Carlquist et al., 2010). Other studies on the other hand, have shown that high numbers of *S. aureus* do not necessarily result in SE formation (Notermans and van Otterdijk, 1985). Additional documentation including measurements of the number of bacteria and analyses for SE formation combined with relevant variables is needed to verify these findings and enable more reliable predictions in the future.

For the meat industry, relevant input variables are: temperature history, pH profile, salt concentration and Sodium nitrite. Salt concentration is converted to WPS because salt is dissolved in the water phase and the bacteria proliferate in the water phase. For the user, it is convenient to apply the salt concentration directly from the recipe. The relevant outputs are: the increase in number of *S. aureus* during the heat treatment or fermentation and if SE formation can occur.

The challenge in modelling dynamic processes such as fermentation and heat treatment is to quantify change in the number of microorganisms and whether SE can be formed at any time during the process. The change in the number of microorganisms is normally described in secondary models that quantify the effect of environmental factors (temperatures, pH, preservatives, etc.) (McKellar and Lu, 2004). For the secondary modelling, artificial neural networks (ANN) have been successfully used for modelling the growth of *L. monocytogenes* and *C. botulinum* under static conditions (Mejlholm et al., 2010; Gunvig et al., 2013). An ANN is a supervised non-linear regression technique, with the ability to take potential interactions between parameters into account. Like any regression method, the neural network must be trained on a set of experimental data to have predictive power. The environmental factors are used as independent variables, and the resulting maximum growth rate is the dependent variable that is to be estimated. In this study, the ANN is trained on maximum growth rates and lag phases, based on measured growth under static conditions. However, the model is subsequently used under dynamic conditions in which the process parameters change by allowing the trained ANN to predict maximum growth rates at each discrete point in time during the process and then integrating over the entire course of the production process.

Fujikawa and Morozumi (2006) developed a model that expresses a linear correlation between SEA formation and temperature in the range of 15 to 32 °C. Kim et al. (2009) developed an equation for the correlation between toxin production and the cell number. Models for toxin production in food that include more than one factor will produce better estimates for the probability of SE formation in complex foods.

In Gunvig et al. (2013), the probability of growth of *C. botulinum* was modelled as a function of five independent variables. The approach is similar to the present work, in which a logistic regression model using a logit transform is applied to estimate if any SE is formed. The objective has been to develop a mathematical model which predicts if SE formation is possible based on the production process and product composition combined with the number of *S. aureus*, either during mild heat treatment or fermentation of meat.

2. Materials and methods

2.1. Bacterial strains and preparation

For inoculation, a cocktail containing four strains of *S. aureus* was used. Strain one produced SEA, strain two SEB and SED and strain three SEC, and these were provided by the National Food Institute, Danish Technological University, Denmark. The fourth strain was methicillin-resistant and produced SEG, SEI, SEM, SEN, SEO, SEU and was provided by Statens Serum Institut, Denmark. In a preliminary test, the methicillin-resistant strain was positive for SEA-SEE. Later findings have indicated, this might have been due to cross-reaction of the antibodies in the ELISA kit. Strain one to three were all positive for one or more of the SEA-SEE. Therefore, all four strains were included in the cocktail.

In a meat model system, the four-strain cocktail were measured positive for SE formation with the Ridascreen SET Total kit (R-Biofarm, Art. no. R4105), which detects SEA-SEE. The meat model system was made from muscles from pork leg mixed with a brine containing 14.4% WPS, phosphate (3.5%) and dextrose (1%). Furthermore, a preliminary test showed that all four strains had similar maximum growth rates when grown in the meat model system at 25 °C and with varying pH and Sodium nitrite content.

The strains were maintained as frozen stock at −80 °C. Initially, the cultures were thawed and streaked on Brilliance agar (Oxoid). One colony from each culture was grown overnight at 37 °C in Brain Heart Infusion broth (BHI) (PO1186A, Oxoid). Two mL of each strain were mixed, and the cocktail was centrifuged at 5000 x g for five minutes. The supernatant was discarded and the pellet was resuspended in 10 mL sterile 0.85% saline with peptone (0.1%). The process was repeated, and the washed and resuspended cells were diluted to a final concentration of approximately $5{\cdot}10^4$ CFU/mL. Before mixing with the pork, 20 mL of the inoculation cocktail was added to the brine, corresponding to approximately 10^3 CFU/g in the final meat batter.

2.2. Production of meat model systems

To mimick a semiprocessed ham product, trimmed muscles from the pork leg were cut using a double kidney knife plate. The pork (87%) was mixed in a mixer (Varimixer, "Bear") with the brine (11%). The basic brine contained 81.1% water, 3.5% phosphate (Carmal 346, SFK, DK), 14.4% WPS and 1% dextrose. The final mixture contained approximately 2% WPS. Meat model systems with WPS concentrations of 4.0 and 5.5% were obtained by dry curing the pork at 5 °C for 48 h with 1.15% and 2.33% NaCl, respectively, prior to mixing with the brine.

In meat model systems containing 80 or 150 ppm Sodium nitrite, 0.62 g Sodium nitrite/kg brine or 1.16 g Sodium nitrite/kg brine were added to the basic brine, respectively. The pH was adjusted to 5.8, 5.3

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