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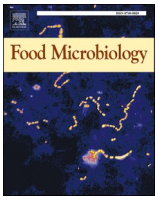

# Development and validation of a regression model for *Listeria monocytogenes* growth in roast beefs

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ABSTRACT

Food business operators are responsible for food safety and assessment of shelf lives for their ready-to-eat products. For assisting them, a customized software based on predictive models, ListWare, is being developed. The aim of this study was to develop and validate a predictive model for the growth of *Listeria monocytogenes* in sliced roast beef. A challenge study was performed comprising 51 different combinations of variables. The growth curves followed the Baranyi and Roberts model with no clear lag phase and specific growth rates in the range <0.005–0.110 $hr^{-1}$. A linear regression model was developed based on 528 observations and had an adjusted R-square of 0.80. The significant predictors were storage temperature, sodium lactate, interactions between sodium acetate and temperature, and MAP packaging and temperature. The model was validated in four laboratories in three countries. For conditions where the model predicted up to + log 2 cfu/g *Listeria* concentration, the observed concentrations were true or below the predicted concentration in 90% of the cases. For the remaining 10%, the roast beef was coated with spices and therefore different from the others. The model will be implemented in ListWare web-application for calculation of "Listeria shelf life".

## 1. Introduction

*Listeria monocytogenes* is a ubiquitous bacterium that causes the severe illness listeriosis (Gandhi and Chikindas, 2007). The number of listeriosis cases in Europe is about 2500 per year and has the highest case fatality rate and hospitalization rate among all foodborne zoonotic diseases surveilled in the EU (EFSA, 2013; 2017; 2018; 2019; Maertens de Noordhout et al., 2014).

*L. monocytogenes* is one of the few foodborne pathogens that can adapt and grow slowly under refrigeration temperatures (Tasara and Stephan, 2006; Evans and Redmond, 2019). Therefore, *L. monocytogenes* is of special concern in ready-to-eat (RTE) foods that are not heat treated before consumption and served cold (Allen et al., 2016). Most people can ingest some *L. monocytogenes* without being sick. According to the microbial criterium for *L. monocytogenes* in RTE foods in the EU law (EU regulation 2073/2005), the maximum limit of the bacterium in such products is 2 log cfu/g (100 cfu/g) at any time during the shelf life.

Surveillance studies in Europe in 2010–2012 revealed that the occurrence of *L. monocytogenes* in RTE food categories ranged from 0.09% for hard cheeses made from pasteurized milk up to 3.1% for RTE

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bovine meat (EFSA, 2013; European Food Safety Authority, 2018). Among positive samples, most were compliant with the criteria in the legislation of maximum 2 log cfu/g by the end of the shelf life, but a fraction contained 6 log cfu/g or even higher (EU 2073/2005; European Food Safety Authority, 2013). Several products have been the sources of large outbreaks of invasive listeriosis, such as cheeses, smoked fish, frozen vegetables, cold cuts, etc. (EFSA, 2019; Omer et al., 2018). The largest registered outbreak with more than 1000 cases of illness and 200 fatalities was traced back to a meat product in South Africa (Smith et al., 2019; Olanya et al., 2019).

The EU Food legislation places the responsibility for assessment and documentation of food safety on the food business operators (FBOs). In the case of RTE foods able to support the growth of *L. monocytogenes*, compliance with the legislation is obtained either by documenting that the concentration does not exceed 2 log cfu/g during the entire shelf life under reasonably foreseeable conditions of distribution, storage and use, or by absence of *L. monocytogenes* before the product has left the immediate control of the FBO. This documentation shall result from a continuous sampling plan, challenge studies or from relevant predictive models. In case of product variation, conditions representing the worst-case conditions for growth (Luber et al., 2011; Beaufort et al., 2014; Alvarez-Ordóñez et al., 2015). Challenge studies are costly, and as many companies often change their product recipes, it will demand a new challenge study for each substantial change in recipe. A cheaper way is simulation using predictive models for testing the impact on shelf life for a new recipe, package method or storage condition. In the ListWare project, several Norwegian FBOs are joining to develop a new software to assist them in this work.

It has been well documented that the growth rate of *L. monocytogenes* in foods depends largely on temperature, atmosphere, food matrix, water activity, pH, additives like lactate and acetate and interfering microbes (Beaufort et al., 2014; Augustin et al., 2005, 2011; Devlighere et al., 2001; Mejlholm et al., 2010; Dussault et al., 2016; Gimenez; Dalgaard 2004; Mellefont et al., 2008; Cornu et al., 2011). Results from such studies have been used to develop primary models (growth along timeline) and secondary models that predict the most likely bacterial growth based on the main growth factors. However, it is generally agreed that predictive model tools are currently underutilized by the food industry. A possible reason for the low use is that the early-developed models overestimated the growth. The models were developed using single cell cultures in lab-based growth media where the mobility of nutrients is higher than in a solid food matrix effect and therefore often lead to a higher growth rate than in food. During the last decade, the models have become more precise, but it is still pointed out that validation of models is important (Guillier 2016; Mejlholm et al., 2010; De Cesare et al., 2018).

Roast beef is a popular RTE product and a model for products made of whole muscles that undergo relatively mild heat-treatments. Roast beef has few additives but is a non-homogenous product due to the raw inside, the cooked surface and the spices added on the surface only. Prediction of the growth of *L. monocytogenes* in roast beef stored at various conditions is therefore highly relevant, but a validated model for predicting the growth is, to our knowledge, missing.

The aim of the current study was to explore the variation of commercial roast beefs, and to develop and validate a predictive growth model by producing a dataset from growth experiments with experimental design including storage temperature, packaging methods, maximum meat core temperature, and addition of sodium lactate and sodium acetate as predictor variables.

## 2. Materials and methods

### 2.1. Study approach

The study was designed in five steps.

1) Mapping of product characteristics for roast beefs available on the Norwegian market was done in order to select suitable conditions in the challenge study.
2) The identified variables were used in a challenge study for *L. monocytogenes* growth using experimental design.
3) On basis of the challenge study results, best fitting of primary model was investigated for each of the variable combinations of the growth experiments.
4) On basis of the challenge study results, a secondary model was developed by linear regression analysis.
5) Finally, the secondary model was validated with similar products from three countries in four different laboratories.

### 2.2. Mapping of roast beef characteristics

Ten commercial roast beef products were purchased from local shops in Norway or provided from FBOs participating in the project (ListWare). Information on the label regarding the shelf life, packing conditions, meat percentage, salt content, fat content and type of additives was collected. The roast beefs were analyzed for lactic acid, acetic acid, water activity, pH with the same analyzing procedures as described for the challenge study.

### 2.3. Challenge study

#### 2.3.1. Roast beefs

Custom made roast beefs were produced by an industrial project partner. Totally, 24 meat cuts of *M. semimembranosus* of 1.2 kg each, in total 30 kg, were added salt (0.3 g per 100 g product), spices (pepper, sugar, union, aroma), colour (E150b), and netted. Before heat treatment, the thermometer (Center 370 RTD Thermometer) was calibrated, and the probes were put in the core of the roast beefs. The beefs were placed in the middle of the cooking cabinet (Rational AG SelfCookingCenter 5Senses, size 0.8 × 1.7 m) and pre-cooked for 30 min at 240 °C, and then heated at 110 °C until core temperatures reached either 48, 55 (most beefs reached 59) or 63 °C. The core temperature was read manually every 10 min. Core temperatures of 48 °C were reached after 60–70 min, 55–59 °C after 70–90 min, and 63 °C after 78–104 min of cooking. After cooking, the roast beefs rested at room temperature before being placed in a chiller overnight at <4 °C. The next day, the roast beefs were vacuum packed and kept refrigerated during transport (by car, less than 2 h duration) to the analyzing laboratory. The next day, the roast beefs were sliced in 3 mm thick slices under aseptic conditions using a sterilized slicing machine (Berkel RP-A355CE) before inoculation and packaging. The roast beefs were produced in three series (batches) over a period of five months. The variable combinations were randomly distributed on the three batches, and there were 8 roast beefs with core temperatures of 48, 59 or 63 °C in each batch.

#### 2.3.2. L. monocytogenes strains

Four L. *monocytogenes* strains were selected for the challenge studies (Table 1) and inoculated as a cocktail. Apart from the reference strain (12MOB089LM), the other isolates were from production facilities or typical ingredients for local RTE meat foods and chosen because of their rapid growth. In the validation studies in three countries, strains of *L. monocytogenes* from national meat products were used (Table 1).

#### 2.3.3. Study design

The chosen variables in the challenge study were packaging methods (air, modified atmosphere (MAP), vacuum), core temperature (48–63 °C), storage temperature (4–12 °C) and addition of sodium lactate (0–4000 ppm) and sodium acetate (0–1000 ppm). The study was carried out using experimental design testing all the variable simultaneously, in order to elucidate both the main effects and the interactions of several independent variables (Augustin et al., 2011). In total, 51 combinations of input variables were used (Table 1). The continuous

**Table 1**
*Listeria monocytogenes* strains used in the challenge study for model development and in the validation studies.

| Name | Use of the strain in the present study | Remarks and sources |
|---|---|---|
| 12MOB089LM | Challenge and validation | Recommended reference strains for challenge studies in product with meat ( Beaufort et al., 2014) |
| VI 55766 O113-131 | Challenge | Isolated from ready to eat chicken meat for use in salads. De Cesare et al. (2018), Stratakos et al. (2016). |
| VI 59793 | Challenge and validation | Isolated from wiener sausage Pettersen et al. (2020) |
| VI 59792 | Challenge | Isolated from meat balls Pettersen et al. (2020) |
| 12MONO13045 | Validation | Reference strain |
| LMCIZS155 | Validation | Isolated in rabbit cuts |
| LM28 | Validation | Isolated from speak |
| LMIW | Validation | Isolated from meat mixture for wurstel |
| 970 | Validation | Isolated from the food environments of a meat cutting facility |
| 1702 | Validation | Isolated from the food processing environment of a meat cutting facility. |

variables included were represented by a maximum and minimum value, in addition to a midpoint, to define the multivariable valid "space" as described by Esbensen (2001) and Montgomery (2013). The packing methods were kept as category variables, while the other variables were continuous. The core temperature was included as the growth kinetics and interfering matrix effects may be different in raw and cooked meat. Sodium salts of lactate and acetate were included as additives as they are commonly used as preservatives in Norway. The storage temperatures covering the expected area for cold and abuse storage were included. Low and high levels of the continuous variables for each of the three packaging methods made 48 factor combinations. In addition, there were three factor combinations for the middle levels.

### 2.3.4. Laboratory analyses

The growth experiment followed the steps in Fig. 1. To secure randomization of slices between variable combinations, the blocks of slices from each roast beef were divided in four parts and two parts were exchanged. The roast beef slices were bisected to produce samples of approximately 10 g. Each sample (10 g) was transferred to a marked Petri dish with lid.

One ml of solution with sodium lactate (Sigma L7002) or sodium acetate (Merck 1.06268) or a mix was added to the relevant roast beef samples. Then, the samples were packed in air (Petri dish with a lid), MAP, or vacuum. The applied MAP reflected the commercial ones and consisted of 50% $CO_2$ and 50% $N_2$ (Aga Gas, Norway), while the vacuum after packing was 200 Pa (2 mbar). The petri dishes with the air samples were boxed in stomacher bags without filter and stacked on racks, while MAP- and vacuum-packed samples were placed in bags of Oriented Polypropylene and Polyvinyl Alcohol using a packing machine (Multivac Sepp Haggenmuller GmbH & Co, chamber machine C200, Wohlfertschwenden, Germany).

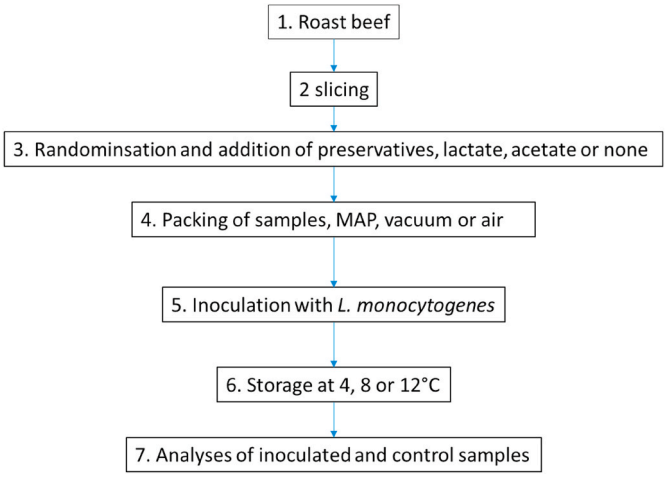


**Fig. 1.** Steps in the main challenge experiment. Specifications are given in Table 2.

After packaging, the samples were inoculated with 100 μL of the mixed cultures of *L. monocytogenes*. For MAP and vacuum-packed samples, a needle (0.6*25 mm) through a septum (septum 15 mm white/hard, PBI Dansensor A/S, Ringsted, Denmark) was used. This procedure avoids spread of pathogens in aerosols from the packing machine. A cold adapted inoculum of *L. monocytogenes* was prepared and inoculated as described in the guidelines of the European Union Reference Laboratory for L. *monocytogenes* technical document for conducting shelf-life studies on *L. monocytogenes* in RTE foods (Beaufort et al., 2014). The roast beef samples were incubated at 4 °C, 8 °C or 12 °C. For each variable combination, 10–15 sampling points were distributed during the storage period to obtain at least 9 sampling points in the expected exponential phase. The storage time and sampling points were selected based on growth observed in pre-trials. For samples stored at 4 °C in air, vacuum or MAP the storage periods were 21, 28 and 28 days, respectively. For samples stored at 12 °C the corresponding numbers were 11–14, 12 and 12 days.

Control samples from all test batches were analyzed at the beginning and end of each of the series. Unintended contamination of *L. monocytogenes* was analyzed using the ISO 11290-1 method. Listeria was quantified using the ISO 11290-2 method. The total aerobic count and lactic acid bacterial count of the challenged roast beef test batches were analyzed. After stomaching, the samples were diluted 1:10 in Unbuffered Peptone Water (UPW), (Becton Dickinson and Company, Sparks, USA). Samples for enumeration of lactic acid bacteria were plated three plates of either MRS-AB Agar (NMKL 140) and incubated anaerobically at 25 °C for 72 h or on petrifilm (3 M 6461) and incubated at 37 °C for 48 h. For the total aerobic count, dilutions were transferred (1 ml) to an empty Petri dish prior to addition of 20 ml Plate Count Agar (PCA) (Becton, Dickinson and Company, Sparks, USA). Plates were incubated at 20 °C for three days before colony counting. Petrifilm (3 M, 6400) plates, incubated at 30 °C for 72 h, were used as an alternative method for total aerobic count as well. The water activity (NMKL No. 168) and the pH (AOAC 981.12 (1982)) were analyzed at the start and at the end of the storage period by a commercial laboratory. In addition, the pH was measured in selected samples throughout storage according to ISO 2917; 90 ml UPW was added to 10 g roast beef and mixed with a stomacher. The concentration of lactate and acetate (in the analytical method measured as the sum of dissociated and undissociated lactic acid and acetic acid) in the samples were measured at the beginning of the trials by Eurofins using High Performance Liquid Chromatography (HPLC).

## 2.4. Primary model

The data for each of the 51 factor combinations in the challenge study were analyzed using DMFit (www.combase.cc) to assess which primary models for growth curves along time axis fitted the obtained data best. The length of the lag phases, growth rates and maximum cell densities were registered, as well as the primary model that fitted the data best, based on the highest R square and SE of fit. Predicted numbers for growth rates, $\mu_{max}$, for the same test conditions applied in the experiments were obtained by inserting the test conditions in the software Food Spoilage and Safety Predictor (FSSP version 4.0), DMRI predict (DTU university in Denmark, www.dmripredict.dk), and Combase (www.combase.cc) were changed from log-scale to ln-scale (multiplied by 2.3) before comparison.

### 2.5. Secondary model

#### 2.5.1. Data quality assurance

The results from the challenge study were collected in an Excel sheet. A quality assurance of the data was performed, and exclusion criteria of data were set. *L. monocytogenes* results above $5*10^9$ cfu/g were omitted in further analyses. Three observations of less than $5*10^9$ cfu/g were omitted in the model analysis because they were measured after several observations above $5*10^9$ cfu/g and therefore expected to have reached the death phase. A selection of test data and training data was done before statistical analyses in R tool (R Studio version 1.0.136 (http://cran.r-project.org). The significance level was set at $P \leq 0.05$.

#### 2.5.2. Development of a linear regression model

For the growth phase in the primary model the exponential growth rate, $\mu_{max}$. Was calculated:

$$\mu_{max} = \frac{\log(y_t) - \log(y_0)}{t} \quad (1)$$

where $y_0$ is *Listeria* start concentration ($t = 0$) when entering the growth phase, and $y_t$ is concentration at time t, where t is the time in the exponential growth phase. The mean (215 cfu/g, range 90–300) *Listeria* density observed at day of inoculation (i.e. $\log(y_0) = 2.33$) was used as a constant over all samples.

The secondary model is based on the linear regression model, i.e.

$$\mu_{max} = \beta_0 + \beta_t x_t + \beta_a x_a + \beta_l x_l + \beta_m z_m + \beta_v z_v + \beta_{t:a} x_t x_a + \beta_{t:m} x_t z_m + \beta_{t:v} x_t z_v + \varepsilon, \varepsilon \sim N(0, \sigma^2) \quad (2)$$

where predictor variables $x_t$ is storage temperature (°C), and $x_a$ and $x_l$ are added sodium acetate and sodium lactate g/kg (ppm/1000). Furthermore, the predictors $z_m$ and $z_v$ are indicator variables taking a value of 1 if packing method is "MAP" or vacuum, respectively, and a value of 0 else. Associated regression parameters are given by the β′s and the parameter $\sigma^2$ gives random error variance.

The model/variable selection was done by R tool using a stepwise variable selection tool combining "forward" and "backward" variable selection, applied with the Bayesian Information Criteria (BIC). The most complex, not chosen, model was the model containing all primary predictors (x's and z's) and their interaction terms of second degree. Significance level of 5% was used as threshold values for significance. In cases where an interaction effect was significant, the underlying single predictors were included in the model even though the single effects were not significant. Core temperature was omitted as predictor based on the BIC criteria.

### 2.6. Validation studies

Validation studies were performed in four laboratories, at i) University of Bologna (Unibo), Italy, ii) University of Leon, Spain, iii) NMBU (Norwegian Veterinary School), Oslo, Norway, and iv) NVI (Norwegian Veterinary Institute), Oslo, Norway (same lab as for the challenge study).

#### 2.6.1. Roast beefs and L. monocytogenes strains

For validation studies, roast beefs were purchased from supermarkets or companies near to the labs in each country. The roast beefs analyzed in Norway and Italy had salt and pepper as the only spices, while the ones analyzed in Spain were coated with a demi-glace containing vegetables and green spices. At NVI in Oslo, roast beefs produced by the same producer and in the same ways as in the challenge study were used. The slicing was however carried out by laboratory staff in the canteen without using gloves and without disinfection of the slicing machine, and thus, contained more background flora than in the challenge study.

The samples were analyzed at Unibo, Leon and NMBU in all cases inoculated with a cocktail of national strains and a reference strain. In NVI single strains were used in the validation test (Table 1).

#### 2.6.2. Design of validation studies

The labs were given instructions about which storage temperatures to use (Table 2) and storage periods according to the secondary model. In total, 20 variable combinations were tested. The temperatures tested were 4, 6, 7, 10, and 12 °C. The same procedures for preparation of inoculum and inoculation as in the challenge experiment was used.

The number of sampling points were limited to maximum 4, but 3 parallel samples were taken at each sampling point.

#### 2.6.3. Model validation

The validation of the secondary model was performed based on comparisons of predicted and observed concentrations of *L. monocytogenes* in roast beef products. The predicted times for when the *Listeria* growth reached a 2-log increase were calculated for each variable combination based on predictions from Eq. (2) using the estimated probability of approximately 10 or 50% were included as the minimum number of sampling points. However, in case of the highest storage temperatures where a 2-log increase were predicted within 2 days, additional sampling points were included. Extra sampling points were added also for cases when an early onset of stationary phase were observed. Samples were analyzed, and *Listeria* concentrations were calculated at each given time point. The equations used for validation were mean error and root mean square error of prediction (RMSEP):

$$Mean\ error = \frac{\sum_{i=1}^{n} \left( \mu_{\max\ i} - \widehat{\mu}_{\max\ l} \right)}{n} \quad (3)$$

$$RMSEP = \frac{\sum_{i=1}^{n} \left( \mu_{\max\ i} - \widehat{\mu}_{\max\ l} \right)^2}{n} \quad (4)$$

## 3. Results

### 3.1. Mapping of roast beef characteristics

The mapping of roast beefs on the market was included in order to cover the variability of products as much as possible (Table 3). All tested commercial roast beefs were produced of round steak of beef. The content of meat was at least 99.4% meat for 8 products and around 90% for two products. The latter two also contained spices and additives i.e. glucose, yeast extract, onion, pepper, and oil. The fat content in the meat was 0.6–2.4%. The salt content was 0.6–2.2%. Only one of the products was smoked, but "aroma" was declared as an additive on four products. Some of the products had a raw appearance in the middle of the slices, while others, in particular those with aroma added, did not. All packed products were packed in MAP or vacuum, while a product purchased from a deli counter was stored in air. The shelf life given on the package was 1–3 weeks after the day the products were purchased. The additives according to E-number categories were colour: E150 or E150c (sugar based colour) in 5 products, preservatives: E250 (sodium nitrite) in 4 products, E261 (potassium acetate) in 5 products, antioxidant and acid

**Table 2**
Variable combinations and sampling points applied in the challenge and validation studies.

| | Temperature °C | Packing | Sodium lactate (Ppm) | Sodium acetate (ppm) | Number of variable combinations | Number of sampling points[1] |
|---|---|---|---|---|---|---|
| Roast beef challenge study in NVI, Norway for development of the model | 4 | Air, vacuum, MAP | 0, 4000 | 0, 1000 | 24 | 199 |
| | 8 | Air, vacuum, MAP | 2000 | 1000 | 3 | 171 |
| | 12 | Air, vacuum, MAP | 0, 4000 | 0, 1000 | 24 | 194 |
| Validation UNIBO, Italy | 4 | MAP | 0 | 0 | 1 | 9 |
| | 7 | Air, vacuum, MAP | 0 | 0 | 3 | 27 |
| Validation University of Leon, Spain | 7 | Air, vacuum | 0 | 0 | 2 | 36 |
| Validation NMBU, Norway | 4 | Air | 0, 2000 | 0, 500 | 2 | 18 |
| | 6 | MAP | 0, 2000 | 0, 500 | 2 | 18 |
| | 12 | MAP | 2000 | 500 | 1 | 9 |
| Validation NVI, Norway | 4 | Air, vacuum, MAP | 0, 125, 250 | 0, 125, 250 | 7 | 44 |
| | 10 | Air, vacuum, MAP | 0 | 0 | 3 | 24 |

[1] Sampling points represent number of combinations * number of sampling days *replicates.

**Table 3**
Summary of product characteristics of 10 roast beefs bought in Norwegian shops. Lactic acid, acetic acid, water activity and pH are analyzed data. The two former represent the sums of dissociated and undissociated acids. Data for fat, NaCl and energy contents are information from the product labels.

| | Lactic acid (mg/kg) | Acetic acid (mg/kg) | Water activity | pH | Fat /100 g | NaCl /100 g | Energy (Kcal /100 g) |
|---|---|---|---|---|---|---|---|
| Mean | 12,082 | 853 | 0.97 | 5.8 | 1.8 | 1.3 | 112.8 |
| lowest value | 6500 | 62 | 0.96 | 5.2 | 0.6 | 0.6 | 94.0 |
| highest value | 18,000 | 2600 | 0.98 | 6.0 | 2.4 | 2.2 | 126.0 |
| standard deviation | 3800 | 802 | 0.01 | 0.2 | 0.5 | 0.6 | 9.0 |

regulators: E301 (sodium ascorbate) in 1 product, E326 (potassium lactate) in 4 products, E331 (potassium citrate) in 1 product, and emulsifiers, stabilizers, thickeners and gelling agents: E471 (MDG) in 1 product.

The water activity was in the range 0.96–0.98 in all products and remained stable during storage. The pH was in the range 5.2–6.0 when the packages were opened within a week after the production date. The variation of pH and $a_w$ was assessed as small and therefore these factors were not included as variables in the challenge study. The total content of lactic acid, including potassium lactate, was 6500 mg/kg or higher in all products. The highest concentrations were found in the products where E326 was added. Acetic acid (as acetate) was detected in all products where E250 was declared.

### 3.2. Challenge study

Totally, 528 observations of *L. monocytogenes* were found in the challenge study. There were 51 variable combinations.

### 3.3. Primary model

The growth curves for test conditions at 4, 8, and 12 °C are given in Fig. 2. Visual inspection of the growth curves indicated no or minimal lag-phase, more rapid growth at 12 than at 4 °C, and more rapid growth in air and vacuum packed than in MAP-packed samples. The time until a 100-fold increase varied from less than 3 days to more than 28 days, depending on the packing conditions, storage temperature and quantity of additives.

The pH changes in the samples in the challenge experiment during the storage periods were of less than 0.3 units. The concentrations of lactic acid bacteria were with few exceptions below the detection limit (100 cfu/g) for the analyses.

The data for the *L. monocytogenes* concentrations for each variable combination in the challenge study were fitted using DMFit. For all variable combinations the Baranyi and Roberts model (1994) fitted the data best, except for one where best fit was linear model (for air storage at 4 °C with added sodium acetate). The complete model, i. e with a lag phase, an exponential phase and a stationary phase, gave the best fit for nearly all variable combinations at 12 °C, while the no asymptotic model, which means that the stationary phase was not reached during the storage period, gave the best fit for datasets obtained at 4 °C and for some of the datasets of MAP packed samples with preservatives at higher temperatures. The median value for R-square for single growth curves was 0.97 (range 0.51–0.99), while the mean R-square was 0.94±0.10 standard error. Only two of the test combinations returned R-square values below 0.8 (MAP at 4 °C with sodium lactate or both sodium lactate and sodium acetate).

The durations of the lag phases at 12 °C were less than 24 h. At 4 °C, the estimated lag-phases were in the range 0–90 h for all growth series, except for the combinations where the growth was so slow that growth was hardly seen. The growth rates obtained were compared with those estimated with the tools FSSP, DMRI-predict and Combase predictor (Fig. 3). The data from our datasets were in all cases between the highest and lowest rates estimated by these tools.

### 3.4. Secondary model

A linear regression model of growth rate for *L. monocytogenes* in roast beefs was developed from data obtained in the challenge studies. The significant variables were storage temperature, added sodium lactate, interactions of MAP*temperature, and sodium acetate*temperature. Meat core temperature at cooking (48–63 °C) was not significant for the model and therefore not included in the model (Table 4). Parameter estimates for the model defined in equation (2) are given in Table 4. The model was fitted based on n = 528 observations and contains p = 9 regression parameters, leaving 519 degrees of freedom and having an adjusted R-square of 0.80.

The temperature was the variable with the largest influence on

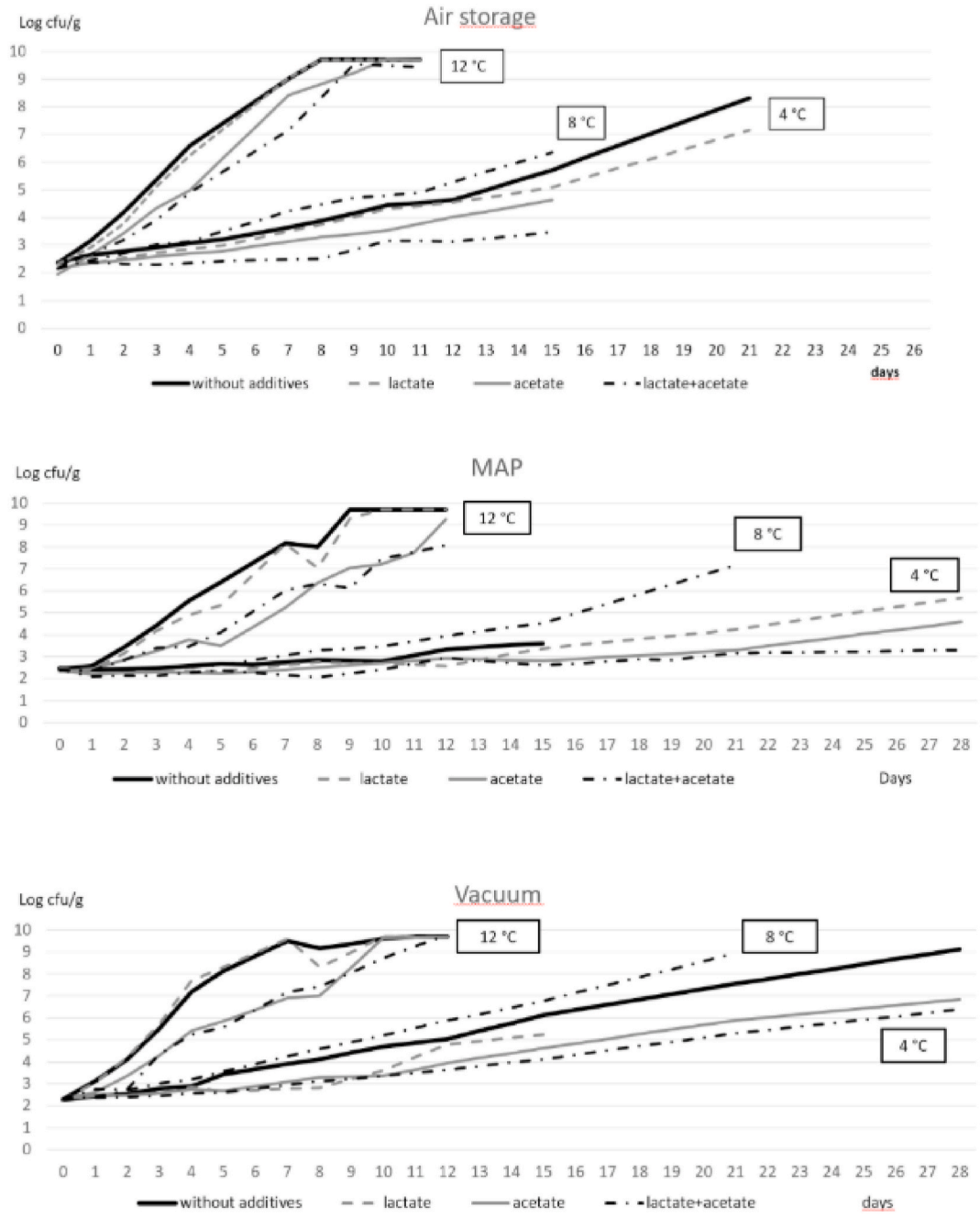


**Fig. 2.** Growth curves of *L. monocytogenes* in roast beef obtained at 4, 8, and 12 °C in air storage (top figure), MAP packed (in the middle), and vacuum packed (bottom figure). Black lines indicate no additives, grey dotted lines indicate added lactate, grey lines indicate added acetate and black dotted lines indicate added lactate and acetate.

*L. monocytogenes* growth. The added sodium lactate had a significant negative impact on pathogen growth rate. Packing in MAP reduced the growth and showed an interaction with higher temperatures. According to the model, sodium acetate had no significant impact on the growth rate when considered alone but an interaction effect with temperature, where the growth hampering effect of acetate increases with temperature. Due to this interaction effect that acetate has a larger impact on the growth rate at higher storage temperatures than lactate. The effects of sodium acetate and sodium lactate appear opposite as the single effects have different signs. However, the acetate single effect is not significant it is listed in the table as significant combination effects should be given together with the single effects, even if the latter are not significant.

### 3.5. Validation studies

The validation of the secondary model was based on comparisons of predicted and observed concentrations of *L. monocytogenes* in roast beef products and of growth rate ($\mu_{max}$).

#### 3.5.1. Laboratory analyses

All observed results for conditions which according to the model should lead to less than a 2 log increase of the Listeria counts, i.e. up to predicted conditions log 5 cfu/g, gave correct or lower observations than predicted for three of the participating labs (Fig. 4). For the last lab, the observations for the last sampling points were higher than the predicted, possibly due to that the roast beefs they tested were covered by spices

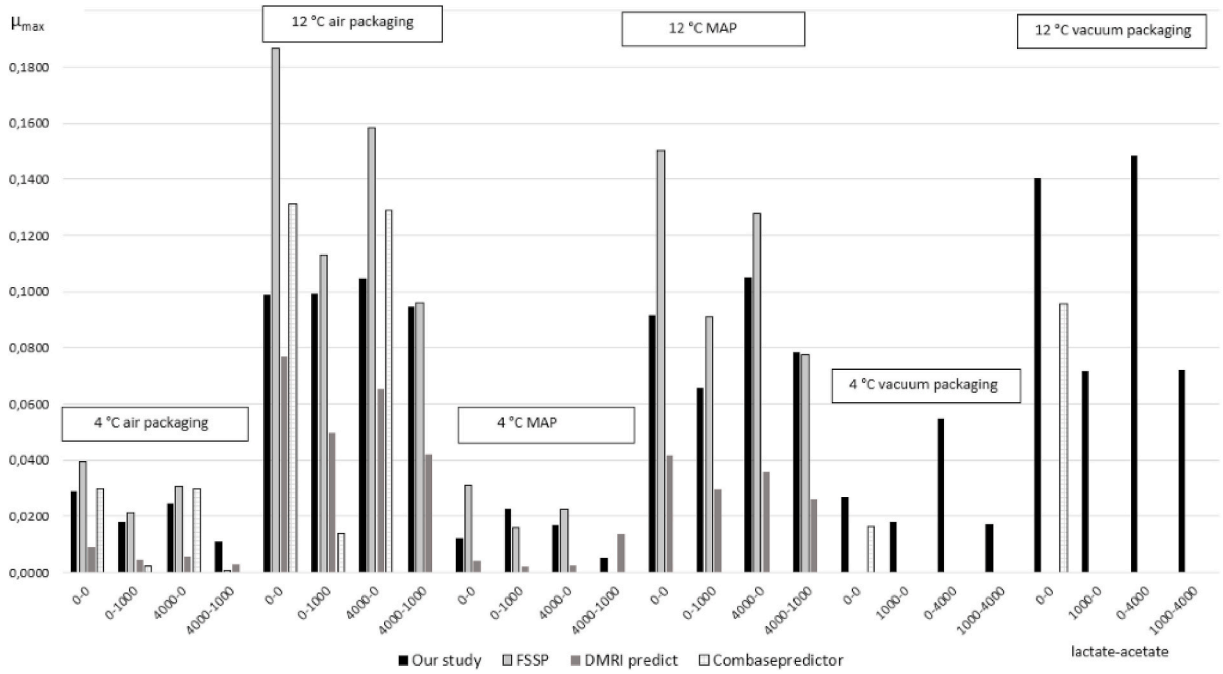


**Fig. 3.** Predicted specific growth rates (μmax) for *L. monocytogenes* at 4 and 12 °C stored in air, MAP, and vacuum for our study estimated by DMFit (black bars), by software FSSP (white bars), DMRI predict (grey bars), and Combase predictor (dotted bars). The x-axis shows the quantity of added lactate (0 or 4000 ppm) and acetate (0 or 1000 ppm) to roast beefs.

**Table 4**
Parameter estimates for the linear regression model (equation (2)). Single variables are included when their interactions are significant for the model.

| Parameter | Estimate±standard error | Significance level |
|---|---|---|
| $\widehat{\beta_0}$ (intercept) | −0.177±0.0321 | 0.001 > p |
| $\widehat{\beta_m}$ (MAP) | −0.0208±0.0377 | p > 0.05 |
| $\widehat{\beta_v}$ (vacuum) | −0.0213±0.037 | p > 0.05 |
| $\widehat{\beta_t}$ (temperature) | 0.0900±0.0038 | 0.001 > p |
| $\widehat{\beta_a}$ (acetate) | 0.0094±0.0303 | p > 0.05 |
| $\widehat{\beta_l}$ (lactate) | −0.0113±0.0036 | 0.01 > p > 0.001 |
| $\widehat{\beta_{t:m}}$ (temp*MAP) | −0.0177±0.0045 | 0.001 > p |
| $\widehat{\beta_{t:v}}$ (temp*vacuum) | 0.0062±0.0044 | p > 0.05 |
| $\widehat{\beta_{t:a}}$ (temp*acetate) | −0.031±0.0036 | 0.001 > p |
| $\widehat{\sigma^2}$ | 0.0181 | |

and therefore had areas with slightly better growth conditions. For conditions leading to higher predicted growth than plus 2 log cfu/g, there was a larger diversity of results. The under predicted observations from NMBU, with predicted concentration around log 6 cfu/g, had been stored at 12 °C until the end of shelf life, but had a deviating quality as they were slimy and the pH had increased 0.4 units compared to the initial value due to growth of moulds in the roast beef.

The validation experiments at NVI were performed with roast beef produced by the same manufacturer as in the challenge studies. No samples gave higher concentrations than predicted, but some were one or more log units below the predicted concentrations (Fig. 4). In these cases, the growth of *L. monocytogenes* changed from exponential phase to stationary phase at concentrations in the range 2–5 log cfu/g. These samples were MAP- and vacuum-packed stored at 10 °C temperature and contained high concentrations of lactic acid bacteria.

### 3.5.2. Evaluation of the validation results

The results from the validation study tested at temperature range

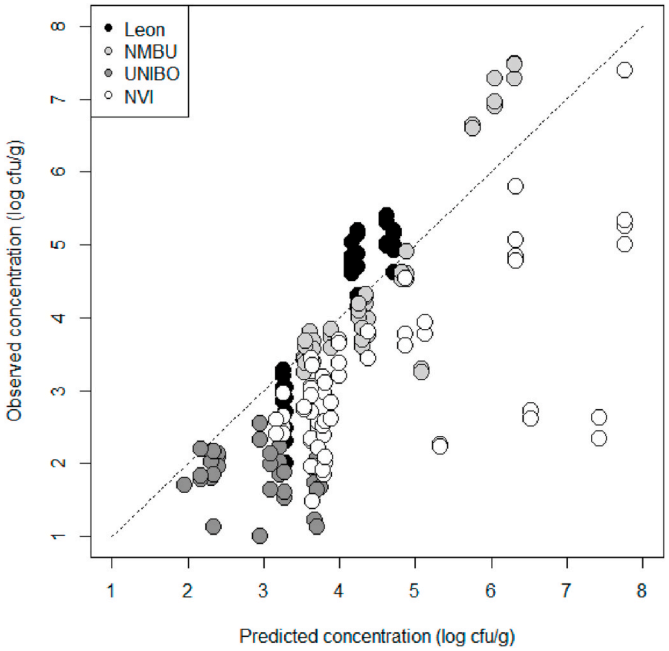


**Fig. 4.** Predicted versus observed *Listeria* counts in log scale, log cfu/g, independent of days of growth for data in the validation study. Different colours of the circles indicate different laboratories.

4–12 °C showed good correlation with the regression model developed with the data from the challenge study (Table 4). The mean error, indicating bias compared to the model, were negative for all temperatures, which means that the observed concentrations were lower than the predicted ones. The temperatures with the least bias were 12, 6 and 4 °C, and largest at 10 °C. The latter was due to that the samples with high concentrations of lactic acid bacteria were tested at this temperature. Also, at 7 °C, there was a systematic over-prediction (Fig. 4) as the

**Table 5**
Estimated mean error, indicating bias, and RMSEP, indicating accuracy, of observations versus predicted concentrations in the validation study.

| Temperature (°C) | Mean error | RMSEP | Observations (n) |
|---|---|---|---|
| 4 | −0.13 | 0.17 | 71 |
| 6 | −0.09 | 0.15 | 18 |
| 7 | −0.22 | 0.42 | 63 |
| 10 | −0.46 | 0.50 | 24 |
| 12 | −0.03 | 0.16 | 9 |

results from UNIBO at this temperature were all lower than the predicted ones. The RMSEP values for each temperature reflected the mean errors, and were lowest and in the range 0.15–0.17 for 4, 6 and 12 °C (Table 5). Reasons for over or under-prediction were identified for all deviating observations, and it can be concluded that if no biological deviation of the samples, the model predicts safe-fail concentrations.

## 4. Discussion

### 4.1. Variation in roast beef food matrix

The variations of roast beef products available in the Norwegian market are mainly related to raw or cooked inside, additives used, and packing conditions applied. The concentrations of acetate are mostly lower than the lactate concentrations. This is reasonable, as acetate influence the taste of the product more than lactate does. The pH and water activity of the analyzed roast beefs showed very limited variation, also when potassium and sodium salts of acetate and lactate were added. When the roast beefs were stored until the end of shelf life, hardly any pH and water activity variation was observed unless the samples contained high amounts of lactic acid bacteria or visible moulds. This observation indicates that roast beefs have a high buffer capacity and, if sliced and packed under good hygienic conditions, the background microbiota does not influence the pH after outgrowth. It should be noted that pH and water activity are considered as main variables for the prediction of microbial growth (Augustin et al., 2011). The results obtained in this study do not contradict this statement. On the contrary, the importance of pH was observed in deviating samples in the validation study. Even though pH and water activity are not required as input variables in a predictive model to cover the variability of roast beef, it is necessary that the user of the model confirms that the roast beef has the expected pH.

In the validation study, some of the roast beef samples which were sliced under realistic hygienic conditions in a canteen where the slicer was used at irregular intervals developed high concentrations of lactic acid bacteria during storage. In these samples, the observed *L. monocytogenes* concentrations were significantly lower than predicted. The effect was larger for samples stored at abuse temperature and packed in vacuum or MAP. The inhibitory effect of lactic acid bacteria on other pathogens, including Listeria, is well described (Dalgaard and Mejlholm 2019; Giménez and Dalgaard, 2004; Cornu et al., 2011; Leroi et al., 2015; Jameson 1962), and further, the inhibitory effect of lactic acid bacteria on *L. monocytogenes* is higher at abuse temperatures than at chilled storage conditions (Stratakos et al., 2016). In order to cover the variability of roast beef, we suggest that a predictive model does not need to include the concentration of lactic acid bacteria, but the user needs to check if the product does contain high loads of lactic acid bacteria. The presence of lactic acid bacteria will lead to an over prediction of *L. monocytogenes* growth, i.e., a safe-fail prediction. According to the literature, inhibiting effects of lactic acid bacteria on *L. monocytogenes* is not seen at lactic acid bacteria concentrations below 6 log cfu/g (Østergaard et al., 2014).

In predictive models developed and validated for other but similar meat products to roast beef, additives and packing conditions were used as input variables (Devlighere et al., 2001; Dussault et al., 2016; Mejlholm et al., 2010).

### 4.2. Observed growth of L. monocytogenes in roast beef

Rapid growth of *L. monocytogenes* was observed for all variable combinations during storage at 12 °C, but hardly any growth was seen at 4 °C in MAP-packed samples. The growth kinetics demonstrated good correlation with established primary models (Baranyi and Roberts, 1994). Growth potentials for *L. monocytogenes* of <0.6 log cfu/g during 12 days of storage at 8 °C have been reported for steak tartare (Torlini et al., 2020). This is lower than observed in the present study, but lower growth potentials for raw meat than for cooked meat have also been observed by Skjerdal et al. (2010). The observed growth rates for *L. monocytogenes* in roast beef were in most cases lower than the estimated growth rates found using the FSSP tool, probably because data for all the organic acids were not inserted. On the other hand, the observed growth rates were higher than those estimated by the DMRI tools. The predicted time until a 100-fold doubling, referred to as the *Listeria* shelf life, differed with these tools up to a week of chilled storage, which illustrates the relevance an of a model specific for roast beef.

The impact of additives on the growth rates of *L. monocytogenes* in roast beef found by using DmFit followed this order: (1) No additives gave the most rapid growth rate; (2) low level of lactate gave some inhibition, followed by (3) high level of lactate, (4) low level of acetate, and finally (5) high level of acetate, which inhibited the growth significantly. This order of growth inhibiting impact correlated with the concentration of undissociated acids in each of the cases. Zulani et al. (2007) and Wemmenhove et al. (2016) have previously reported the higher importance of undissociated acids compared to the dissociated acids in terms of growth inhibiting effects. The $pK_a$ values of lactic acid and acetic acid are 3.86 and 4.75, respectively. When 1000 ppm of the sodium salts of these acids are added to roast beef at pH 5.9 the concentrations of undissociated acids correspond to 0.07 and 0.72 mmol/g for lactic acid and acetic acid, respectively. The concentrations in the meat liquid phase will be higher, and, according to the minimum inhibitory concentrations of organic acids reported by Wemmenhove et al. (2016), the concentration of undissociated acetic acid at pH 5.9 will have a significant growth inhibiting effect.

### 4.3. Development of the secondary model

The motivation for building the linear regression model was assisting FBOs to determine correct shelf lives, i.e. to predict the consequences of changes in additives, packing methods and storage temperature, on the concentration of *L. monocytogenes* until exceeding 2 log cfu/g, which is the limit value in the legislation for RTE foods (EU regulation 2073/2005). The focus was therefore on the early phase of the growth curves of *L. monocytogenes*. The lag phase was ignored because it was short, and because it was considered better and safer that the model overestimated than underestimated the concentrations of *L. monocytogenes*.

Effects of interaction were observed between sodium acetate and temperature, and between MAP and temperature, thus, interaction effects were seen for the single variables with the highest impact on the growth of *L. monocytogenes*. A surprising observation was that sodium acetate alone was not a significant factor, however, in interaction with temperature the effect was significant. This may be due to the fact that growth rates at low temperature were generally low, and differences less clear, while the growth at higher temperatures was more rapid and this made it possible to detect different impacts of additives. Therefore, the interactions could be due to the stronger signal compared to the random errors in the dataset. However, interactions between dissolved $CO_2$ concentrations, organic acids and temperatures have been reported previously (Devlighere et al., 2001; Dussault et al., 2016). The packing atmosphere was found to have a large impact on the growth rate, in particular modified atmosphere. This is also in line with the literature

(Mejlholm et al., 2010; De Cesare et al., 2018).

The effect of $CO_2$ enriched atmosphere is related, firstly, to the fact that facultative anaerobic bacteria like *L. monocytogenes* grow slowly without oxygen than with oxygen, and secondly, to the fact that anaerobic conditions stimulate growth of lactic acid bacteria which in turn inhibit growth of *Listeria* (Harris et al., 1989). As lactic acid bacteria were not present in high amounts in the roast beefs in the main experiments, an interaction effect between packing condition and temperature was not observed.

### 4.4. Validation with practical experiments

The validation experiments illustrated the strong and weak points of the developed model. The model predicted true or over predicted results, indicating that the model is solid for roast beef as a product. The roast beef products used in the validation experiments were from different countries and the applied *L. monocytogenes* strains were local and recently isolated. Some deviations were observed in cases where the background microbiota was large and caused either competing growth inhibition or changes in pH. Other differences between countries cannot be ruled out, but a larger study with the same conditions tested in each country, including the same inoculated strains, would need to be tested to further investigate this.

### 4.5. Outlook perspectives

The model developed in this study is a regression model, based on a data obtained by testing roast beefs covering the reasonable variation of roast beef products available in shops. The regression approach has the benefit that some chosen variables can be included, while others can be omitted and the number of input data can then become less than in models based on product characterisation, like the gamma models and a regression model where several variables included. The model developed in the present study is built on a large dataset, it is built on those variables the users have knowledge about, it is validated as described in this study, and will be implemented in the web-tool ListWare. In the tool, it will be noted that the model is suited for roast beef provided that the pH is within the variation expected for beef meat, and that the presence of lactic acid bacteria may lead to an over prediction of the *Listeria* concentrations.

## 5. Conclusion

A regression model has been successfully developed for predicting *L. monocytogenes* growth in roast beefs. The model is fit for roast beefs stored at temperatures ranging from 4 to 12 °C in air, vacuum, and MAP packaging, and also when sodium lactate and/or sodium acetate are added. The model will be implemented in the new ListWare web tool.

## Contribution of authors

Taran Skjerdal: lead researcher, design of experiment, collection of data, interpretation of results, writing manuscript; Lars Erik Gangsei: modelling and statistical analyses, interpretation of results, writing manuscript; Ole Alvseike: design of experiment, interpretation of results, writing manuscript; Kyrre Kausrud, modelling and statistical analyses, interpretation of results, writing manuscript; Alessandra De Cesare, Elena Alexandra Alexa and Avelino Alvarez-Ordóñez: validation studies, discussions during the work, interpretation of result, writing manuscript; Lena Haugland Moen and Ane Mohr Osland, design of experiments, laboratory experiments, interpretation of results; Cecilie From: preparation of the roast beef for challenge studies, contributed with food industry knowledge; Toril Lindbäck: validation study; Janne Kvello, Beate Folgerø and Sissel Dommersnes, contributed with food industry knowledge, discussion partners, Sigrun Hauge, project leader, design of experiment, participated in experiments, interpretation of results, writing manuscript.

## Declaration of competing interest

The authors have no conflicting interest.

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