Extension of growth/no growth predictive models for the preservation of low-acid pasteurized sauces by incorporating water activity and model validation in sauces

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Abstract

Combined preservation methods have awakened a growing interest in low-acid pasteurized sauces under ambient storage, aiming to produce more 'natural' foods with enhanced microbial stability. However, limited information and predictive models are available to assess the microbial stability of this kind of products, for which the spoilage is mainly caused by acid-tolerant spore-forming spoilage bacteria (ATSSB). In this study, a set of growth/no growth (G/NG) models developed previously (Sun, R., Vermeulen, A., Devlieghere, F., 2021a. Modeling the combined effect of temperature, pH, acetic and lactic acid concentrations on the growth/no growth interface of acid-tolerant *Bacillus* spores. International Journal of Food Microbiology) for spores of two ATSSB strains (*Bacillus velezensis* and *Bacillus subtilis*) from pasteurized sauces were upgraded to incorporate the effect of water activity (a_w). The growth from heat-treated spores (80 °C, 10 min) was assessed during three months in Nutrient Broth under 320 combinations of storage temperatures (22 and 30 °C), a_w (0.95 and 0.965), pH (4.4-5.6), total acetic acid (0.0-0.3% (w/w)), and total lactic acid (0.00-1.00% (w/w)). Twelve replicates were tested for each combination. After merging the dataset collected previously at a_w 0.98, a set of 4-variate (4V) models were developed for each strain under a single temperature after 30, 60 and 90 days separately. Additionally, the developed models were validated by challenge tests in different industrially produced low-acid pasteurized sauces for three months.

The results showed that lowering a_w significantly enhanced the antimicrobial effect of decreased pH and the addition of acetic and lactic acid against the ATSSB spores, while the enhancement was less evident at high acetic acid concentrations. In the challenge tests, the developed models exhibited accurate predictions for the sauces, with the % correct-predicted values ≥ 90%. The developed models can be used in microbiological risk assessments or the innovation of preservation strategies for low-acid pasteurized sauces.

Keywords: *Bacillus*; spores; organic acid; pH; spoilage

1. Introduction

Intensive thermal treatments are traditionally used in the food industry for their efficacy in microbial inactivation, but the high temperature during the heating process can negatively affect the nutritional and sensory qualities of food products. Therefore, manufacturers are seeking alternatives to the intensive treatment, to meet the rising demand for high-quality food. The use of non-thermal preservation factors combined with mild pasteurization is becoming an appealing strategy (Arioli et al., 2019; Katiyo et al., 2017; Kim and Rhee, 2020; Silva and Gibbs, 2010; Sobral et al., 2017). As for the sauce industry, efforts have been made to apply milder pasteurization on low-acid sauces (e.g., pepper sauce, processed cheese sauce and mushroom sauce) with a limited amount of additions. However, the ambient storage ability of low-acid pasteurized sauces is challenged by acid-tolerant spore-forming spoilage bacteria (ATSSB), as their spores can survive pasteurization and initiate growth in low-acid conditions.

The application of organic acids such as acetic and lactic acid has exhibited excellent antimicrobial effects against bacterial spores in various food products (Cho and Chung, 2017; Franceschini et al., 2020; Redondo-Solano et al., 2021; Theron and Lues, 2010). Nevertheless, the addition of acetic and lactic acid should be limited, as the abuse of them can result in an off-taste of the products (Ramirez-Hernandez et al., 2018). Other than acidic conditions, lowering water activity (a_w) is also an effective strategy to inhibit microbial growth in food products. On one hand, at low a_w conditions, the metabolic activities of microorganisms are slowing down, leading to an extended lag phase and decreased growth rate or even a total inhibition of specific microorganisms (Tapia et al., 2020). On the other hand, the adjustment of a_w should also respect the organoleptic properties of food products. An adequate combination with decreased a_w can limit the addition of acids and salts in low-acid pasteurized sauces, leading to a better organoleptic and nutritional quality. To elaborate an applicable combination of these preservation factors, predictive models can be a convenient tool, especially when successfully validated in the target food products. As a critical factor influencing microbial growth, decreased a_w has been included in many predictive microbiology models (Debonne et al., 2019; Jin et al., 2018; Kuroda et al., 2019; Tapia et al., 2020). However, the influence of a_w on the antimicrobial effect of decreased pH and the addition of acetic and lactic acid against ATSSB spores is still not clearly quantified. The growth/no

growth (G/NG) models that predict the growth probability of certain microorganisms under different preservative conditions are a useful tool for defining microbial stable products. In spite of the extensive application of G/NG models, bare of them are applicable to low-acid pasteurized sauces (Debonne et al., 2019; Gunvig et al., 2013; Gysemans et al., 2007; Huang et al., 2018; Kuroda et al., 2019; Tsuruma et al., 2021; Vermeulen et al., 2012; Vermeulen et al., 2007a). A set of growth/no growth (G/NG) models have been established recently for low-acid pasteurized sauces under ambient storage, which aimed to predict the growth probability of ATSSB spores as a function of temperature, pH, acetic and lactic acid concentrations at a_w 0.98 (Sun et al., 2021a). However, applying the models built at a_w 0.98 can lead to an overestimation of the growth of ATSSB, as many acidic sauces have lower a_w values. Therefore, the former models were extended by incorporating a_w as an extra variable. Furthermore, the models were developed in culture media, while food products are more complicated systems including more factors that may influence bacterial growth, like food structures, initial contamination level, and food microbial interaction (Augustin et al., 2011; Lianou and Koutsoumanis, 2011; Park et al., 2007). The difference between culture media and food products can lead to unrealistic outcomes, highlighting the importance of validating the models in real food systems.

Two strains (*Bacillus velezensis* BS127 and *Bacillus subtilis* subsp. *subtilis* BS128) were used as representative of ATSSB for modeling, as they were isolated from low-acid pasteurized sauces and exhibited high tolerance to low pH and high concentrations of acetic and lactic acid in a previous screening experiment (Sun et al., 2021b). The objective of this study is therefore to upgrade the G/NG models of the representative ATSSB spores by (*i*) incorporating the effect of decreasing a_w (from 0.980 to 0.950) on the growth probabilities, and (*ii*) validating the established models in low-acid pasteurized sauces. To the authors' knowledge, this is one of the first models including the effect of a_w, pH, acetic and lactic acid on the growth from *Bacillus* spores, which contributes to determining preservation strategies for low-acid pasteurized sauces under ambient storage.

2. Materials and Methods

2.1. Preparation of the spore suspensions

B. velezensis (BS127) and *B. subtilis* (BS128) were stored in Brain Heart Infusion (BHI, Oxoid, Oxford, England) broth with 15% sterile glycerol at - 75 °C in the FMFP-UGent culture collection (Research Unit Food Microbiology and Food Preservation, Ghent University). Spore suspensions of the selected strains were produced according to Coroller et al. (2001) with little modification. In brief, strains were re-cultivated on Tryptone Soya Agar (TSA, Oxoid) from the glycerol stocks and inoculated into sterile Tryptone Soya Broth (TSB, Oxoid) tubes by a sterile inoculation loop. After incubation at 30 °C for 24 h, 100 µL culture of each strain was spread plated on strengthened Nutrient Agar (sNA) (28 g/L Nutrient agar (Oxoid), 0.04 g/L $MgCl_2$ (Sigma-Aldrich, St. Louis, USA) and 0.10 g/L $CaCl_2$ (Sigma-Aldrich)). Six plates were prepared for each isolate. After incubation at 30 °C for 7 days, sterile 0.85% (w/v) sodium chloride (Sigma-Aldrich) solution was used to collect spores. Then the collected spores were washed three times by centrifugation at 10000 g for 15 min at 4 °C, followed by suspension in 10 mL ethanol-water (1/1). After incubation at 2 °C for 12 h to eliminate vegetative cells, the centrifugation process was repeated three times to wash the spores. Finally, the pellet was suspended in 10 mL sterile distilled water and stored at 2 °C. For enumeration, spore suspensions were activated in a hot water bath at 80 °C for 10 min, then serially diluted in Peptone Physiological Solution (PPS) (1 g/L peptone (Oxoid) and 8.5 g/L sodium chloride), plated out on TSA and incubated at 30 °C for 48 h.

2.2. Data generation

Growth experiments were performed in modified Nutrient Broth (NB, Oxoid) with 320 combinations of temperature, a_w, pH, acetic and lactic acid concentrations, and 12 replicates were performed for each combination. A full factorial design of the G/NG experiments was developed with two levels of temperature (22 and 30 °C), two levels of a_w (0.950 and 0.965), five levels of pH (4.4, 4.7, 5.0, 5.3 and 5.6), four concentrations of total acetic acid (0.0, 0.1, 0.2 and 0.3% (w/w)) and four concentrations of total lactic acid (0.00, 0.35, 0.70 and 1.00% (w/w)). The procedures of media preparation and spore inoculation were the same as described in (Sun et al., 2021a). The heat-activated (80 ˚C, 10 min) spores were serially diluted to 5.8±0.2 log spores/ml in the different modified NB media. For each isolate, 20 µL of the spore inoculums were transferred aseptically into 180 µL of the different media in 96 wells microplates. To avoid the evaporation of acetic and lactic acid, the inoculated microplates were covered

with a sterile polyurethane film (Breath-easy film, Fiers NV, Kuurne, Belgium). Then the filmed microplates were closed with the lids and sealed with Parafilm® to avoid dehydration. After that, the sealed microplates were placed in a closed jar containing KNO_3-solution with the same a_w as the media in the microplates, and incubated at 22 °C or 30 °C for 90 days. Growth of the spores was assessed regularly by optical density (OD) measurement at 380 nm using a Versamax Microplate Reader (Molecular Devices, San Jose, USA) during incubation. On each day of analysis, the growth or no growth was determined in each single well of the microplates under investigation. A well was considered as growth when the OD of this well was consistently higher than three times the standard deviation of the OD_{blank} (pure medium). Besides, the G/NG in each well was visually verified on each day of analysis. After incubation, the growth wells were streaked onto TSA to check their purity. The obtained data, corresponding to pure cultures, were used to evaluate the growth probability initiated by bacterial spores in different conditions

2.3. Model development

The ordinary logistic regression models were used to describe the G/NG data collected after 30, 60 and 90 days of incubation: one model for each strain under a fixed temperature (Eq. 1). All the developed models consist of a polynomial equation (right-hand side) and logit (p) = ln (p/ (1-p)) (left-hand side). "p" is the growth probability at 30, 60 or 90 days.

$$\text{Logit}(p) = b0 + b1 * pH + b2 * AW + b3 * TLAW + b4 * TAAW + b5 * pH * AW + b6 * pH * TLAW + b7 * pH * TAAW + b8 * AW * TLAW + b9 * AW * TAAW + b10 * TLAW * TAAW + b11 * pH * pH + b12 * TLAW * TLAW + b13 * TAAW * TAAW \quad (1)$$

Where bi (i=0, …, 18) are the parameters to be estimated, pH is the pH value of the media, AW is the water activity of the media, TAAW is the concentration of total acetic acid in the water phase, and TLAW is the concentration of total lactic acid in the water phase. The importance of expressing antimicrobial agents on the water basis for G/NG model development has been proven (Dang et al., 2011). Two levels of incubation temperatures (22 and 30 °C) were tested, while incorporating a variable in the models requires at least 3 levels, so separate models were developed at each single temperature.

148 The models were fitted to the data by using the SPSS Statistics 26 software (SPSS, Inc., Chicago, IL,
149 USA). A binary logistic regression was selected to build the models according to the procedure described
150 in Vermeulen, et al. (2007b), where a combination of the 'Enter' method and 'Forward Stepwise LR'
151 method was used to build these models. The predicted G/NG interfaces were plotted in MATLAB®9.9
152 software (The Matworks, Inc., Natick, MA, USA).

153 **2.4. Evaluation of the model performance**

154 The model performance was assessed with several statistics based on the G/NG data. First, the overall
155 model performance was evaluated by Nagelkerke R^2, which quantifies the variability in outcomes that
156 can be explained by the model predictions (Nagelkerke, 1991). Normally, a high Nagelkerke R^2 value
157 indicates that the dependent variable can be well explained by the independent variables. Then the
158 discriminative ability of each model was accessed by the c-value (concordance index), % correct-
159 predicted and % fail-dangerous. The c-value can be calculated by the area under the receiver operating
160 curve (ROC), which is obtained by plotting the sensitivity of a model against the complement of the
161 specificity for consecutive cutoffs for the probability of an outcome. In detail, the sensitivity of a model
162 means the portion of positive observations that are correctly predicted while the specificity means the
163 portion of correctly predicted negative observations (Tienungoon et al., 2000). Therefore, a higher value
164 of c-index means that the predicted growth and no growth cases have a better concordance with the
165 observations. A c-index value of 1.0 indicates a perfect predictive power while 0.5 implies that the
166 predictions are not better than random. The % correct-predicted indicates the percentage of the overall
167 correct predictions where the observations of "growth" or "no growth" are in compliance with the
168 predictions, and the % fail-dangerous is the percentage of growth cases observed in the predicted no
169 growth conditions. In terms of calibration, Hosmer–Lemeshow (HL) goodness-of-fit test is used to
170 measure the overall calibration error, which compares observations to predictions by decile of the
171 predicted probability (Steyerberg, 2010). Normally, a smaller value of HL statistic (corresponding to a
172 P-value higher than 0.05) indicates the model fits the data well.

173 **2.5. Challenge test in pasteurized acidified sauce: validation**

2.5.1. Sauce production and sample collection

Eight combinations of a_w, pH, lactic and acetic acid concentrations covered by three types of low-acid pasteurized sauces were considered: satay sauce, cheese sauce and beef sauce (Table 1). To perform a strict validation, all the conditions of sauces were chosen near or in the G/NG zone. The test sauces were industrially manufactured and heated in a steam oven at 90 °C for 40 min. After that, the sauces were delivered to FMFP-UGent for the challenge test within 24 hours. Typical product composition and ingredients for the sauces are as follows: (1) Satay sauce: hydrolyzed vegetable protein, modified maize starch, water, spices, guar gum, xanthan gum, garlic puree, peanut butter, soy sauce, pea protein isolate, tomato concentrate, sugar and salt; (2) Beef sauce: water, onion, concentrated beef broth, rapeseed oil, modified maize starch, maltodextrin, xanthan gum, carrots, paprika powder, ground caraway seeds, white pepper, dried parsley flakes, garlic powder, chili powder, sugar and salt; (3) Cheese sauce: Cream, water, cheese spread, crème fraiche, modified maize starch, maltodextrin, xanthan gum, hydrolyzed wheat protein, dried onion, milk protein, white pepper, sugar and salt. For all the sauces, vinegar or/and lactic acid were used as acidulants if needed. The sauces were packaged in sealed plastic bags that were pasteurized by the food company, and divided into sterile sample bottles (150 ± 1 g) as soon as arrived at FMFP-UGent.

2.5.2. Physicochemical and microbiological analyses

For each kind of sauce, the pH was measured with a Seven easy pH meter (Mettler Toledo GmbH, Schwerzenbach, Switzerland), while the a_w was determined by an aw-kryometer (TypAWK-20, NAGY Messysteme GmbH, Gaüfelden, Germany). Moreover, the dry matter content of the sauce was determined by vaporization in a drying oven at 105 °C. Three independent measurements of pH, a_w and dry matter content were performed for each condition of the homogenized sauce.

The microbiological quality of the pasteurized sauce was determined according to the method of International Organization for Standardization (ISO) with minor modifications. For each sample, 20 ± 1 g of sauce was removed into a sterile stomacher bag and homogenized with PPS. Subsequently, a decimal dilution series was prepared in PPS and 100 μL of appropriate dilutions were spread plated on

TSA and Mannitol Egg Yolk Polymyxin B (MYP, Oxoid) to enumerate the total aerobic bacteria and presumptive *Bacillus*, respectively. Reinforced Clostridial Agar (RCA, Oxoid) plates were used to enumerate the total anaerobic bacteria. The cell counts of lactic acid bacteria and sulfite-reducing bacteria were determined by pour plating on deMan, Rogosa Sharpe agar (MRS, Oxoid) and Tryptose Sulfite Cycloserine (TSC, Oxoid) plates, respectively. Yeast and molds were enumerated on Yeast Glucose Chloramphenicol (YGC) agar (Merck, Germany). Moreover, the spore counts were determined by heating the sauce dilutions in PPS at 80 °C for 10 minutes and then spread plating on TSA. The TSA, MYP and YGC plates were incubated aerobically at 30 °C for up to 3 days, while the RCA, MRS and TSC plates were incubated anaerobically at 30 °C for up to 5 days. Three independent samples were analyzed for each condition of sauce.

2.5.3. Inoculation

The mixed spore suspensions of *B. velezensis* and *B. subtilis* were appropriately diluted in sterile distilled water and pasteurized in a hot water bath (80 °C for 10 min), followed by rapidly cooling down in the ice. Then each sample (150 ± 1 g) was inoculated with 150 µL of the mixed spore suspension, which resulted in a final concentration of 4.8 ± 0.2 log spores/g. Thereafter, the inoculated samples were manually mixed to ensure uniform distribution of spores. Each condition of sauces includes five replicated samples.

2.5.4. Assessment of growth and model validation

All the inoculated samples were stored at 30 °C for 90 days with a visual check every five days. For each condition of sauces, all five replicated samples were analyzed at day 0, day 30, day 60 and day 90 or until visual spoilage. On each day of analysis, the cell count in each sample was enumerated in triplicate on MYP plates, which is a selective medium for isolation of *Bacillus* species (Balcazar and Rojas-Luna, 2007). If the cell count of the sample at the day of analysis is higher than 0.5 $\log_{10}$ of the cell count at "day 0", the sample is considered as showing "growth", otherwise "no growth" was assigned. The model performance in the sauce was evaluated by c-value (concordance index), % correct-predicted, % fail-safe, and % fail-dangerous.

3. Results and discussion

3.1. The developed G/NG models

From the 320 combinations of the temperature, a_w, pH, TLAW and TAAW concentrations, 3840 G/NG data (320 * 12 replicates) were generated on each day of analysis. Based on the generated G/NG data in the current study and the former G/NG data collected by Sun et al. (2021a), three models were built for each strain under a fixed temperature to fit the G/NG data collected at 30, 60 and 90 days separately, describing the influence of a_w, pH, and TLAW and TAAW concentrations on the growth probability for three time points. The ordinary logistic regression models were used to model the data in this research, whose superiority in describing the G/NG interface has been proved previously (Gysemans et al., 2007; Vermeulen et al., 2007a).

The parameter estimates and the model performance of the models are presented in Tables 2-5. For these 4-variate (4V) models, the Nagelkerke R^2 values varied from 0.804 to 0.885, implying that the growth probability of the ATSSB spores can be well predicted by the independent variables. The HL statistics of most models were quite low with p-values > 0.05, indicating that most models could fit the data adequately. All these models exhibited high c-values (> 0.98) and high % correct-predicted values (≥ 95.8%), which represented a good predictive power. Moreover, the highest % fail-dangerous value among all the models was no more than 2.2%, which indicated that the no growth conditions can be predicted well by the models. Some cross-sections at D30 and D90 generated by the models were selected to show the important outcomes (Figs. 1 and 2). As shown in the figures, the observed G/NG responses have a good consistency with the model predictions, indicating that the growth behavior of the strains can be accurately predicted by their corresponding models. Besides, the G/NG interface at D30 and D90 only exhibited little differences, which is in accordance with the observed G/NG data, indicating that only a small portion of cases transformed from no growth to growth during 30-90 days. According to the observed G/NG data, most of the growth cases occurred during the first 30 days, while only a small portion of cases transformed from no growth to growth during 30-60 days, and nearly no changes took place after 60 days. This fact can be proved in Table 3, where the model parameter estimates and standard errors for 90 and 60 days were practically the same, while they were markedly

different from that for 30 days. These similar model parameter estimates reflected the similar G/NG behavior for 90 days and 60 days. It has been observed that for several types of food products and microorganisms when stable conditions are found for a period of 3 months, they are normally stable for a period of 6-12 months (Marvig et al., 2015; Kuroda et al., 2019; Vermeulen et al., 2012). Therefore, the models based on the G/NG data during three months should be reliable to make predictions for the shelf-stable acidified sauces, whose expected shelf life is generally 6 months to 1 year.

3.2. Influence of water activities

From the overall results, a_w exerted an evident influence on the G/NG interface of *B. velezensis* and *B. subtilis* spores. The combined inhibitory effect of temperature, pH, acetic and lactic acid on the spores of *B. subtilis* and *B. velezensis* at a_w 0.98 was in accordance with the former models (Sun et al., 2021a), while the growth probabilities at a_w 0.965 and 0.95 significantly decreased in the same combination of pH and organic acid concentrations.

With constant organic acid concentrations, reduced a_w levels allowed higher pH values to prevent the growth of ATSSB spores. At higher a_w levels (0.98), even at the lowest pH (4.4) a high growth probability was predicted in the absence of organic acids. Especially for *B. velezensis* spores, the growth probabilities under both temperatures were higher than 90% regardless of the pH (Fig. 1a), while decreasing a_w to 0.95 dramatically changed the growth probability at both temperatures, as no growth ($P < 0.1$) occurred below pH 4.7 (Fig. 1b). At constant pH levels, the antimicrobial effect of lactic acid was strongly influenced by a_w in the absence or at low concentrations of acetic acid (Fig. 3). For example, at pH 5.3 and in the absence of acetic acid, 0.1% TLAW absolutely inhibited the growth from *B. velezensis* spores at a_w 0.95 irrespective of the incubated temperatures, while at a_w 0.98 even 1.0% TLAW was insufficient to prevent the growth under 22 °C (Figs. 3 a, b). On the other hand, the interactive effect between a_w and acetic acid concentrations was less demonstrated. As shown in Figure 3, the a_w change only resulted in a narrow shift of the no growth boundary on the TAAW concentrations compared with the shift on TLAW concentrations. Especially for *B. velezensis* spores at pH 5.3 and 22 °C (Fig. 3a), the no growth boundary maintains at around 0.2% TAAW regardless of the a_w change (0.95-0.98). The no growth boundaries of spores from *B. velezensis* and *B. subtilis* at some selected

conditions are presented in Figure 4. Obviously, decreasing a_w narrowed the growth area of both strains, while more evident changes took place in the growth of *B. velezensis,* which mainly resulted from the decreased resistance to lactic acid. The difference between these two strains regarding a_w stress can be illustrated at pH 5.3 (Fig. 4), where the potential growth area ($P > 0.1$) of *B. velezensis* was broader than *B. subtilis* at a_w 0.98, while *B. subtilis* spores had more chance to grow at lower a_w values (0.95 and 0.965). It implied that the acid resistance of *B. subtilis* spores was less influenced by a_w changes than *B. velezensis* spores during the evaluated range (a_w 0.95-0.98), which might be due to the higher resistance of *B. subtilis* spores to low a_w. Sun et al. (2021b) reported that the growth from *B. velezensis* spores was started at a_w 0.96 in NB, while the minimum a_w value allowing *B. subtilis* spores to initiate growth was around a_w 0.95. This also illustrated the importance of incorporating several strains of specific spoilage organisms, when developing a preservation strategy. This is especially the case with *Bacillus* species having a very diverse resistance towards different preservation strategies.

Furthermore, changing a_w at high acetic acid concentrations had less effect on the minimum growth pH (Figs. 1 and 2) and the no growth concentrations of TLAW (Fig.3). It revealed that the enhancement of lowering a_w on the antimicrobial effect of decreased pH and lactic acid might be diminished at higher TAAW concentrations. Linked with the phenomenon that changing a_w only slightly influenced the inhibitory effect of acetic acid, lowering a_w might be more beneficial in the absence or at low concentrations of acetic acid, which could be explained by the dominant antimicrobial effect of acetic acid when it was present at high concentrations. The dominant antimicrobial effect of acetic acid was also observed by Vermeulen, et al. (2007a), towards inhibiting the growth of spoilage bacteria (*Lactobacillus fructivorans*) in acidified sauces. They mentioned that the addition of lactic acid was more beneficial in the absence or low concentrations of acetic acid. Otherwise, the inhibition mainly resulted from the combinatory effect of pH and acetic acid. Another reason could be that the addition of NaCl (to decrease a_w) under certain acetic acid conditions exerted a protective effect on the ATSSB spores. A similar phenomenon was observed by Cervenka et al. (2004), who reported that a decrease in a_w (0.993 to 0.977) enhanced the antimicrobial effect of 0.1% (w/v) acetic acid on a food-borne pathogen

306 (*Arcobacterbutzleri*), while the same a_w change resulted in an adverse effect when 0.2 and 0.3% acetic
307 acid was present.

308 Lowering a_w of food systems can not only inhibit the germination of bacterial spores but also prevent
309 the growth of germinated spores (Rao et al., 2018). Although the lowest a_w value in the investigated
310 range could not individually prevent the growth from ATSSB spores without other hurdles, a narrowed
311 growth area was observed accompanied by deceased a_w levels. The combined effect of a_w, pH, and
312 organic acids on microbial growth has been observed by several studies (Franceschini et al., 2020;
313 Kuroda et al., 2019; Nyhan et al., 2018). For instance, Kuroda et al. (2019) observed that decreasing a_w
314 levels (0.94 to 0.88) increased the minimum growth pH (around 5.0 to 6.5) for the *B. subtilis* spores at
315 0.1% acetic acid in broth. Franceschini et al. (2020) observed a clearly combined action of pH, a_w,
316 temperature, and organic acid on inhibiting the spore germination and growth of *Bacillus*. Ignoring the
317 influence of a_w can lead to an overestimation of the growth probability. By doing this food manufacturers
318 may utilize too stringent processes on the products, such as more intensive heat treatments and excessive
319 addition of acids or salts, resulting in adverse effects on the food quality. It highlighted the significance
320 to incorporate a_w in the predictive models for low-acid pasteurized sauces, which can generate more
321 accurate predictions, limiting overzealous processes. A synergistic effect between a_w, pH, TAAW and
322 TLAW concentrations on the growth of ATSSB were observed in the current study. However, it needs
323 to be noted that lowering a_w combined with the addition of organic acids can generate a synergistic
324 effect, no significant effect, or even antagonistic effect on inhibiting microbial growth (Kim and Lee,
325 2021). It might be due to the fact that the decrease in a_w relies on the addition of solutes, like sodium
326 chloride, sucrose or glycerol (Ben Braïek and Smaoui, 2021; Casey and Condon, 2002; Kim and Lee,
327 2021; Lee and Kang, 2009; Yoon and Lee, 2018). Therefore, utilizing the antimicrobial agents all
328 present at high levels could not always result in a better additive antimicrobial effect. In addition,
329 although acetic and lactic acid exhibited a clearly antimicrobial effect against ATSSB spores, high
330 concentrations of them can result in inappropriate flavors of the food product. A combination with a
331 moderately reduced pH and a_w seems to be a good strategy to reduce the addition of organic acids,
332 avoiding off-flavors. The consumers' demand for more 'natural' foods also highlights the importance

of exploring a suitable combination of the preservative factors to guarantee the microbial stability of food products by utilizing mild preservation treatments.

3.3. Validation of the developed models in real food systems

Eight industrially produced low-acid pasteurized sauces covering eight combinations of a_w, pH, acetic and lactic acid concentrations and three types of sauces (satay sauce, cheese sauce and beef sauce) were used for the validation challenge tests. The sauces were produced under factory conditions, and their physical, chemical and microbial characteristics are presented in Table 1. It is not surprising that some bacteria were still detected in several sauces after industry pasteurization, as some bacterial spores have high resistance to stress conditions, including heat, acidity and salinity, which allows their survival during the processing and preservation treatments in the food industry (den Besten et al., 2018; Wells-Bennik et al., 2016). A comparison between the prediction of the G/NG models and the observed growth probabilities of the ATSSB spores in the sauces after 30, 60 and 90 days is presented in Table 6 and the "fitted v. observed" line is put into the appendix (Fig. A1). Five replicated samples were prepared for each condition of sauces. Overall, the % correct-predicted values at D30, D60 and D90 were all higher than 90%, which indicated that most growth and no growth cases were well predicted by the developed models. There were no fail-dangerous cases occurring after 30 days, while the % fail-dangerous cases were increased to 2.5% after 60 days and 5.0% after 90 days, indicating that slightly more growth cases were observed in the predicted no growth conditions. Besides, to perform a strict validation, all the validated conditions were chosen near or in the G/NG zone, which can lead to a low % correct predictions(e.g., < 70%) even for an accurate model (Mejlholm and Dalgaard, 2009). Despite this, the model performance was still acceptable, indicating that validation with randomly chosen conditions should have better results.

A reliable predictive model that has been validated with challenge tests in representative food products can rapidly obtain the information regarding microbial safety and stability of a product and reduce costs, compared with the laborious challenge studies (Membré and Lambert, 2008). According to the authors' knowledge, (Sun et al., 2021a) is the only research focusing on the predictive models typically for low-acid pasteurized sauces under ambient storage, in which ATSSB would be the specific spoilage bacteria.

However, the models were based on a single a_w value and lacked the validation in real food systems, limiting their application. Therefore, the current study enlarged the models by considering the variation in a_w values typical for low-acid pasteurized sauces. The reliability and accuracy of the developed G/NG models were evaluated in pasteurized sauces produced under industrial conditions, which made the models more applicable to the food industry. However, it should be noted that the predictive models focus on ATSSB spores that survived pasteurization, which means that the models should be applied to food products that are pasteurized under good manufacturing practices. If not, the presence of other acid-tolerant spoilage microorganisms, such as lactic acid bacteria, yeasts and molds, will result in an inappropriate application of the developed models.

4. Conclusion

In conclusion, the G/NG models for ATSSB spores as a function of a_w, pH, TLAW and TAAW concentrations were developed under two temperatures in laboratory broth and were validated in different pasteurized sauces. The results of this study demonstrated that lowering a_w enhanced the antimicrobial effect of decreased pH and the addition of acetic and lactic acid against the ATSSB spores. However, the influence of a_w changes was less pronounced at high acetic acid concentrations. The G/NG models were based on the conditions typically encountered in the low-acid pasteurized sauces and accurately predicted the growth probabilities of ATSSB spores in the real sauces. This work contributes to determining milder preservation strategies for this type of food products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Physicochemical and microbial characteristics of the low-acid pasteurized sauces for challenge test

No.	Sauce types	Physico-chemical characteristics					Microbial background (log cfu/g)						
		Aw	pH	TA AW[a]	TLA W[b]	Moisture /%	TAB[c]	PB[d]	Spores	TANB[e]	LAB[f]	SRB[g]	Y&M[h]
1	Satay sauce	0.947	5.27	0.0	0.35	50.89	-[i]	-	-	-	-	-	-
2	Satay sauce	0.948	5.36	0.1	0.00	52.60	-	-	-	-	-	-	-
3	Cheese sauce	0.965	5.14	0.0	0.47	63.32	3.3	2.9	2.5	2.6	2.3	-	-
4	Cheese sauce	0.966	5.20	0.2	0.00	64.68	2.8	2.2	2.4	2.0	2.2	-	-
5	Beef sauce	0.982	5.12	0.0	0.00	86.06	-	-	-	-	-	-	-
6	Cheese sauce	0.983	5.33	0.1	0.00	73.82	2.8	2.4	2.4	2.2	-	-	-
7	Cheese sauce	0.981	4.75	0.2	0.36	73.54	-	-	-	-	-	-	-
8	Cheese sauce	0.982	4.95	0.3	0.00	73.53	2.1	2.0	2.6	-	-	-	-

[a] Total acetic acid in the water phase expressed in % (w/w)

[b] Total lactic acid in the water phase expressed in % (w/w)

[c] Total aerobic bacteria

[d] Presumed *Bacillus* spp.

[e] Total anaerobic bacteria

[f] Lactic acid bacteria

[g] Sulfite reducing bacteria

[h] Yeast and molds

[i] Cell counts below the detection limit of 100 cfu/g

Table 2. Parameter estimates with their standard errors and performance statistics for the models of *B. velezensis* (BS127) spores at 22 °C

Parameter	4V model-30 days	4V model-60 days	4V model-90 days
Intercept	-353.4 ± 31.6	-287.9 ± 25.9	-185.5 ± 33.9
AW	332.3 ± 30.3	273.4 ± 25.2	236.3 ± 22.3
pH	6.78 ± 0.67	5.13 ± 0.53	-20.38 ± 10.26
TLAW	-500.8 ± 77.3	-442.3 ± 65.8	-414.7 ± 59.9
TAAW	1147 ± 171.9	959.3 ± 151.8	899.3 ± 140.4
pH*pH	NS [a]	NS	2.44 ± 1.03
TAAW*TAAW	-198.4 ± 27.8	-194.4 ± 25.3	-179.0 ± 22.8
TLAW*TLAW	NS	NS	NS
AW*pH	NS	NS	NS
AW*TAAW	·-1479 ± 176.6	-1327 ± 160.1	-1234 ± 148.0
AW*TLAW	502.0 ± 78.7	443.6 ± 67.0	416.0 ± 61.1
pH *TAAW	55.20 ± 7.88	62.97 ± 7.64	57.13 ± 8.14
pH *TLAW	NS	NS	NS
TAAW*TLAW	NS	NS	NS
Statistics			
Nagelkerke R^2	0.873	0.859	0.848
% correct-predicted	95.8	96.2	95.9
% fail-dangerous	2.2	1.7	1.9
Hosmer–Lemeshow	6.8	6.6	13.7
(HL) p-value	0.55	0.59	0.09
c-value (ROC curve)	0.994	0.992	0.99
−2 ln (L)	372.7	430.0	478.9

[a] Not significant

Table 3. Parameter estimates with their standard errors and performance statistics for the models of *B. velezensis* (BS127) spores at 30 °C

Parameter	4V model-30 Days	4V model-60 Days	4V model-90 Days
Intercept	426.9 ± 256.6	-64.25 ± 29.14	-65.19 ± 28.80
AW	-402.3 ± 255.7	200.2 ± 19.0	199.8 ± 18.6
pH	-145.2 ± 56.0	-55.90 ± 10.77	-55.18 ± 10.59
TLAW	-804.8 ± 108.1	-841.0 ± 109.0	-823.6 ± 106.8
TAAW	199.6 ± 244.9	-268.2 ± 35.7	-269.5 ± 34.7
pH*pH	2.73 ± 1.34	6.06 ± 1.10	5.97 ± 1.08
TAAW*TAAW	-159.1 ± 25.8	-163.4 ± 19.5	-141.8 ± 18.4
TLAW*TLAW	NS [a]	NS	NS
AW*pH	128.6 ± 53.0	NS	NS
AW*TAAW	-557.5 ± 222.4	NS	NS
AW*TLAW	808.5 ± 110.1	847.9 ± 111.0	830.4 ± 108.8
pH *TAAW	65.22 ± 12.57	53.19 ± 7.10	52.84 ± 6.90
pH *TLAW	NS	NS	NS
TAAW*TLAW	13.05 ± 7.74	NS	NS
Statistics			
Nagelkerke R^2	0.874	0.863	0.861
% correct-predicted	97.4	97.1	97.0
% fail-dangerous	1.5	1.8	1.5
Hosmer–Lemeshow	20.9	1.0	2.7
(HL) p-value	0.01	0.86	0.95
c-value (ROC curve)	0.994	0.992	0.992
−2 ln (L)	393.8	442.5	456.0

[a] Not significant

Table 4. Parameter estimates with their standard errors and performance statistics for the models of *B. subtilis* (BS128) spores at 22 °C

Parameter	4V model-30 Days	4V model-60 Days	4V model-90 Days
Intercept	368.1 ± 286.0	237.9 ± 185.3	-138.6 ± 14.3
AW	-414.2 ± 295.6	-271.6 ± 191.5	113.7 ± 13.2
pH	-104.6 ± 54.5	-68.89 ± 35.5	5.70 ± 0.71
TLAW	-9.71 ± 1.11	-7.82 ± 0.80	14.71 ± 9.01
TAAW	-1033 ± 306.4	-322.9 ± 56.0	-204.8 ± 46.2
pH*pH	NS [a]	NS	NS
TAAW*TAAW	-1279 ± 380.2	-542.8 ± 71.2	-444.9 ± 59.8
TLAW*TLAW	NS	NS	NS
AW*pH	114.9 ± 56.4	76.35 ± 36.7	NS
AW*TAAW	NS	NS	NS
AW*TLAW	NS	NS	NS
pH *TAAW	230.6 ± 68.7	77.65 ± 12.4	52.29 ± 10.07
pH *TLAW	NS	NS	-4.09 ± 1.72
TAAW*TLAW	-378.9 ± 111.6	-146.2 ± 23.2	-116.0 ± 19.3
Statistics			
Nagelkerke R^2	0.885	0.834	0.813
% correct-predicted	98.5	96.9	97.2
% fail-dangerous	1.1	1.9	2.1
Hosmer–Lemeshow	0.9	0.2	5.4
(HL) p-value	1.00	0.99	0.72
c-value (ROC curve)	0.996	0.991	0.988
−2 ln (L)	221.3	358.1	412.6

[a] Not significant

Table 5. Parameter estimates with their standard errors and performance statistics for the models of *B. subtilis* (BS128) spores at 30 °C

Parameter	4V model-30 Days	4V model-60 Days	4V model-90 Days
Intercept	-199.1 ± 17.0	-47.74 ± 24.1	-29.18 ± 23.4
AW	176.5 ± 15.9	173.3 ± 14.9	178.8 ± 15.7
pH	5.48 ± 0.65	·-51.38 ± 9.9	-61.75 ± 10.0
TLAW	-8.82 ± 0.94	-9.00 ± 0.87	5.19 ± 7.07
TAAW	-141.8 ± 35.0	-118.6 ± 27.7	-93.86 ± 25.84
pH*pH	NS [a]	5.45 ± 1.00	6.56 ± 1.02
TAAW*TAAW	-292.4 ± 29.3	-283.2 ± 27.6	-294.2 ± 8.9
TLAW*TLAW	NS	NS	NS
AW*pH	NS	NS	NS
AW*TAAW	NS	NS	NS
AW*TLAW	NS	NS	NS
pH *TAAW	37.50 ± 7.2	32.70 ± 5.75	28.35 ± 5.41
pH *TLAW	NS	NS	-2.80 ± 1.37
TAAW*TLAW	NS	-31.10 ± 8.47	-29.40 ± 8.87
Statistics			
Nagelkerke R^2	0.819	0.801	0.807
% correct-predicted	97.5	96.7	97.1
% fail-dangerous	1.5	1.9	1.7
Hosmer–Lemeshow	2.2	2.6	4.6
(HL) p-value	0.97	0.96	0.80
c-value (ROC curve)	0.989	0.987	0.986
−2 ln (L)	402.7	468.0	463.4

[a] Not significant

Table 6. The observed and predicted growth responses in the low-acid pasteurized sauces

No.	Sauce Types	Growth probability D30			Growth possibility D60			Growth probability D90		
		Predicted /%		Observed	Predicted /%		Observed	Predicted /%		Observed
		M1[a]	M2[b]	/%	M1	M2	/%	M1[a]	M2[b]	/%
1	Satay sauce	0	0	0	0	0	20	0	0	20
2	Satay sauce	53	66	20	59	66	40	55	68	40
3	Cheese Sauce	0	1	0	0	1	0	0	1	20
4	Cheese Sauce	3	14	0	4	14	0	6	13	0
5	Beef Sauce	100	91	100	99	91	100	99	90	100
6	Cheese Sauce	100	100	100	100	100	100	100	100	100
7	Cheese Sauce	0	0	0	0	0	0	0	0	0
8	Cheese Sauce	0	0	0	0	0	0	0	0	0
Performance Statistics		M1	M2		M1	M2		M1	M2	
c-value		0.994	0.994		0.942	0.949		0.912	0.912	
% correct-predicted		95.0	92.5		95.0	92.5		92.5	90.0	
% fail-safe		5.0	7.5		2.5	5.0		2.5	5.0	
% fail-dangerous		0.0	0.0		2.5	2.5		5.0	5.0	

[a] Predictions generated by the 4V models for *B. velezensis* spores.

[b] Predictions generated by the 4V models for *B. subtilis* spores.

Figure 1

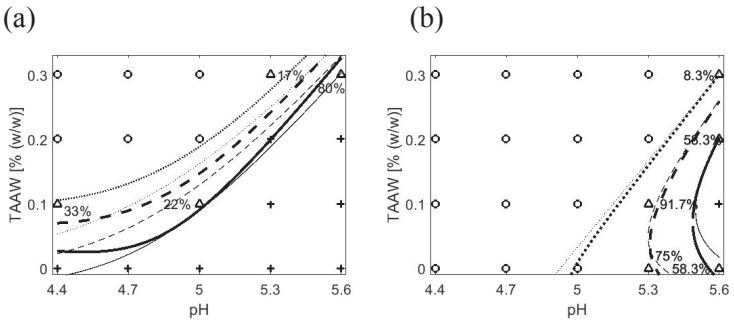


Figure 2

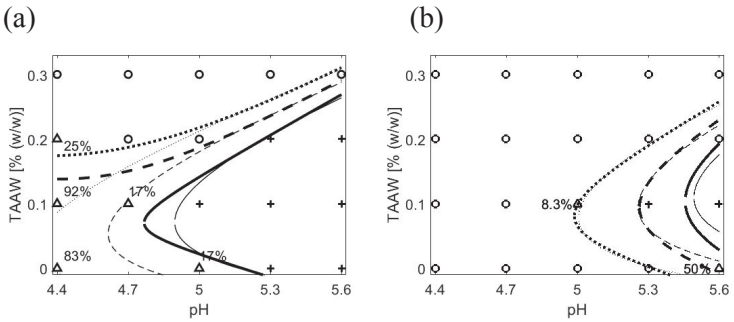


Figure 3

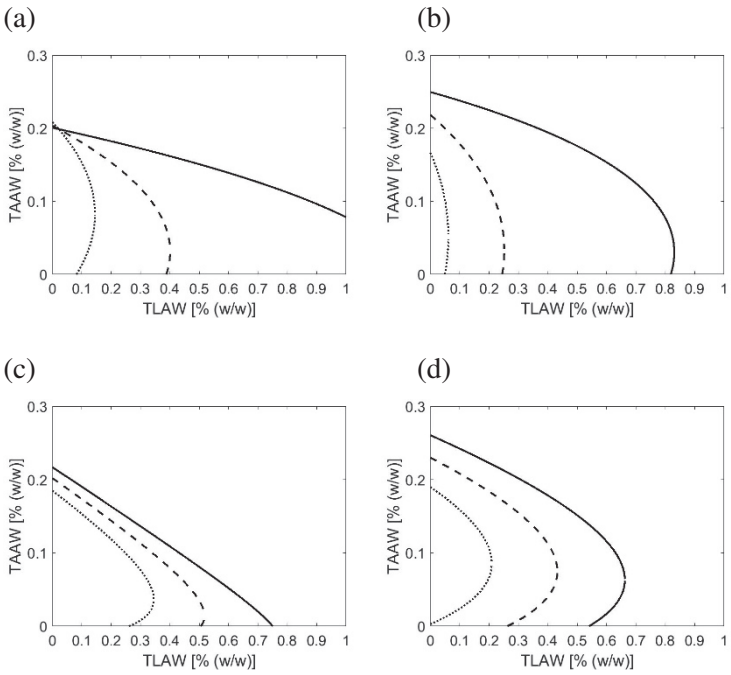


Figure 4

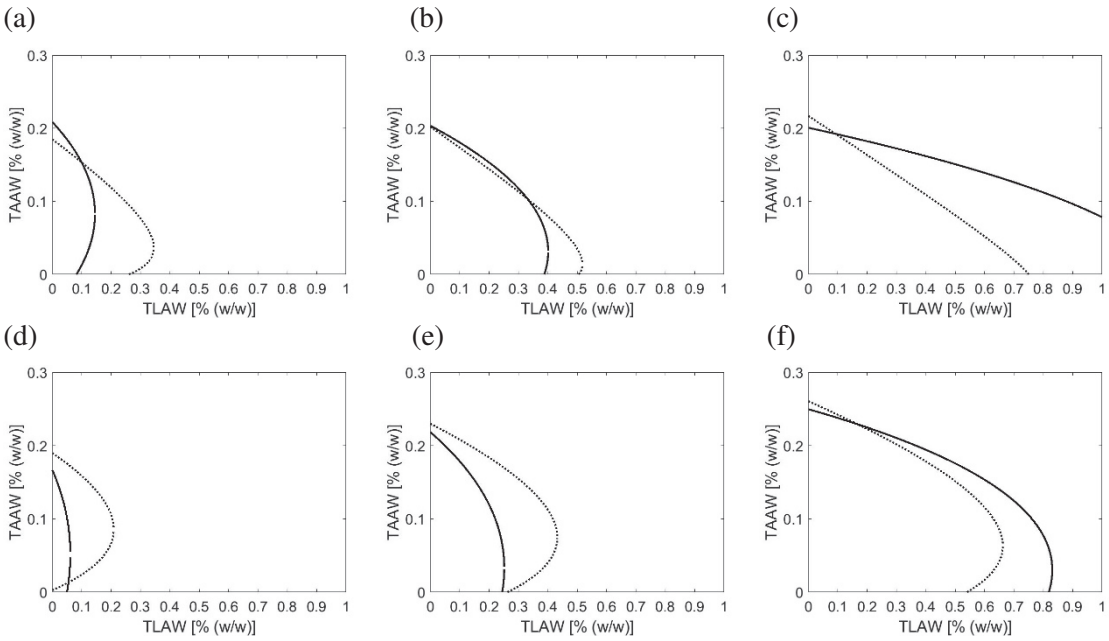
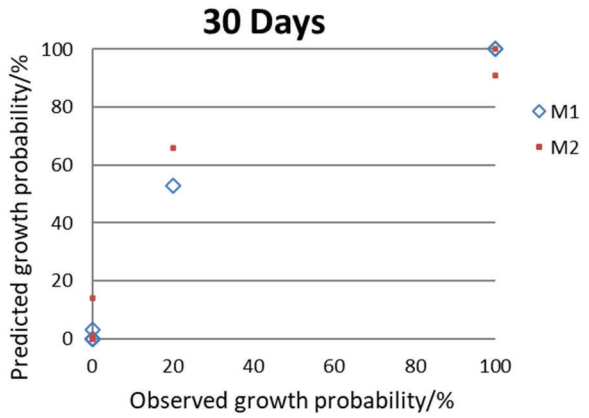
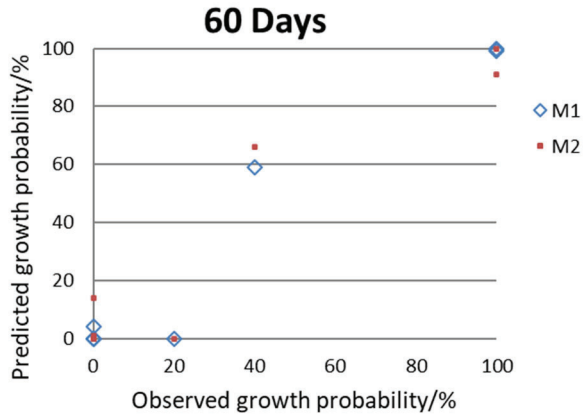


Figure A1

(a)



(b)



(c)

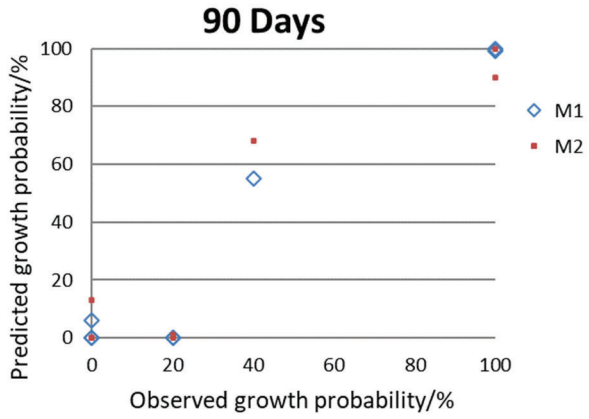


Figure Captions

Figure 1. G/NG interface for *B. velezensis* (BS127) spores at (a): a_w 0.98 and (b): a_w 0.95 generated by the 4V models after 30 days (thin lines) and 90 days (bold lines) without lactic acid at 30 °C. TAAW is the concentration of total acetic acid in the water phase. Symbols indicate the observed data at 90 days: (+) 100% of growth, (O) 0% of growth and (△) growth between 0-100% (with the indicated percentage). Lines represent the ordinary logistic regression model predictions: p=0.9 (—), p=0.5 (- - -), p= 0.1 (. . . .).

Figure 2. G/NG interface for *B. subtilis* (BS128) spores at (a): a_w 0.98 and (b): a_w 0.95 generated by the 4V models after 30 days (thin lines) and 90 days (bold lines) without lactic acid at 30 °C. TAAW is the concentration of total acetic acid in the water phase. Symbols indicate the observed data at 90 days: (+) 100% of growth, (O) 0% of growth and (△) growth between 0-100% (with the indicated percentage). Lines represent the ordinary logistic regression model predictions: p=0.9 (—), p=0.5 (- - -), p= 0.1 (. . . .).

Figure 3. No growth boundaries (P=0.1) for spores of *B. velezensis* (BS127) (a, b) and *B. subtilis* (BS128) (c, d) at pH 5.3 generated by the 4V models after 90 days at 22 °C (left) and 30 °C (right) with different a_w levels: a_w 0.98 (—), a_w 0.965 (- - -), a_w 0.95 (. . . .). TAAW is the concentration of total acetic acid in the water phase and TLAW is the concentration of total lactic acid in the water phase.

Figure 4. Cross sections at 10% growth probability generated by the 4V models after 90 days for spores from *B. velezensis* (BS127) (—) and *B. subtilis* (BS128) (. . . .) at 22 °C (a, b, c) or 30 °C (d, e, f) with pH 5.3 and different a_w levels: a and d: a_w 0.95; b and e: a_w 0.965; c and f: a_w 0.98. TAAW is the concentration of total acetic acid in the water phase and TLAW is the concentration of total lactic acid in the water phase.

Figure A1. The "fitted v. observed" lines of the growth responses in the low-acid pasteurized sauces after (a) 30, (b) 60 and (c) 90 days.