MIRIAN PEREIRA DA SILVA

MODELLING OF THE *Staphylococcus aureus* GROWTH AND ENTEROTOXIN A PRODUCTION BASED ON DIFFERENT CONDITIONS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Doctor Scientiae*.

Orientador: Luís Augusto Nero

Coorientador: Antônio Fernandes de Carvalho

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Luís Augusto Nero
Orientador

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RESUMO

SILVA, Mírian Pereira da, D.Sc., Universidade Federal de Viçosa, novembro de 2023. **Modelagem da multiplicação de *Staphylococcus aureus* e produção de enterotoxina A frente a diferentes condições.** Orientador: Luís Augusto Nero. Coorientador: Antônio Fernandes de Carvalho.

Staphylococcus aureus é um dos patógenos de maior envolvimento em surtos alimentares. *S. aureus* enterotoxigênicos produzem enterotoxinas estáveis a altas temperaturas. Isto é preocupante para produtos lácteos, como o queijo Minas frescal, uma vez que enterotoxinas podem ser liberadas pelo microrganismo no leite cru e não serem destruídas por processos térmicos, como a pasteurização. Portanto, é necessário adotar medidas adequadas para prevenir a contaminação e a eventual produção de toxinas. A microbiologia preditiva é uma ferramenta poderosa para a segurança e qualidade dos alimentos. A partir dela, modelos matemáticos são capazes de prever o comportamento microbiano em condições específicas de desenvolvimento. Considerando a elevada frequência de *S. aureus* em queijo Minas frescal, modelos matemáticos são relevantes para demonstrar como a sua multiplicação e a produção de enterotoxinas são influenciados por condições de produção e armazenamento. Assim, este estudo teve como objetivo caracterizar os aspectos físico-químicas e a presença de *S. aureus* em queijo Minas frescal, e a partir desses dados, criar modelos preditivos de multiplicação e produção de enterotoxina A por este patógeno. Amostras de queijo Minas frescal (n = 50) foram obtidas e submetidas a análises de pH, concentração de cloreto de sódio e temperatura. A presença e caracterização de *Staphylococcus* spp. foram avaliadas e os isolados coagulase-positivo foram investigados quanto à presença de gene *nuc* para identificação da espécie e genes de enterotoxinas (*sea*, *seb*, *sec*, *sed*, *see*). A partir das informações obtidas, modelos de multiplicação (*Combase* e modelo superfície de resposta) e produção de enterotoxinas (modelo de probabilidade de regressão logística) foram desenvolvidos e validados estatisticamente e experimentalmente. Todos os isolados coagulase-positivo testados foram confirmados como *S. aureus* e nenhum apresentou genes relacionados à enterotoxina. O pH, a temperatura e a concentração de NaCl variaram de 5,80 a 6,62, de 5 °C a 12 °C e de 0,85% a 1,70%, respectivamente. O modelo gerado pelo *Combase* avaliou a influência da temperatura, pH e concentração de cloreto de sódio na cinética de multiplicação de *S. aureus* e encontrou valores variando de 0,012 a 0,419 log UFC/mL/h de taxa de multiplicação e tempo de adaptação de 4,60 a 159,24 h. A temperatura foi o fator que mais influenciou na cinética de multiplicação e

o cloreto de sódio não influenciou significativamente nas respostas. Posteriormente, modelos de superfície de resposta e de regressão logística foram desenvolvidos para avaliar as respostas de multiplicação de *S. aureus* e a probabilidade de produção de enterotoxina A (SEA), respectivamente. Além dos fatores avaliados na cinética de multiplicação, diferentes níveis de contaminação inicial de *S. aureus* e o tempo foram incorporados aos modelos. Com exceção da concentração de cloreto de sódio, todos os fatores afetaram significativamente a multiplicação e a produção de SEA pelo microrganismo. A população máxima obtida variou de 8,27 log UFC/mL a 9,36 log UFC/mL a 25 °C e de 3,90 log UFC/mL a 8,27 log UFC/mL a 15 °C. A 10 °C, no menor nível de contaminação avaliado. *S. aureus* (10^0 UFC/mL) permaneceu abaixo do limite de detecção (<1,0 UFC/mL) durante toda a incubação e não houve produção de SEA, enquanto para as demais concentrações (10^3 UFC/mL e 10^5 UFC/mL), a máxima população foi de 7,79 UFC/mL, demonstrando a importância de manter o queijo Minas frescal a temperaturas de refrigeração e controlar o nível de contaminação inicial. SEA foi produzida em todas as condições avaliadas a 15 °C e 25 °C, mas em diferentes tempos. Os modelos apresentaram bom ajuste aos dados experimentais, com valores satisfatórios de R^2 (0,90), fator de precisão (1,09) e fator bias (0,99) para o modelo de multiplicação e, alta porcentagem de concordância (94,4%), R^2 de Nagelkerke (0,92) e teste de Hosmer e Lemeshow ($p > 0,05$) para o modelo de produção de SEA. Além disso, a validação experimental em meio de cultura, leite e queijo confirmou que modelos são apropriados para prever as respostas de multiplicação e a probabilidade de produção de SEA por *S. aureus* no queijo Minas frescal.

Palavras-chave: *Staphylococcus aureus*. Enterotoxina A. Queijo Minas frescal. Microbiologia preditiva. Modelos matemáticos. Cinética de multiplicação.

ABSTRACT

SILVA, Mírian Pereira da, D.Sc., Universidade Federal de Viçosa, November, 2023. **Modelling of the *Staphylococcus aureus* growth and enterotoxin A production based on different conditions.** Adviser: Luís Augusto Nero. Co-adviser: Antônio Fernandes de Carvalho.

Staphylococcus aureus is one of the pathogens most involved in food outbreaks. Enterotoxigenic *S. aureus* produce enterotoxins stable at high temperatures. This is a concern for dairy products, like Minas frescal cheese, since enterotoxins can be produced in early stages of production (raw milk) and remain stable after thermic treatments, like pasteurization. Therefore, it is necessary to adopt appropriate measures to prevent contamination and the eventual toxins production. Predictive microbiology is a powerful tool for food safety and quality. Using mathematical models, it is possible to predict the behavior of microorganisms under specific conditions. Given that Minas frescal cheese is strongly associated with *S. aureus*, mathematical models are relevant to demonstrate how bacterial growth and enterotoxins production are influenced by production and storage conditions. Thus, this study aimed to characterize the physicochemical aspects and the presence of *S. aureus* in Minas frescal cheese and, based on this data, create predictive models of growth and the production of enterotoxin A by *S. aureus*. Samples of Minas frescal cheese (n = 50) were obtained and subjected to pH, sodium chloride concentration, and temperature analysis. The presence and characterization of *Staphylococcus* spp. were evaluated, and coagulase-positive isolates were investigated for the presence of the *nuc* gene for *S. aureus* identification and enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*). Based on this data, growth models (*Combase* and response surface model) and enterotoxin production models (probabilistic logistic regression model) were developed and statistically and experimentally validated. All coagulase-positive isolates tested were confirmed as *S. aureus*, and none presented genes related to enterotoxins. The pH, temperature, and salt concentration varied from 5.80 to 6.62, from 5 °C to 12 °C, and from 0.85% to 1.70%, respectively. The model generated by *Combase* assessed the influence of temperature, pH, and salt concentration on the growth kinetics (maximum growth rate and lag time) of *S. aureus* and found values ranging from 0.012 to 0.419 log CFU/mL/h of growth rate and adaptation time from 4.60 to 159.24 hours. Temperature was the factor that most influenced the growth kinetics, and salt concentration did not significantly influence the responses. Subsequently, response surface models and logistic regression models were developed to assess the growth responses of *S. aureus* and the probability of enterotoxin A (SEA) production, respectively. In addition to

the factors assessed in the growth kinetics, different levels of initial contamination of *S. aureus* and time were incorporated into the models. With the exception of sodium chloride concentration, all factors significantly affected the growth and production of enterotoxin A by *S. aureus*. The maximum population obtained ranged from 8.27 to 9.36 log CFU/mL at 25 °C and from 3.90 to 8.27 log CFU/mL at 15 °C. At 10 °C, at the lowest contamination level evaluated, *S. aureus* (10^0 CFU/mL) remained below the detection limit (<1.0 CFU/mL) throughout incubation, and no SEA production occurred, while for the other concentrations (10^3 CFU/mL and 10^5 CFU/mL), the maximum population was 7.79 CFU/mL, highlighting the importance of maintaining Minas frescal cheese at refrigeration temperatures and controlling the initial contamination level. SEA production occurred for all conditions evaluated at 15 °C and 25 °C, but at different times. The models showed a good fit to the experimental data, with satisfactory values for R^2 (0.90), accuracy factor (1.09), and bias factor (0.99) for the growth model; a high agreement percentage (94.4%), Nagelkerke's R^2 (0.92), and the Hosmer and Lemeshow test ($p > 0.05$) for the SEA production model. Additionally, experimental validation in culture media, milk, and cheese confirmed that the models are suitable for predicting the growth and the probability of SEA production by *S. aureus* in Minas frescal cheese.

Keywords: *Staphylococcus aureus*. Enterotoxin A. Minas frescal cheese. Predictive microbiology. Mathematical models.

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INTRODUÇÃO

O gênero *Staphylococcus* é constituído por bactérias coagulase positivas e negativas, baseado na produção da enzima coagulase, capaz de coagular plasma sanguíneo. Ambos possuem espécies patogênicas, capazes de produzir enterotoxinas. Dentre as espécies enterotoxigênicas, *S. aureus* se destaca pela incidência de surtos de origem alimentar, especialmente em produtos de origem animal.

Nos anos de 2009 a 2021, *S. aureus* representou cerca de 2,1% dos surtos alimentares notificados ocorridos nos EUA (CDC, 2021). No Brasil apesar da escassez de dados epidemiológicos no sistema de coleta, foi registrado que nos anos de 2013 a 2022, *S. aureus* foi o 3º patógeno de maior incidência em alimentos, representando 10,8% dos surtos de doenças de transmissão hídrica e alimentar (Brasil, 2023).

S. aureus se desenvolvem em ampla faixa de condições ambientais: é capaz de se multiplicar em temperaturas de 6 a 48 ºC, pH entre 4,2 e 9,3 e em atividade de água mínima de 0,83. Quando em condições favoráveis, podem produzir as enterotoxinas estafilocócicas (SEs). Quando ingeridas, SEs podem causar náusea, vômito, dores abdominais e diarreia.

S. aureus são suscetíveis a tratamentos térmicos e antimicrobianos, além de serem considerados fracos competidores em relação a outras bactérias. No entanto, as enterotoxinas estafilocócicas são resistentes a altas temperaturas e às enzimas proteolíticas do trato digestório, o que permite a permanência da sua atividade mesmo após pasteurização ou ultrapasteurização do leite. Este fato causa grande preocupação para inocuidade, principalmente de leite e seus derivados, como queijo Minas frescal.

O queijo Minas frescal, por apresentar valores de pH e umidade elevados, apresenta alta suscetibilidade à multiplicação bacteriana. Diversos estudos relatam a ocorrência de índices de contaminação e surtos alimentares associadas ao consumo de queijo Minas frescal pela presença de enterotoxinas produzidas por *S. aureus* (Arcuri *et al*., 2012; Cândido *et al*., 2020; Carmo *et al*., 2002; Júnior *et al*., [2023?]). Desse modo, cuidados higiênicos durante o processamento desses alimentos devem ser tomados, visando a prevenção da disseminação de *S. aureus* e consequente produção de SEs na cadeia alimentar.

Apesar do grande número de estudos focados na avaliação da cinética de multiplicação de *S. aureus* nos alimentos, não há muitos registros de pesquisas que analisam a produção de suas enterotoxinas sob diferentes condições de processamento e armazenamento de queijo Minas frescal. Neste contexto, a microbiologia preditiva é um instrumento fundamental na garantia de controle e inocuidade alimentar. Esta ferramenta tem como objetivo desenvolver

modelos matemáticos capazes de prever o comportamento microbiano sob condições específicas, como diferentes valores de pH, temperaturas, conservantes, microbiota competidora e atividade de água (a_w) e outras alterações importantes para multiplicação ou inativação de microrganismos. Nesse sentido, o presente estudo tem como objetivo identificar a presença de *S. aureus* em queijo Minas frescal e avaliar a capacidade de multiplicação de *S. aureus* e a produção de enterotoxinas estafilocócicas frente a diferentes temperaturas, pH, concentração de cloreto de sódio (NaCl), concentração microbiana inicial e tempo.

REVISÃO BIBLIOGRÁFICA

1. Queijo Minas frescal

A produção de queijo constitui uma das mais importantes atividades na indústria de laticínios. Atualmente, 36% da produção de leite no Brasil é destinada à fabricação de queijo. O consumo médio anual *per capita* de queijos no país é de cerca de 5,6 kg. Durante a pandemia, o queijo foi o derivado lácteo de maior consumo entre os brasileiros. Em 2022, a produção brasileira deste alimento foi de 795 mil toneladas (USDA, 2022). Entre os principais queijos, destaca-se o Minas frescal, o qual representou 11% do valor total de vendas do mercado de queijos em 2020 (USDA, 2020). Isso se deve às vantagens do ponto de vista tecnológico, como, o maior rendimento obtido de sua elaboração, preços acessíveis e por ser de fácil aceitação no mercado consumidor (Oliveira *et al*., 2017; Scudino *et al*., 2023).

O queijo Minas frescal é de origem brasileira, com sua produção iniciada em Minas Gerais no século XVIII, nas regiões de predominância do gado leiteiro (Vinha *et al*., 2010). O regulamento Técnico de Identidade e Qualidade do queijo Minas frescal o define como um produto fresco obtido por coagulação enzimática do leite com coalho e/ou outras enzimas coagulantes apropriadas, adicionada ou não de bactérias láticas específicas (Brasil, 1997).

Diversas alternativas são utilizadas para fabricação desse queijo, como a adição de ácido lático industrial, substituição parcial do fermento por ácido lático, adição de culturas láticas, diferentes temperaturas de coagulação e formas mais modernas de produção, como a ultrafiltração, processo que confere à massa uniformidade de sabor, além de maior tempo de vida útil (Furtado; Lourenço, 1994).

Os parâmetros físico-químicos do queijo Minas frescal são influenciados pelos processos de fabricação, mas no geral trata-se de um queijo semi-gordo, de muito alta umidade, pH entre 5,8 e 6,5, em torno de 0,8 a 1,6% de NaCl e validade de 20 a 30 dias (Carvalho; Viotto; Kuaye, 2007; Dias *et al*., 2016; Silva *et al*., 2021).

Fatores intrínsecos, como o pH próximo ao neutro, presença de nutrientes e altos valores de a_w, favorecem a sobrevivência e multiplicação dos microrganismos no queijo Minas frescal, acarretando em uma curta vida de prateleira, mesmo sob temperaturas de refrigeração (De Paula *et al*., 2021; Silva *et al*., 2023). Dessa forma, a extensão da vida útil e consequentemente, da qualidade deste produto é dependente de condições higiênicas adequadas desde a matéria-prima, até a etapa de armazenamento. O descumprimento de boas práticas higiênico-sanitárias, acarreta no desenvolvimento microbiano (Abreu *et al*., 2021).

De acordo com os padrões microbiológicos vigentes, queijos com umidade superior a 55% como o Minas frescal devem atender os critérios apresentados no Quadro 1.

Quadro 1. Requisitos microbiológicos para o queijo Minas frescal.

Microrganismos	**Critérios de aceitação**
Coliforme/g 30º	n=5 c=2 m=100 M=1.000
Coliforme/g (45ºC)	n=5 c=2 m=50 M=500
Estafilococos Coag. Pos./g	n=5 c=1 m=100 M=500
Fungos e Leveduras/g	n=5 c=2 m=500 M=5.000
Salmonella sp/25g	n=5 c=0 m=0
Listeria monocytogenes/25g	n=5 c=0 m=0

Fonte: Brasil, 1997.

De acordo com a Agência Nacional de Vigilância Sanitária (ANVISA), no comércio o queijo Minas frescal deve atender aos critérios apresentados no Quadro 2.

Quadro 2. Requisitos microbiológicos para o queijo Minas frescal no comércio.

Microrganismos/Toxina	**Critérios de aceitação**
Enterotoxinas estafilocócicas (ng/g)	n=5 c=0 m=Aus
Salmonella/25 g	n=5 c=0 m=Aus
Estafilococos Coagulase Positiva/g	n=5 c=2 m=10^2 M=10^3
Escherichia coli/g	n=5 c=1 m=10^2 M=10^3

Fonte: Brasil. 2022.

Dados da literatura revelam um quadro desfavorável da qualidade microbiológica do queijo Minas frescal. Diversos estudos relatam a ocorrência de altos índices de contaminação e doenças associadas ao consumo desse alimento pela presença de bactérias patogênicas, tais como, *S. aureus* (Abreu *et al*., 2021; Arcuri *et al*., 2012; Carmo *et al*., 2002), *Escherichia coli* (Gonzalez *et al*., 2000; Júnior *et al*., 2019; Mottin *et al*., 2015), além de *Salmonella* spp. (Brant *et al*., 2007; Queiroz *et al*., 2017) e *Listeria monocytogenes* (Carvalho et al., 2007; Falardeau; Trmčić; Wang, 2021; Valiatti *et al*., 2015). Entretanto, têm-se maior preocupação com *S. aureus* devido a sua maior incidência neste produto.

2. O gênero *Staphylococcus*

2.1. Características gerais

O gênero *Staphylococcus* está amplamente distribuído na natureza. Apresenta grande habilidade de adaptação, sendo desse modo, frequentemente isolado de fontes ambientais e alimentares (Rong *et al*., 2023; Zieliński *et al*., 2020). Pertencentes à família *Staphylococcaceae*, estes microrganismos são caracterizados como cocos Gram positivos, com 0,5 a 1,5 µm de diâmetro, sem motilidade e não formadores de esporos. Em sua maioria são anaeróbios facultativos, catalase positivos e coagulase negativos (Becker *et al*., 2015).

Staphylococcus sp. são os principais microrganismos colonizadores do corpo humano: são predominantemente encontrados na pele, mucosas de mamíferos e nos tratos respiratório e digestório (Johnson, 2018). Este gênero é composto por 75 espécies e 30 subespécies (LPSN, 2023), subdividindo-se em: coagulase-positivas e coagulase-negativas, baseado na produção da enzima coagulase que converte o fibrinogênio em fibrina, fornecendo à bactéria a habilidade de coagular o plasma sanguíneo, formando o coágulo (Chajęcka-Wierzchowska; Zadernowska, 2016; Deresinsk, 2005).

Embora *Staphylococcus* coagulase positiva sejam patógenos bem estabelecidos, *Staphylococcus* coagulase negativa têm emergido no reconhecimento como patógenos produtores de enterotoxinas (Beukes; Schmidt, 2018; Helak; Daczkowska-Kozon; Dłubała, 2020; Salamandane *et al*., 2022). Treze espécies são coagulase positivas: *S. aureus* (DE LA FUENTE; SUAREZ, 1985), *S. hyicus* (Devriese *et al*., 1978), *S. intermedius* (Hajek, 1976), *S. pseudintermedius* (Devriese *et al*., 2005), *S. lutrae* (Foster *et al*., 1997), *S. schleiferi* subsp. *coagulans* (Igimi; Takahashi; Mitsuoka, 1990), *S. delphini* (Varaldo *et al*., 1988), *Staphylococcus agnetis* (Taponen *et al*., 2012), *Staphylococcus argenteus*, *Staphylococcus*

schweitzeri (Tong *et al*., 2015), *Staphylococcus cornubiensis* (Murray *et al*., 2018), *Staphylococcus roterodami* (Schutte *et al*., 2021) *e Staphylococcus singaporensis* (Chew *et al*., 2021), sendo as demais coagulase variável ou coagulase negativas. No entanto, apenas *S. aureus*, *S. intermedius* e *S. hyicus* são envolvidas em casos de surtos de intoxicação alimentar pela produção de SEs (Gandra *et al*., 2016).

Dentre as espécies enterotoxigênicas, *S. aureus* se destaca pela incidência de surtos de origem alimentar, especialmente produtos de origem animal (Alves *et al*., 2018; Jamali *et al*., 2015; Johler *et al*., 2018; Li *et al*., 2022).

2.1.1. *Staphylococcus aureus*

S. aureus é uma bactéria Gram positiva, em forma de cocos, anaeróbia facultativa, capsulada ou não e não formadora de esporos (Jay, 2005). Esse microrganismo é mesófilo, com temperatura ótima entre 35 e 37 ºC, podendo se multiplicar em uma faixa de temperatura entre 6 e 48 ºC. O pH ótimo de multiplicação da bactéria é de 6,0 a 7,0, mas se desenvolvem em pH entre 4,2 e 9,3. A atividade de água mínima que permite a multiplicação de *S. aureus* é de 0,83 (Baird-Parker, 1990; Schelin *et al*., 2011). Além disso, são halotolerantes, sobrevivendo em concentrações de NaCI de 10 a 20% (m/v).

Tais micro-organismos são ubíquos, amplamente distribuídos na pele e mucosas de seres humanos e animais. A capacidade de se multiplicar em condições adversas associada a versatilidade nutricional, permitem a multiplicação de *S. aureus* em uma ampla variedade de alimentos, em especial leite e seus derivados (Ahmed, 2019; Ferreira *et al*., 2016; Gajewska *et al*., 2023; Jamali *et al*., 2015; Johler *et al*., 2018).

A transferência de *S. aureus* para o alimento pode ocorrer através do contato deste com o animal infectado ou por práticas impróprias durante obtenção da matéria-prima, produção e armazenamento dos produtos, sendo o manipulador a principal causa de contaminação (Bhunia, 2018; Seow *et al*., 2021; Yap *et al*., 2019).

S. aureus é um dos patógenos de maior envolvimento em surtos alimentares. Sua patogenicidade é devido a produção de SEs no alimento, potencialmente tóxicas para o homem (Zhang *et al*., 2022). Nos anos de 2009 a 2021, *S. aureus* representou cerca de 2,1% dos surtos alimentares notificados ocorridos nos EUA, o que corresponde, a 4.412 doentes, 199 hospitalizações e 2 mortes (CDC, 2021). No Brasil, apesar da deficiência quanto ao sistema de coleta de dados epidemiológicos, há registros de que *S. aureus* foi o 3º patógeno de maior

incidência em alimentos entre 2013 e 2022, representando 10,8% dos surtos de doenças de transmissão hídrica e alimentar (BRASIL, 2023).

Alimentos como leite e queijo Minas frescal são frequentemente associados às infecções e intoxicações de origem alimentar, representando 1,3% dos alimentos incriminados em surtos no Brasil (BRASIL, 2023). Dias *et al.* (2016) constataram que 70% das amostras de queijo Minas frescal avaliadas apresentaram contagens de *S. aureus* acima do preconizado pela legislação vigente (Brasil, 1996, 2022). Souza *et al.* (2017) verificaram a presença de *S. aureus* em 20% de queijo Minas frescal, todos com contagens em níveis inaceitáveis, acima de 10^5 UFC/g. Segundo Nunes e Caldas (2017), quando essa bactéria atinge níveis elevados de contaminação (10^5 UFC/g) pode ocorrer a produção de SEs, ocasionando a intoxicação alimentar. Entretanto, apesar do perigo relacionado e da incidência de *S. aureus* no país, na maioria dos casos as intoxicações não são investigadas ou notificadas.

2.1.2. Enterotoxina estafilocócica

As enterotoxinas estafilocócicas (SEs) são um grupo de proteínas extracelulares, de massa molecular de 22 a 29 KDa, estruturadas em uma única cadeia polipeptídica, apresentando duas cisteínas que formam uma ponte dissulfeto (Hennekinne *et al*., 2012; Hennekinne, 2018; Le Loir, 2003). Elas exibem resistência a altas temperaturas e às enzimas proteolíticas do sistema digestório (Balaban; Rasooly, 2000; Campos *et al*., 2022; Gonet *et al*., 2023). As SEs podem ainda comportar-se como superantígeno com liberação de citocinas e toxinas gastrointestinais que causam intoxicação através dos alimentos (Le Loir *et al.*, 2003; Umeda *et al.*, 2021).

Foram identificadas uma gama de SEs que são denominadas de acordo com as letras do alfabeto correspondente à ordem cronológica de sua descoberta. Hoje são conhecidas 23 enterotoxinas distintas (Tabela 1), mas com função, estrutura e relação filogenética comum, além de homologia de sequência (Wu *et al*., 2016). Apenas as que induziram vômito após administração oral em experimentos com macacos são designadas como SEs. As enterotoxinas que não promoveram emese nos experimentos citados ou que ainda não foram testadas recebem a denominação de *staphylococcal enterotoxin-like* (SEl) (Hu; Nakane, 2014; Ono *et al*., 2015).

Tabela 1. Características biológicas das enterotoxinas estafilocócicas (SEs).

Toxina	Peso Molecular (Da)	Característica Genética	Modo de ação	
			Superantígeno	Emética
SEA	27100	Profago	+	+
SEB	28336	Cromossomo, plasmídio, ilha de patogenicidade	+	+
$SEC_{1,2,3}$	27500	Plasmídio	+	+
SED	26360	Plasmídio	+	+
SEE	26425	Profago	+	+
SEG	27 043	Enterotoxin gene cluster (*egc*), cromossomo	+	+
SEH	25 210	Transposon	+	+
SEI	24 928	*egc*, cromossomo	+	(+)[a]
SEIJ	28 565	Plasmídio	+	n.c.[b]
SEK	25 539	ilha de patogenicidade	+	n.c.
SEIL	25 219	ilha de patogenicidade	+	n.c.
SEIM	24 842	*egc*, cromossomo	+	n.c.
SEIN	26 067	*egc*, cromossomo	+	n.c.
SEIO	26 777	*egc*, cromossomo	+	n.c.
SEIP	26 608	Profago	+	n.c.
SEIQ	25 076	Ilha de patogenicidade	+	-
SER	27 049	Plasmídio	+	+
SES	26 217	Plasmídio	+	+
SET	22 614	Plasmídio	+	(+)
SEIU	27 192	*egc*, cromossomo	+	n.c.
$SEIU_2$	26 672	*egc*, cromossomo	+	n.c.
SEIV	24 997	*egc*, cromossomo	+	n.c.
SEIX	19343	Genoma central	+	n.c.[c]

[a] fraco positivo [b] não conhecido [c]Wilson *et al*. (2011). Fonte: Adaptado de Hennekinne *et al*. (2012) e Hu e Nakane (2014).

As principais enterotoxinas envolvidas em surtos alimentares são SEA, SED, SEC, SEB e SEE, na ordem decrescente de decorrência. As toxinas SEC e SED estão relacionadas à contaminação de origem animal, enquanto SEA e SEB são frequentemente associadas à contaminação humana (Freitas *et al*., 2009; Wu *et al*., 2016).

O gene para a produção de SEA está localizado em um profago (Tabela 1) e sua produção ao contrário da maioria das SEs, não é controlado pelo gene regulador do sistema acessório (*agr)*. A expressão de SEA é regulada principalmente por processos ligados ao ciclo de vida do profago e é afetado pela natureza do profago, pela multiplicação microbiana e pelas mudanças ambientais (Schelin *et al*., 2011; Zeaki *et al*., 2015).

Além das enterotoxinas SEA-SEIX, existe a toxina antes denominada SEF, reconhecida atualmente como toxina da síndrome do choque tóxico (TSST-1), doença aguda grave, cujos sintomas são febre alta, erupção cutânea, hipotensão, insuficiência de órgãos e diferentemente das demais toxinas, esta não induz emese (Hu; Nakane, 2014; Koosha *et al*, 2016).

A produção das SEs normalmente está associada com espécies produtoras das enzimas coagulase e termonuclease. Entretanto, estudos comprovam que *Staphylococcus* coagulase negativos podem produzir SEs e causar intoxicações (Rajkovic, 2016).

A intoxicação estafilocócica ocorre a partir da ingestão da toxina pré-formada no alimento. Na maioria dos casos, a recuperação ocorre em 24 a 48 h e assim, não é necessário o atendimento médico. Desse modo, a intoxicação geralmente não é notificada aos órgãos de Vigilância Sanitária, resultando na subnotificação da doença. Os principais sintomas incluem náuseas, vômito, diarreia, calafrios e fraqueza. O período de incubação é de 30 min a 8 h. (Henekine, 2012; Rajkovic, 2016). A severidade dos sintomas depende da quantidade ingerida e da suscetibilidade do indivíduo. A morte é rara, ocorrendo principalmente em crianças, idosos e imunodeprimidos, dependendo da intensidade dos sintomas ou em casos de complicações (Bhunia, 2018).

O mecanismo de emese das SEs não é muito bem elucidado. Sugere-se que a ação emética das toxinas se dá pela ligação destas aos seus receptores localizados no intestino (Hu *et al*., 2007). Dessa forma, o estímulo é transferido através do nervo vago ao centro do vômito, ocasionando o retroperistaltismo do intestino e estômago, o que causa o vômito. Outro mecanismo, porém, pouco sugerido é o da ação diarreica, em que se relaciona a ativação de um mecanismo que expele sódio e cloro, causando perda excessiva de líquidos e eletrólitos e consequente, inflamação e irritação da mucosa intestinal (Franco; Landgraf, 2009; Henekine, 2012).

Em alimentos, *S. aureus* produzem SEs capazes de causar intoxicação em contagem entre 10^3 e 10^5 UFC/mL (Jablonski; Bohach, 2001).Wong e Bergdoll (2002) e Rajkovic (2016), afirmam haver risco de intoxicação alimentar por *S. aureus* apenas com concentração microbiana a partir de 10^5 UFC/g ou mL. Outros estudos sugerem que as toxinas são produzidas a partir de 10^6 UFC/g ou mL (Hu *et al*., 2018). Entretanto, há evidências tanto da produção das SEs em números menores, quanto da ausência das toxinas em contagens maiores que 10^5 UFC/g ou mL (Rajkovic, 2016). Segundo Hennekinne *et al*. (2009), Pinchuk *et al*. (2010) e Rajkovic (2016), doses de 100 ng de SEs podem causar sintomas da intoxicação estafilocócica. Já Pelisser *et al*. (2009), acreditam ser necessário doses de 20 ng a 1 µg da toxina para quadros de intoxicação. Nunes e Caldas (2017) consideram necessárias a ingestão de 20 a 100 ng de SEs,

enquanto Sirotamarat *et al.* (2022) detectaram risco de intoxicação com concentrações inferiores.

As enterotoxinas de *S. aureus* são normalmente formadas no final da fase exponencial ou início da fase estacionária, exceto a SEA, produzida na fase exponencial (Tremaine *et al*., 1993). A intoxicação ocorre caso o alimento contaminado apresente condições favoráveis para multiplicação da bactéria e da produção de SE, que é influenciada por fatores como pH, temperatura, a_w, concentração do inóculo e concentração de NaCl. Temperaturas entre 37 e 45 ºC são ideais para produção das SEs. Os valores de pH ótimo variam entre 7 e 8 e a atividade de água entre 0,85 e 0,89. Além disso, são produzidas em concentrações de até 10% de NaCl (Hennekinne, 2012).

S. aureus é inativado por tratamento térmico adequado, apresentando valor D de 2 a 15 min quando submetido à temperatura de 60 ºC (Rajkovic, 2016). No entanto, SEs são altamente resistentes a altas temperaturas, o que permite a permanência da sua atividade mesmo após os tratamentos de pasteurização ou ultrapasteurização do leite (Hennekinne, 2018; Le Loir, 2003). Este fato é preocupante para a qualidade microbiológica dos alimentos, principalmente de leite e seus derivados. Nesse sentido, cuidados higiênicos durante a cadeia de produção, processamento e armazenamento dos alimentos devem ser tomados, visando a prevenção da disseminação de *S. aureus* e consequente produção de SEs na cadeia alimentar.

3. Microbiologia preditiva

3.1. Definições e histórico

A microbiologia preditiva é um campo científico que combina elementos da microbiologia, matemática e estatística com a finalidade de desenvolver modelos matemáticos baseados em dados experimentais para predizerem mudanças do comportamento microbiano, como, a multiplicação e inativação de microrganismos quando submetidos a diferentes condições, tais como temperatura, pH, umidade, conservantes, microbiota competidora e a_w (Garre *et al*., 2020; Huang, 2014; Walls; Scott, 1997).

O primeiro modelo preditivo foi desenvolvido por Esty e Meyer em 1922, onde descreveram a inativação térmica dos esporos de *Clostridium botulinum* tipo A por meio de um modelo preditivo. Este modelo é amplamente aceito pela indústria de alimentos, entretanto, não é bem reconhecido como um modelo preditivo (Esty; Meyer, 1922).

Em 1937, Scott compreendeu o uso da microbiologia preditiva para prever a segurança alimentar e a vida de prateleira dos alimentos. Seus estudos sobre os efeitos da temperatura, a_w e concentração de CO_2, possibilitou o embarque de carcaças bovinas não congeladas das antípodas para o Reino Unido (Scott, 1937).

Até 1960, a literatura permaneceu silenciosa em relação à microbiologia preditiva, quando surgiram problemas de deterioração dos alimentos e surtos de intoxicação e infecção alimentar (McMeekin *et al*., 2002). No entanto, os modelos preditivos em alimentos desenvolveram-se significativamente apenas em 1980 como resultado de surtos de intoxicação alimentar e consequente conscientização da população sobre a importância de alimentos seguros, além do surgimento de recursos computacionais e pacotes estatísticos mais sofisticados (Bratchell *et al*., 1989; McMeekin *et al*., 2002; McMeekin *et al*., 2007; Valdramidis, 2016).

Para verificar a inocuidade dos alimentos, a avaliação microbiológica através dos tradicionais *challenge tests* é de extrema relevância. Entretanto, esta abordagem demanda alto custo e tempo, o que dificulta a rápida adoção de medidas preventivas de contaminação. Além disso, o conhecimento adquirido com os resultados obtidos não é cumulativo e quando ocorre alterações de processamento ou na formulação do produto, os testes microbiológicos devem ser repetidos (Huang, 2017). Dessa forma, observa-se um aumento na procura por modelos matemáticos para a microbiologia de alimentos.

A microbiologia preditiva surgiu como uma ferramenta moderna essencial para complementar a microbiologia de alimentos. Essa metodologia é considerada prática, rápida, de baixo custo, onde se tem um conjunto de informações sobre a multiplicação, sobrevivência ou inativação dos microrganismos em alimentos e assim, permite uma maior compreensão do comportamento microbiano. Pode ser aplicada para i) favorecer a tomada de decisões em processos de controle de qualidade de forma pró-ativa, ii) analisar riscos do desenvolvimento de microrganismos patogênicos em alimentos iii) desenvolver novos produtos e processos iv) auxiliar na determinação vida de prateleira dos alimentos (Chaturvedi *et al*., 2023; Fakruddin *et al*., 2011; Nunes; Caldas, 2017; Park *et al*., 2020; Ross; McMeekin, 1994). Os modelos foram ainda estudados no sentido de favorecer a multiplicação de microrganismos desejáveis (Arroyo-López *et al*., 2010; Di Biase *et al*., 2022; Dorota *et al*., 2014).

Algumas limitações devem ser consideradas para utilização dos modelos preditivos no estudo do comportamento microbiano. Os modelos preditivos só podem ser usados para condições em que os níveis dos fatores avaliados (pH, temperatura, por exemplo) estejam no limite utilizado para construção do modelo. Caso esses valores sejam extrapolados, predições

perigosas, que não refletem a realidade serão preditas (Stavropoulou; Bezirtzoglou, 2019). Além disso, experimentos realizados em ambientes laboratoriais e posteriormente validados em alimentos podem gerar imprecisões. Entretanto, estas limitações podem ser controladas, com cautela na seleção e utilização dos modelos e fatores avaliados (Fakruddin *et al*., 2011).

Além disso, a microbiologia preditiva não substitui as análises microbiológicas normalmente realizadas para alimentos. Somente poderá ser utilizada para complementar esses métodos microbiológicos pois são implementadas com base em dados coletados experimentalmente.

3.1.1. Classificação dos modelos

Os modelos matemáticos utilizados na microbiologia preditiva são classificados em modelos mecanísticos e modelos empíricos. Os modelos mecanísticos descrevem os dados sobre o comportamento microbiano com base teórica e contribuem para o conhecimento do comportamento microbiano além de um processo biológico, pois consideram a complexidade da fisiologia dos microrganismos. Estes modelos são vantajosos uma vez que podem extrapolar os dados experimentais. No entanto, não são utilizados frequentemente pois, não são facilmente ajustáveis. Segundo McMeekin (2002), nenhum modelo utilizado é completamente mecanicista. Já os modelos empíricos são os modelos mais utilizados, por serem práticos e descreverem as respostas de multiplicação, inativação ou produção de metabólitos por microrganismos por meio de equações matemáticas (Coffigniez *et al*., 2021; Semenov *et al*., 2010; Fakruddin *et al*.; 2011). Os modelos são também classificados em cinéticos e probabilísticos. Os cinéticos preveem o comportamento microbiano em um determinado período, enquanto os probabilísticos descrevem a probabilidade de ocorrer determinado evento, como a multiplicação de um patógeno, a produção de enzimas, expressão de toxinas ou formação de biofilme em função do tempo (Nakashima *et al*.; 2000; Silva *et al*., 2022; Tarlak; Ozdemir; Melikoglu, 2020).

Whiting e Buchanan (1993), classificam os modelos matemáticos em três níveis: modelo primário, modelo secundário e modelo terciário. O modelo primário descreve parâmetros cinéticos (tempo de adaptação (λ), velocidade específica de multiplicação (μ), taxa máxima de multiplicação (μmáx), tempo de geração (G) ou taxa de mortalidade) do desenvolvimento microbiano em função do tempo. Estes modelos não levam em consideração as variáveis do meio, como temperatura ou pH. São exemplos de modelos primários o modelo

Monod (Monod, 1949), modelo de Gompertz, modelo logístico (Gibson *et al*., 1987), e modelo de Baranyi e Roberts (Baranyi; Roberts, 1994).

O modelo de Baranyi e Roberts (Equação 1) é o modelo primário de maior utilização e importância na microbiologia preditiva. Este modelo inclui parâmetros cinéticos de importância durante a multiplicação dos microrganismos, como a fase linear no crescimento exponencial, a fase de adaptação e o estado fisiológico, se destacando em termos de melhores ajustes em relação a outros modelos primários, como de Gompertz e Gompertz modificado.

$$y(t) = y_0 + \mu_{máx}\, t + \frac{1}{\mu_{máx}} \ln(e^{-v*t} + e^{-h0} - e^{-v*t-h0}) -$$

$$\frac{1}{m} \ln\left(1 \frac{e^{m\mu_{max}(t) + \frac{1}{\mu_{máx}} \ln(e^{-v*t} + e^{-h0} - e^{-v*t-h0})} - 1}{e^{m(y_{max} - y_0)}}\right) \qquad (1)$$

Em que:

y(t) = ln (x(t)) com o x(t) sendo a concentração microbiana no tempo t (log (UFC/mL));

y0 = ln (x0) e ymáx = ln (xmax), sendo o x0 a concentração microbiana inicial no tempo t0 (log (UFC/mL)) e xmax concentração microbiana máxima (log (UFC/mL)).

µmáx= taxa máxima de multiplicação (log (UFC/mL/h));

m = parâmetro de curvatura depois da fase exponencial;

v = parâmetro relacionado à curvatura para caracterizar a transição para a fase exponencial;

h0 = parâmetro que quantifica o estado fisiológico para cálculo do tempo de adaptação (h0 = µmáx).

Os modelos secundários descrevem como a variação dos parâmetros do modelo primário são influenciados por fatores intrínsecos e extrínsecos, tais como temperatura, a_w, microbiota competidora, conservantes e pH. São exemplos de modelos secundários o modelo superfície de resposta e de probabilidade de regressão logística.

O modelo de superfície de resposta permite gerar modelos a partir de um número reduzido de experimentos através do delineamento composto central ou Box-Behnken. São utilizados para otimizar experimentos caros ou demorados para garantir a eficiência desejada. A partir dele, são geradas equações polinomiais que descrevem o efeito de coeficientes lineares, quadráticos e interações na variável dependente de interesse. A equação 2 representa este modelo.

$$Y = \beta 0 + \sum \beta i x i + \sum \beta i x i^2 + \sum \beta i j x i x j \qquad (2)$$

Em que:

Y é a variável resposta do modelo;
β0 e *βi* são os coeficientes do modelo a serem estimados;
xi, xi^2 e *xixj* representam, respectivamente, os efeitos individuais, quadráticos e interativos das variáveis independentes.

O modelo de probabilidade de regressão logística é de grande interesse em microbiologia preditiva pois descreve a probabilidade de ocorrer um determinado evento Y (valores entre 0 e 1), condicionado a um vetor x (variáveis independentes). Esse evento Y pode positivo ou negativo, ou seja, de sucesso (Y = 1) ou de falha, (Y = 0) (Salter *et al*., 2000). Nesse modelo, a influência das variáveis independentes na variável dependente é avaliada e estas podem ser quantitativas ou qualitativas. Os coeficientes obtidos indicam a influência das variáveis em favor do sucesso (Y = 1). O modelo é classificado como empírico, probabilístico e secundário. A Equação 3 representa esse modelo.

$$P(x) = \frac{e^{|\beta 0+\sum \beta ixi|}}{1+ e^{|\beta 0+\sum \beta ixi|}} \quad (3)$$

Em que:
P(x) é a probabilidade de ocorrer ou não determinado evento;
β0 e *βi* são os coeficientes do modelo a serem estimados;
xi são as variáveis independentes.

Para ajuste dos dados ao modelo representado na equação 2, é necessário a realização de uma transformação nesta equação. A transformação logit de *P(x)* está representada na Equação 4.

$$Logit\ (P) = g(x) = Ln\left(\frac{p(x)}{1-p(x)}\right) = \sum \beta ixi \quad (4)$$

Em que:
g(x) é a transformação logit;
β0 e *βi* são os coeficientes do modelo a serem estimados;
xi são as variáveis independentes.

Além das respostas de probabilidade dadas pelas condições estudadas, os valores de xi podem ser substituídos por valores dentro da faixa estudada na equação e novos valores de

probabilidade são encontrados. Além disso, pela substituição dos dados de probabilidade, é possível determinar respostas sobre os níveis críticos das variáveis independentes que afetam a variável dependente (Lopez-Malo; Palou, 2000; Silva *et al*., 2022). Portanto, este modelo avalia os riscos relacionados ao comportamento microbiano de forma abrangente. Estudos comprovam sua efetividade para predição de limites de multiplicação/ausência de multiplicação, produção de biofilme/ausência de produção e produção de toxinas/ausência de produção e, desse modo, é de grande importância na prevenção de contaminação de alimentos por patógenos, garantindo a segurança dos produtos alimentícios (Ding *et al*., 2013; Huang*;* JIA; HWANG, 2022; Moraes *et al*., 2019). Dessa forma, o modelo de regressão logística pode ser utilizado para descrever o efeito de características intrínsecas e extrínsecas relacionadas aos alimentos na probabilidade de produção ou não produção de enterotoxinas por *S. aureus*.

Em relação aos modelos terciários, estes têm como princípio a combinação de modelos primários e secundários com softwares que calculam a cinética microbiana sob condições ambientais (Garre, 2017; Huang, 2014). Cada software apresenta uma base de dados com os microrganismos e os níveis dos fatores a serem avaliados. A escolha do modelo dependerá do microrganismo e das condições que se deseja avaliar. Diversos programas são disponíveis, sendo o *ComBase Predictor* e *Pathogen Modeling Program* (PMP) os mais utilizados para a modelagem preditiva. Esses softwares têm como vantagem a rapidez, praticidade e facilidade de utilização. Desse modo, auxiliam na prevenção da contaminação de alimentos por patógenos (Girbal *et al*., 2021; Koutsoumanis; Lianou; Gougouli, 2016).

3.1.2. Validação de modelos preditivos

A validação matemática consiste em avaliar a eficácia do modelo utilizado em descrever as respostas experimentais. A validação pode ser interna, em que são testados novos dados e combinações de variáveis não utilizados para construção do modelo, mas que estão dentro da faixa estudada, ou externa, que compara os valores preditos pelo modelo utilizado com respostas reais do comportamento microbiano no alimento (Fakruddin *et al*., 2011). Além das validações internas e externas, índices estatísticos são empregados para avaliar o grau de confiabilidade dos modelos, tais como, teste de Hosmer e Lemeshow, porcentagem correta de predição e R^2 de Nagelkerke.

O teste de Hosmer e Lemeshow avalia o bom ajuste do modelo de Regressão Logística, através da comparação dos dados observados com os preditos (Hosmer; Lemeshow, 1989). O teste verifica a existência de diferenças significativas entre as respostas dadas pelo modelo e as

observadas nos experimentos. Têm-se que quanto menor o valor da diferença entre observado-predito, maior a eficácia do modelo em descrever os dados. Nesse contexto, duas hipóteses podem ser observadas: a hipótese de nulidade (H0) que consiste na ausência de diferença significativa entre os valores observados e os preditos e a hipótese alternativa (H1), que é quando há diferenças entre os dados experimentais e os preditos pelo modelo construído. Portanto, caso a hipótese de nulidade seja aceita, o modelo tem bom poder de ajuste.

O coeficiente de determinação (R^2) é amplamente utilizado para avaliação de confiabilidade de dados, já que se aplica a todos os modelos preditivos. Este índice de validação representa a proporção de explicação do modelo através da fração de variação em torno da média. Seus valores variam de 0 a 1 e, quanto mais próximo de 1, melhor o ajuste do modelo aos dados experimentais, ou seja, melhor modelo estudado prediz as respostas do comportamento microbiano (Gonçalves *et al*., 2017). Para modelos probabilísticos de regressão logística, são utilizados pseudos R^2, como de Nagelkerke e Cox e Snell. A diferença é que, como neste tipo de modelo as variáveis respostas são expressas como dados binários, são utilizadas medidas de associação, mas que não são realmente o R^2. Desse modo, os pseudos R^2 são aproximações da variação na variável dependente, devido a variação nas variáveis independentes.

Além dos apresentados, existem diversos índices de validação, tais como, fator de precisão, fator BIAS e erro quadrado médio, comumente utilizados na avaliação do bom ajuste de modelos matemáticos (Ross, 1996). A escolha dependerá do objetivo do estudo, do modelo escolhido e do conhecimento do pesquisador a respeito dos métodos de validação.

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OBJETIVOS

Objetivo Geral

Desenvolver um modelo de superfície de resposta e um modelo de regressão logística capazes de prever a multiplicação e a produção de SEA de *S. aureus* ATCC 13565 em diferentes condições de temperatura, pH, concentração de NaCl, concentração microbiana e tempo.

Objetivos específicos

- Avaliar a qualidade higiênico-sanitária de queijo Minas frescal pela presença de *Staphylococcus* spp;
- Identificar a presença de *Staphylococcus aureus* métodos bioquímicos e moleculares;
- Identificar a presença de genes codificadores de enterotoxinas estafilocócicas nos isolados;
- Monitorar os parâmetros físico-químicos pH, temperatura e concentração de NaCl de queijo Minas frescal;
- Desenvolver um modelo de superfície de resposta para prever a multiplicação de *S. aureus* ATCC 13565 e um modelo de regressão logística para descrever a produção enterotoxina A por *S. aureus* ATCC 13565 em diferentes condições de temperatura (10°C; 15°C; 25°C), pH (5,3; 5,5; 6,0; 6,5; 6,7), concentração de NaCl (0,8%; 1,0%; 1,5%; 2,0%; 2,2%), concentração de *S. aureus* (10^0 UFC/mL; 10^3 UFC/mL; 10^5 UFC/mL) e tempo (24 h; 48 h; 72 h; 96 h; 120 h; 144 h; 240 h);
- Validar estatisticamente os modelos preditivos desenvolvidos através do coeficiente de determinação (R^2) fator bias, fator de acurácia, teste de Hosmer e Lemeshow R^2 de Nagelkerke e porcentagem de concordância;
- Validar experimentalmente os modelos desenvolvidos em meio BHI, leite UHT e queijo Minas frescal.

Capítulo 1. Presence and Growth Prediction of *Staphylococcus* spp. and *S. aureus* in Minas Frescal Cheese, a Soft Fresh Cheese produced in Brazil

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ABSTRACT

Physical-chemical characteristics of Minas frescal cheese (MFC) favor the growth of *Staphylococcus* spp. and allow the production of enterotoxins by specific strains. Here, we aimed to characterize the physical-chemical aspects (pH, storage temperature and salt content) and the presence of *Staphylococcus* spp. in MFC samples (n = 50) to support a modeling study for the growth by this microorganism. Coagulase-positive staphylococci isolates were obtained and also subjected to PCR assays to detect staphylococcal enterotoxin-related genes (*sea, seb, sec, sed, see*). *S. aureus* growth kinetics (maximum growth rate, Grmax, and lag time) was predicted based on ComBase model and MFC physical-chemical aspects. Mean counts of *Staphylococcus* spp. ranged from 3.3 to 6.7 log CFU/g, indicating poor hygiene practices during production. None of the tested Coagulase-positive staphylococci isolates (n = 10) presented enterotoxin-related genes. pH, temperature and salt content ranged from 5.80 to 6.62, 5 °C to 12 °C and 0.85% to 1.70%, respectively. Grmax values ranged from 0.012 to 0.419 log CFU/g/h. Independently of the storage temperature, the lowest Grmax values (0.012 to 0.372 log CFU/h) were obtained at pH 5.80 associated to salt content of 1.7%; independently of the pH and salt content, the best temperature to avoid staphylococcal growth was 7.5 °C. Hygienic conditions during MFC production must be adopted to avoid staphylococcal contamination and storage at temperatures lower than 7.5 °C can prevent staphylococcal growth and the potential production of enterotoxins.

Key words: fresh cheese; *Staphylococcus*; enterotoxins; growth modelling

INTRODUCTION

Minas Frescal cheese (MFC) is characterized as a fresh cheese produced after the enzymatic coagulation of milk with rennet and/or other coagulating enzymes; being alternatively supplemented with specific lactic bacteria (Brasil, 1997). These procedures result in a semi-fat cheese, with high moisture, low sodium content, pH close to neutrality, and not ripened (Brasil, 1997; Nunes, Souza, Pereira, Del Aguila, & Paschoalin, 2016). These characteristics allows the survival and growth of spoilage microorganisms, resulting in short shelf life even when stored at low temperatures (Felicio et al., 2015; Visotto, Oliveira, Prado, & Bergamini, 2011).

MFC production is highly susceptive to microbial contamination, due to heavy manipulation and eventual poor hygienic conditions in some dairy industries (Cândido et al., 2020). As result, foodborne diseases outbreaks were already associated to the consumption of cheeses produced with pasteurized milk, indicating post-processing contamination (O'Brien, Hunt, McSweeney, & Jordan, 2009). Also, the physical-chemical characteristics of MFC can support the survival and growth of pathogenic bacteria that can contaminate this product, such as *Salmonella* spp., *Escherichia coli* and *Staphylococcus aureus* (Carvalho, Viotto, & Kuaye, 2007; Rocha et al., 2020). Staphylococcal food poisoning is particularly relevant for cheeses, being the responsible for 6% of the reported outbreaks in USA between 1998 and 2018 (CDC, 2018). Despite the flaws of epidemiological data in Brazil, *S. aureus* was the 3rd pathogen described as the cause of foodborne diseases outbreaks and highly associated to dairy products, including cheeses (Brasil, 2019b). Several *Staphylococcus* spp. strains can be able to produce enterotoxins, a potential usually associated to coagulase positive species, like *S. aureus*. Coagulase negative *Staphylococcus* were already characterized as harboring enterotoxin related genes and also capable of producing these enterotoxins, being even described as the causative

agent of foodborne diseases outbreaks (Cândido et al., 2020; Carmo et al., 2002; Nunes et al., 2016).

MFC intrinsic and extrinsic characteristics, such as pH, salt concentration and temperature, can influence the behavior of *Staphylococcus* spp. during processing and storage. Given that *Staphylococcus* spp. may growth and produce enterotoxins in MFC, it is mandatory to adopt adequate production procedures to avoid contamination and to establish the ideal conditions to impair the growth and enterotoxin production of eventual contaminant strains. Thus, predictive models have been a fundamental tool to assure the safety and quality of foods (Ozcakmak and Gul, 2017). Based on these mathematical models, it is possible to estimate microbial growth or inactivation under specific conditions, including storage temperatures and other physical-chemical aspects. As MFC is highly associated to *Staphylococcus* spp. (Cândido et al., 2020; Nunes et al., 2016), such mathematical models are extremely relevant to demonstrate how the bacterial growth and enterotoxin product can be avoided during production and storage.

Here, we aimed to characterize the physical-chemical aspects and *Staphylococcus* spp. contamination in MFC in order to support an analysis of *S. aureus* growth prediction in this food.

MATERIALS AND METHODS

MFC samples

MFC samples (n = 50) from different dairy industries (n = 10), belonging to 5 different batches were obtained from retail sale. Dairy industries were inspected by municipal, state and federal inspection organs. Before purchase, MFC samples were checked for integrity of package and visible signs of spoilage, and transported at maintained under refrigeration temperature (4 ºC) during transport and until analysis.

Physical-chemical characteristics

MFC samples were analyzed for temperature, pH, and sodium chloride (salt) content. Temperature was measured directly by contact with MFC samples, using a mercury in glass thermometer (Vidrolabor, São Paulo, SP, Brazil); results were expressed in °C. pH was measured with a digital pH meter (Model pH21, Hanna Instruments, Brazil) (Brasil, 2018). The sodium chloride (NaCl) content was measured based on the Mohr method (ISO/IDF, 2004), modified by Dias, Ferreira, Carvalho, and Soares (2016), resulting in a volumetry of the chloride quantification present in the sample. MFC samples (5 g) were diluted in 100 mL of distilled water, titrated with 0.1M silver nitrate solution (AgNO3) in the presence of potassium chromate indicator; results were expressed in %.

***Staphylococcus* spp. enumeration**

MFC samples were subjected to *Staphylococcus* spp. enumeration according ISO 6888-1 (ISO, 1999). MFC samples (25 g) were transferred to sterile bags with 225 mL of buffered peptone water (0.1%, wt/vol), homogenized in a stomacher, and 10-fold diluted up to 1:100,000 in 0.1% (wt/vol) peptone saline. Selected dilutions of the samples were surface plated onto plates containing Baird Parker agar (Oxoid, Basingstoke, England) supplemented with egg emulsion (5%, vol/vol) and potassium tellurite (1%, wt/vol). Plates were incubated at 36 °C for 48 h, when colonies were analyzed by their morphology (typical: circular, smooth, convex, gray to jet-black, with a light-colored margin surrounded by two halos: one opaque and one light, and atypical: grey to black colonies, without transparent or opaque zones), enumerated, and selected for additional characterization (around 10% of the counts). The selected colonies were subjected to Gram staining and tested for production of catalase, coagulase, and DNAse (ISO, 1999). Isolates with biochemical characteristics consistent with coagulase-positive

staphylococci (CPS) were subjected to DNA extraction (Wizard Genomic DNA Purification kit, Promega Corp., Madison, WI) and a PCR assay targeting *nuc*, according to Baron et al. (2004), for *S. aureus* identification. Primers, product sizes, PCR conditions, and positive controls are detailed in Table 1.

Table 1. Oligonucleotides and conditions of PCR reactions conducted to identify and characterize coagulase-positive *Staphylococcus* isolates and classical staphylococcal enterotoxins in Minas frescal cheeses (MFC).

		PCR[1]					
Target	Oligonucleotide (F, forward; R, reverse)	Denaturation	Annealing	Extension	Product (bp)	Positive control[2]	Reference
nucA	F: TGCTATGATTGTGGTAGCCATC R: TCTCTAGCAAGTCCCTTTTCCA	95°C, 30 s	55°C, 30 s	72°C, 30 s	420	*Staphylococcus aureus* ATCC 8095	Baron et al. (2004)
sea	F: ACGATCAATTTTTACAGC R: TGCATGTTTTCAGAGTTAATC	95°C, 45 s	46.2°C, 45 s	72°C, 30 s	544	*Staphylococcus aureus* ATCC 8095	Rosec and Gigaud (2002)
seb	F: GAATGATATTAATTCGCATC R: TCTTTGTCGTAAGATAAACTTC	95°C, 45 s	46.2°C, 45 s	72°C, 30 s	416	*Staphylococcus aureus* ATCC 14458	Rosec and Gigaud (2002)
sec	F: GACATAAAAGCTAGGAATTT R: AAATCGGATTAACATTATCCA	95°C, 45 s	46.2°C, 45 s	72°C, 30 s	257	*Staphylococcus aureus* ATCC 700698	Rosec and Gigaud (2002)
sed	F: TTACTAGTTTGGTAATATCTCCTT R: CCACCATAACAATTAATGC	95°C, 45 s	46.2°C, 45 s	72°C, 30 s	334	*Staphylococcus aureus* FRI 100	Rosec and Gigaud (2002)
see	F: ATAGATAAAGTTAAAACAAGCAA R: TAACTTACCGTGGACCC	95°C, 45 s	46.2°C, 45 s	72°C, 30 s	170	*Staphylococcus aureus* FRI 326	Rosec and Gigaud (2002)

[1] All reactions were conducted with initial denaturation at 95°C for 5 min; amplification was conducted with 30 (nuc) and 35 (sea, seb, sec, sed, see) cycles, and final extension at 72°C for 5 to 10 min.

[2]ATCC = American Type Culture Collection (Manassas, VA); FRI = Food Research Institute (Madison, WI).

Based on the obtained results, counts of *Staphylococcus* spp. and coagulase-positive *Staphylococcus* were expressed as colony-forming units per g (CFU/g) of MFC. The DNA from isolates carrying the *nuc* gene was subjected to PCR assays targeting genes related to production of SEA (*sea*), SEB (*seb*), SEC (*sec*), SED (*sed*), and SEE (*see*), as described by Rosec and Gigaud (2002).

Prediction of *S. aureus* growth kinetics

Based on the obtained results for temperature, pH and salt content, these parameters were associated considering different values to predict the *S. aureus* growth in MFC, using the ComBase Predictor static growth models (http://modelling.combase.cc/): *S. aureus* behavior was considered as the representative reference for *Staphylococcus* genus in MFC. Temperature values were determined based also on variations of this parameter during MFC production and storage, and 25 °C was included as "inadequate storage temperature". The effect of the conditions found in the MFC samples on the two kinetic parameters, i.e. maximum growth rate (Grmax, expressed in log CFU/g/h) and lag time (λ, expressed in hours) was predicted to indicate the growth or decay kinetics over time. The Grmax is defined as the change in the number of cells in a shorter time interval and the lag time is defined as the adaptation time of the bacteria, during which no growth occurs. The model was used to evaluate the conditions that may favor or inhibit the *Staphylococcus* spp. growth and, consequently, the staphylococcal enterotoxin production. The ComBase Predictor models for *S. aureus* do not allow prediction with temperature values below 7.5 °C; therefore, this was the minimum temperature value used. As for pH and salt content values, values recorded in the MFC samples were considered for the predictions.

Data analysis

A Response Surface Regression method was used to investigate the effects of pH, temperature and salt content on the Grmax values. A second-order polynomial regression model that relates the variables is presented in Equation 1.

$$Y = \beta_0 + \beta_1 T + \beta_2 pH + \beta_3 NaCl + \beta_4 TpH + \beta_5 TNaCl + \beta_6 pHNaCl + \beta_7 T^2 + \beta_8 pH^2 + \beta_9 NaCl^2 \quad (1)$$

Where *Y* is the response variable and the treatment variables are *T* (temperature), *pH* and *NaCl* (salt content). *β*s are regression coefficients. The effects of variables were investigate using the Statistica (version 10.0). The response surface plots for these models were plotted as a function of two variables, while keeping one independent variable (salt content) fixed at constant values.

RESULTS AND DISCUSSION

Temperature, pH, and salt content results for MFC samples are presented in Table 2: at least 20% of the samples were at temperatures below 8 °C, above the storage temperature established by the Brazilian legislation for MFC (Brasil, 1997).

Table 2. Physical-chemical parameters in different samples of Minas frescal cheese

	Physical-chemical parameters		
Samples	pH	Temperature (°C)	% Salt
A	6.31	7	1.69
B	5.82	7	1.14
C	5.98	9	0.95
D	6.23	5	0.88
E	6.22	5	1.15
F	5.82	12	0.91
G	5.97	5	1.14
H	6.35	8	1.02
I	5.84	7	1.07
J	6.46	6	1.15
Range	5.80-6.62	5-12	0.85-1.70

Mean values

The current Brazilian legislation does not establish reference values for pH in MFC (Brasil, 1996), but this parameter can be considered to evaluate the processing conditions and the quality of cheeses. The direct acidification, the method used to produce most of the analyzed MFC samples, results in products with pH higher than 5.5 (Dagostin, Carpine, & Ross, 2013). Most MFC samples presented pH between 5.80 and 6.50, as observed in other studies with this same cheese (Carvalho et al., 2007; Dias et al., 2016; Nunes and Caldas, 2017). Sodium chloride is added to improve flavor and to prevent microbial growth and enzyme activity, which may spoilage the cheese; salt content varied in MFC samples from 0.85% to 1.7% (Table 1). Dias et al. (2016) and Silva et al. (2019) found similar values in MFC, from 0.70% to 1.4% and 1.06% to 1.38%, respectively, while the MFC samples studied by Nunes and Caldas (2017) presented higher salt concentration, varying from 0.64% to 4.6%.

Based on the observed results for the analyzed physical-chemical aspects, most of the MFC samples would allow the *Staphylococcus* spp growth (Schelin, Carlquist, Cohn, Lindqvist, & Barker, 2011). Results for *Staphylococcus* spp. counts in MFC samples are presented in Table 3; it was possible the enumeration of this bacterial group in 45 (90%) samples, but only 2 (4%) presented coagulase positive staphylococci, the current parameter established in Brazilian legislation (Brasil, 2019a). Only one sample presented coagulase positive staphylococci counts higher than 3 log CFU/g. Different studies developed in Brazil with MFC presented similar results (Carvalho et al., 2007; Sangaletti et al., 2009; Silva et al., 2019), while André et al. (2008) and Freitas, Brito, Nero, and Carvalho (2013) demonstrated higher frequencies of MFC samples with high counts of coagulase-positive staphylococci and *S. aureus*. All the 10 coagulase positive staphylococci isolates were positive for *nuc*, being identified as *S. aureus*.

Table 3. Counts of *Staphylococcus* spp. and coagulase-positive *Staphylococcus* in different brands of Minas frescal cheese.

Samples	n.º (samples)	Counts (log CFU/g)			
		Staphylococcus spp.		Coagulase-positive *Staphylococcus*	
		n	Mean	n	Mean
A	5	4	6.66	-	BDL[1]
B	5	5	3.79	-	BDL
C	5	4	5.97	-	BDL
D	5	5	5.72	-	BDL
E	5	5	5.45	-	BDL
F	5	5	6.71	-	BDL
G	5	4	6.63	-	BDL
H	5	5	4.12	1	4.30
I	5	3	6.05	-	BDL
J	5	5	3.32	1	2.11

Mean values in log10
[1]BDL: below the detection limit.

The presence of *Staphylococcus* spp. in MFC can be considered a concern for food safety. Physical-chemical aspects of cheeses, such as water activity (a_w), pH, redox-potential and temperature, can directly influence and contribute to enterotoxigenic staphylococci growth and enterotoxins production, resulting in a potential cause of food poisoning outbreaks (Schelin et al., 2011; Babic et al., 2019). Although *S. aureus* is considered the most important species of the genus, coagulase-negative staphylococci deserves more attention (Cândido et al., 2020). Veras et al. (2008) demonstrated the capacity of coagulase-negative staphylococci, such as *S. saprophyticus*, *S. epidermidis* and *S. conhii* in producing enterotoxins and their enrollment in food poisoning outbreaks. Also, Carmo et al. (2002) described a food poisoning outbreak caused by consumption of food with high counts of coagulase-negative staphylococci capable of producing enterotoxins. Because of this, proper control of safety in foods, including MFC, must include the research of the enterotoxigenic potential of all *Staphylococcus* spp., independently of being coagulase positive or coagulase negative. The enterotoxigenic potential of staphylococci was traditionally associated with the ability of the strains in producing coagulase (Oliveira, Padovani, Miya, Sant'ana, & Pereira, 2011; Rodríguez, Gordillo, Andrade, Córdoba, & Rodríguez, 2016) leading different countries, including Brazil, to establish

reference values for this group to set a safety parameter for cheeses. However, the scientific evidence for the ability of coagulase-negative staphylococci in producing enterotoxins and their enrollment in food poisoning outbreaks are leading to a change in this traditional concept (Vernozy-Rozand et al., 1996; Veras et al., 2008). As consequence, European and Brazilian legislation included in their cheese safety parameters the research of staphylococcal enterotoxins, even being still associated to coagulase-positive staphylococci counts (Brasil, 2019a, European Union, 2005). In the current study, based on the presence of *nuc* gene, the 10 isolates were selected for enterotoxin assays by PCR and none showed positive results, indicating that there would be no potential health risk for consumers related to the classical enterotoxins (SEA, SEB, SEC, SED and SEE). In contrast, Cândido et al. (2020) found enterotoxins-related genes (*seb*, *sec*, *sei* and *sej*) in isolates from Minas Frescal cheese.

High staphylococci counts in MFC are usually associated with poor hygiene during production, typically in processing with heavy handling of ingredients and products, and increased by cross-contamination during processing (Felicio et al., 2015; Dittmann et al., 2017; De Leon et al., 2020). Some production flaws, such as inefficient pasteurization, inadequate storage, and unsatisfactory hygienic-sanitary conditions, can contribute to the prevalence of this bacterial group in cheese processing environments (Rodrigues et al., 2017; Martin et al., 2016; Zeinhom, Abdel-Latef, & Jordan, 2015). Thus, staphylococci counts can be used as a relevant hygiene indicator in cheese production, as well as to indicate inadequate handling and storage conditions during the manufacturing process (Cardozo et al., 2020; Metz, Sheehan, & Feng 2020).

In the present study, most of the samples presented high staphylococci counts, between 3 log CFU/g and 7 log CFU/g, indicating poor hygienic conditions in the MFC production. Similar results were observed in MFC by Cândido et al (2020) and Fontes et al. (2013). Under ideal conditions, enterotoxigenic staphylococci may growth, produce enterotoxins and cause

poisoning in consumers (Nouri, Ahari, & Shahbazzadeh, 2018). In this way, enumeration of *Staphylococcus* spp. is relevant to evaluate potential sources of contamination that may pose as risks to consumers health and, thus, support the search for control strategies to minimize the occurrence of these microorganisms in the food chain (Chaves et al., 2018; Organji, Abulreesh, Elbanna, Osman, & Almalki, 2018).

The predicted values for Grmax and lag time obtained for *S. aureus* based on the reference values established in this study (Table 2) are presented in the Supplementary Table 1. Grmax values ranged from a 0.012 log CFU/g/h to 0.419 log CFU/g/h, corresponding to increases of 0.288 log CFU/g/day and 10.056 log CFU/g/day, respectively. These results indicate that when present in MFC, staphylococci can significantly grow independently of the initial level of contamination, reaching critical levels at the time of consumption and impacting negatively the safety and stability of this cheese (Lima, Conceição, Schaffner, & Souza, 2018). The increase in Grmax values and consequently the decrease in lag times were observed with the increase of the temperature, corroborating with previous studies that found temperature as the environmental factor that most affects microbial growth kinetics in food (Araújo et al., 2017; Possas et al., 2017).

The production and sale of MFC in Brazil are regulated by official government agencies, Ministry of Agriculture (Ministério da Agricultura, Pecuária e Abastecimento) and Ministry of Health (Ministério da Saúde), respectively. Despite being mandatory the use of pasteurized milk to produce MFC (Brasil, 1996), clandestine production and sale of cheeses are common practices in some Brazilian states, including Minas Gerais (Moraes, Vicosa, Yamazi, Ortolani, & Nero, 2009; Okura & Marin, 2014). As consequence, these cheeses are usually characterized as not being adequate for human consumption due to poor microbiological quality and presence of potential hazards, as consequence to flaws during production and storage (Arruda, Nicolau, Reis, Araújo, & Mesquita, 2007; Vinha, Pinto, & Chaves, 2018). Abusive temperatures are

common storage flaws for cheeses, leading us to include the temperature of 25 °C in the predicting models. Comparing the growth kinetics of *S. aureus* at 25 °C and 7.5 °C, an increase of 0.362 log CFU/g/h in Grmax and a decrease of 112.53 h in lag time at 25 °C were observed; the increase in temperature, even for short periods, may increase staphylococci counts and allow the potential production of enterotoxins by enterotoxigenic strains (Chaves, Silva, Alvarenga, Pereira, & Khaneghah, 2017). Considering the temperature of 12 °C, the predictions showed an increase of up to 0.03 log CFU/g/h in the value of Grmax and a decrease of up to 102.55 h in the lag time of *S. aureus* in relation to the lowest temperature studied (7.5 °C). Inadequate storage temperatures, as from 10 °C, may accelerate the bacteria growth and consequently, the enterotoxin production in a shorter time. *S. aureus* was capable to growth and to produce enterotoxins in pasteurized milk under abusive temperatures ranging from 15 °C to 22 °C (Babic et al., 2019). Inadequate storage temperature control in supermarkets may reduce food shelf life (Zeaki, Johler, Skandamis, & Schelin, 2019). Official Codex Alimentarius Commission declares to be inappropriate storage temperature one of the most common causes of foodborne diseases (Codex Alimentarius Commission, 2003). Storage in domestic refrigerators is another important point in the *S. aureus* growth, what is directly influenced by the consumer behavior (Tirloni, Stella, Bernardi, Dalgaard, & Rosshaug, 2019).

As show in Table 4, it has been found that temperature, pH and temperature associated to pH significantly affected the Grmax values ($P < 0.05$). Grmax values increased when temperature associated to pH increasing. The salt content factor, regardless of the other factors, did not influence the Grmax values for *S. aureus* ($P = 0.524$) (Figure 1). The highest Grmax represents the best associated conditions that favored the *S. aureus* growth, and it was observed when pH 6.35 was associated to salt content of 1.7%, while the lowest Grmax, what indicates the best conditions to inhibit the *S. aureus* growth, was obtained when pH 5.8 was associated to salt content of 1.7%. Independently of all associated pH and salt content conditions, the

Grmax values were low at 7.5 °C, indicating that it is the best temperature to avoid *Staphylococcus* spp. growth (Figure 1).

Table 4. Significant coefficients (95% confidence interval) of pH, temperature and salt content (independent variables) of the regression fitting model for maximum growth rate (dependent variable) from *S. aureus*.

	Parameters	
Factor	**EC**	***P*-value**
Constant	-1.243	0.000
T	-0.034	0.000
pH	0.454	0.000
NaCl	-0.021	0.524 [ns]
T*T	0.001	0.000
pH*pH	-0.039	0.000
NaCl*NaCl	-0.002	0.756 [ns]
T*pH	0.003	0.000
T*NaCl	0.000	0.282 [ns]
pH*NaCl	0.004	0.396[ns]
R^2	0.99	

EC: Estimated coefficient;T:Temperature; NaCl: Salt content
ns: no significant effect at level <5%; R^2: adjusted square coefficient of the fitting model.

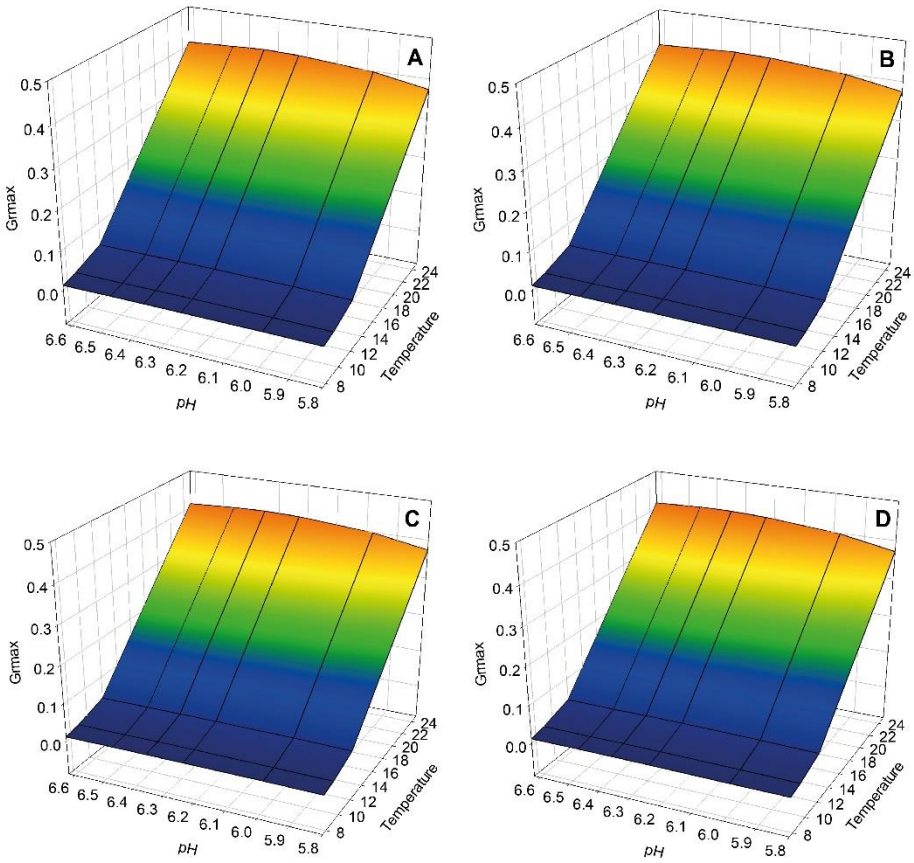

Figure 1. Maximum growth rate (Grmax, expressed in log CFU/g/h) values of S. aureus against different combinations of pH and temperatures at salt content (A) 0.85%; (B) 0.95%; (C) 1.14% and (D) 1.70%.

S. aureus growth and enterotoxin production are affected by several factors, including the food storage environment, temperature, pH, salt and water activity (Elahi and Fujikawa, 2019). *S. aureus* strains are capable to growth in foods with salt content up to 10% and pH in the range of 4.2 to 9.3, although the optimum growth is usually observed in pH near 7 (Dias et al., 2016; Tango et al., 2018), which explains the conditions under which highest Grmax value was predicted in the present study (Figure 1). The tolerance of *S. aureus* to environments with high salt concentration confers an advantage in their growth when compared to other microorganisms (Hajmeer, Ceylan, Marsden, & Fung, 2006). Chaves et al. (2017), when studying prediction models for staphylococcal enterotoxin production in chicken meat, observed that even at lower population levels, enterotoxin production was recorded at

temperatures ranging from 13 °C to 37 °C, pH between 5.3 and 6.7 and between 0.6% and 2.2% salt content. Tsutsuura, Shimamura, and Murata (2013) detected the production of staphylococcal enterotoxin A in BHI at 10 °C-37 °C. Based on these evidences, here we were able to demonstrate that the physical-chemical characteristics of MFC samples can support the staphylococcal growth and consequent enterotoxin production, once enterotoxigenic strains are present. These data reinforce the relevance of controlling the hygienic and sanitary practices during the production, handling, storage and transport of MFC, avoiding potential staphylococcal growth and enterotoxin production, what can pose as risks for consumers.

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Supplementary Table 1. Predicted maximum growth rate (Grmax) and lag time of *S. aureus* in Minas frescal cheese under different combinations of temperature, pH, and sodium chloride.

Temp (°C)[a]	pH	% NaCl	Grmax (log CFU/g/h)[b]	Lag time (Hours)
7.5	5.80	0.85	0.012	159.24
9.0	5.80	0.85	0.018	106.16
12.0	5.80	0.85	0.04	47.77
25.0	5.80	0.85	0.374	5.11
7.5	5.80	0.95	0.012	159.24
9.0	5.80	0.95	0.018	106.16
12.0	5.80	0.95	0.04	47.77
25.0	5.80	0.95	0.374	5.11
7.5	5.80	1.14	0.012	159.24
9.0	5.80	1.14	0.018	106.16
12.0	5.80	1.14	0.04	47.77
25.0	5.80	1.14	0.374	5.11
7.5	5.80	1.70	0.012	159.24
9.0	5.80	1.70	0.018	106.16
12.0	5.80	1.70	0.039	49.00
25.0	5.80	1.70	0.372	5.14
7.5	5.98	0.85	0.013	146.99
9.0	5.98	0.85	0.019	100.57
12.0	5.98	0.85	0.042	45.50
25.0	5.98	0.85	0.398	4.80
7.5	5.98	0.95	0.013	146.99
9.0	5.98	0.95	0.019	100.57
12.0	5.98	0.95	0.042	45.50
25.0	5.98	0.95	0.398	4.80
7.5	5.98	1.14	0.013	146.99
9.0	5.98	1.14	0.019	100.57
12.0	5.98	1.14	0.042	45.50
25.0	5.98	1.14	0.399	4.79
7.5	5.98	1.70	0.012	159.24
9.0	5.98	1.70	0.019	100.57
12.0	5.98	1.70	0.041	46.61
25.0	5.98	1.70	0.398	4.80
7.5	6.23	0.85	0.013	146.99
9.0	6.23	0.85	0.02	95.54
12.0	6.23	0.85	0.043	44.44
25.0	6.23	0.85	0.413	4.63
7.5	6.23	0.95	0.013	146.99
9.0	6.23	0.95	0.02	95.54
12.0	6.23	0.95	0.043	44.44
25.0	6.23	0.95	0.413	4.63
7.5	6.23	1.14	0.013	146.99
9.0	6.23	1.14	0.02	95.54
12.0	6.23	1.14	0.043	44.44
25.0	6.23	1.14	0.415	4.60
7.5	6.23	1.70	0.013	146.99
9.0	6.23	1.70	0.02	95.54
12.0	6.23	1.70	0.043	44.44
25.0	6.23	1.70	0.415	4.60
7.5	6.35	0.85	0.013	146.99
9.0	6.35	0.85	0.02	95.54

Supplementary Table 1. *Continued*

Temp (°C)	pH	% NaCl	Grmax (log CFU/g/h)	Lag time (Hours)
12.0	6.35	0.85	0.043	44.44
25.0	6.35	0.85	0.416	4.59
7.5	6.35	0.95	0.013	146.99
9.0	6.35	0.95	0.02	95.54
12.0	6.35	0.95	0.043	44.44
25.0	6.35	0.95	0.416	4.59
7.5	6.35	1.14	0.013	146.99
9.0	6.35	1.14	0.02	95.54
12.0	6.35	1.14	0.043	44.44
25.0	6.35	1.14	0.418	4.57
7.5	6.35	1.70	0.013	146.99
9.0	6.35	1.70	0.02	95.54
12.0	6.35	1.70	0.043	44.44
25.0	6.35	1.70	0.419	4.56
7.5	6.46	0.85	0.013	146.99
9.0	6.46	0.85	0.019	100.57
12.0	6.46	0.85	0.042	45.50
25.0	6.46	0.85	0.413	4.63
7.5	6.46	0.95	0.013	146.99
9.0	6.46	0.95	0.019	100.57
12.0	6.46	0.95	0.042	45.50
25.0	6.46	0.95	0.413	4.63
7.5	6.46	1.14	0.013	146.99
9.0	6.46	1.14	0.019	100.57
12.0	6.46	1.14	0.043	44.44
25.0	6.46	1.14	0.416	4.59
7.5	6.46	1.70	0.013	146.99
9.0	6.46	1.70	0.019	100.57
12.0	6.46	1.70	0.042	45.50
25.0	6.46	1.70	0.419	4.56
7.5	6.62	0.85	0.012	159.24
9.0	6.62	0.85	0.019	100.57
12.0	6.62	0.85	0.042	45.50
25.0	6.62	0.85	0.408	4.68
7.5	6.62	0.95	0.012	159.24
9.0	6.62	0.95	0.019	100.57
12.0	6.62	0.95	0.042	45.50
25.0	6.62	0.95	0.408	4.68
7.5	6.62	1.14	0.012	159.24
9.0	6.62	1.14	0.019	100.57
12.0	6.62	1.14	0.042	45.50
25.0	6.62	1.14	0.411	4.65
7.5	6.62	1.70	0.012	159.24
9.0	6.62	1.70	0.019	100.57
12.0	6.62	1.70	0.042	45.50
25.0	6.62	1.70	0.414	4.62

[a] Minimum temperature permitted by ComBase Predictor for use in the predictions of *S. aureus* is 7.5 °C

[b] Grmax, maximum growth rate. The Grmax predictions were obtained using the ComBase predictor static growth models (http://modelling. combase.cc/).

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Capítulo 2. Predictive Modeling of the Staphylococcal Growth and Enterotoxin Production in Minas Frescal Cheese Environments

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ABSTRACT

Predictive models were adjusted to investigate the effect of temperature, pH, NaCl concentration, inoculum concentration, and time on the growth and enterotoxin A (SEA) production by *S. aureus*. Combinations of three levels of temperature (10-25 °C), five levels of pH (5.3-6.7), five levels of NaCl (0.8-2.2%), three levels of inoculum concentration (10^0-10^5 CFU/mL) in Brain Heart Infusion broth were studied. Colonies were counted and SEA production was assessed at 24 h intervals for up to 240 h. Surface response and logistic regression models were adjusted to describe the growth of *S. aureus* and productions of SEA, respectively. The growth and production of SEA by *S. aureus* were influenced by all factors, except NaCl concentration. *S. aureus* produced SEA in all samples at 25 °C (Counts of 8.27 to 9.36 log CFU/mL), while at 15 °C (Counts of 3.90 to 8.27 log CFU/mL), production ocurred in all conditions at 144 h. Temperature of 10 °C (Counts of <1.0 CFU/mL to 7.79 CFU/mL) delayed the growth and SEA production of *S. aureus* at initial contamination levels of 10^3 CFU/mL and 10^5 CFU/mL, and prevent it at 10^0 CFU/mL. The models were statistically and experimentally validated, demonstrating a good fit, with satisfactory values for R^2 (0.90), accuracy factor (1.09), and bias factor (0.99) for the growth model; a high agreement percentage (94.4%), Nagelkerke's R^2 (0.92), and the Hosmer and Lemeshow test ($p > 0.05$) for the SEA production model. The experimental validation confirmed the effectiveness of the models in predicting the growth and the SEA probability production by *S. aureus* under conditions of production and storage of Minas frescal cheese.

1. INTRODUCTION

Staphylococcus aureus is one of the pathogens most commonly involved in foodborne outbreaks, being currently characterized as a high-level priority II pathogen (WHO, 2017). From 2009 to 2021, staphylococcal toxins were responsible for 2.1% of foodborne outbreaks in the United States, affecting 4,412 individuals, resulting in 199 hospitalizations and 2 deaths (CDC, 2021). In Brazil, *S. aureus* was the third most frequently occurring pathogen in food between 2013 and 2022, accounting for 10.8% of waterborne and foodborne disease outbreaks (Brasil, 2023). Milk and dairy products are often associated with foodborne infections and intoxications (De Buyser, Dufour, Maire, & Lafarge, 2001; Johler et al., 2015; Keba et al., 2020) representing 1.3% of foods incriminated in outbreaks in Brazil (Brasil, 2023).

The prevalence of *S. aureus* in milk and dairy products is a public health concern. A study conducted between 1992 and 2021 investigated the presence of *S. aureus* in these products. The prevalence in raw milk, dairy products, and pasteurized milk was 33.36%, 16.82%, and 11.07%, respectively. *S. aureus* can multiply and produce staphylococcal enterotoxins (SE) in these foods. The main staphylococcal enterotoxins involved in foodborne outbreaks are SEA, SED, SEC, SEB, and SEE, in descending order of occurrence. They are heat resistant, maintaining their stability at 121°C for 28 minutes (Anderson, Beelman, & Doores, 1996) . This is a concern for products such as Minas frescal cheese, as SEs remain stable even after heat treatments, like pasteurization and ultra-high temperature.

Furthermore, Minas frescal cheese is a fresh food, with high moisture and the production involves excessive handling, which facilitates the prevalence, growth and production of enterotoxins by *S. aureus* (Cândido et al., 2020). The detection of enterotoxigenic *S. aureus* in Minas frescal cheese has been frequently reported (Arcuri et al., 2010; Martin et al., 2016; Silva et al., 2021). Therefore, it is essential to control the growth of *S. aureus* in cheeses to prevent the production of enterotoxins that cause outbreaks.

The incidence of *S. aureus* can be reduced through effective food safety control measures. To achieve this, it is essential to know the growth dynamics and production of enterotoxins of this microorganism under conditions to which the food may be exposed during its production and storage. Predictive microbiology is a powerful tool for predicting the behavior of microorganisms through mathematical models. These models can be primary, in which they predict the kinetics of microbial growth over time. Secondary models can predict how microorganisms behave under the influence of intrinsic and extrinsic food factors. Tertiary models combine primary and secondary models, using software to calculate microbial behavior

under specific conditions (Whiting & Buchanan, 1993). Primary and secondary models that can predict *S. aureus* growth and SEA production have been previously proposed (Elahi & Fujikawa, 2019; Fujikawa & Morozumi, 2006; Tango et al., 2018; Valík et al., 2018; Yu et al., 2020). However, these models mainly focus on culture media, milk and meat products. The secondary logistic regression model is an advanced method that predicts the probability of microbial growth or metabolite production in response to combinations of factors such as temperature, pH, and preservatives (Morassi et al., 2022; Oladeinde et al., 2023; Silva et al., 2022). To the best of the author's knowledge, there has been no development and validation of probability models for SEA production under the influence of Minas Frescal cheese production and storage conditions.

This study aimed to adjust models of SEA growth and production with different combinations of temperature, pH, sodium chloride (NaCl) concentrations, initial *S. aureus* concentration and time.

2. MATERIAL AND METHODS

2.1. Strain and inoculum preparation

For the experiment, a strain of *S. aureus* ATCC 13565, producer of SEA, was used. The strain was activated in Brain Heart Infusion (BHI, Oxoid Ltd., Basingstoke, UK) broth and incubated at 35 °C for 24 h. Inoculum standardization was performed by reading the optical density (OD) in a spectrophotometer (Kazuaki IL-227) at 600 nm, where the absorbance was adjusted to 0.100, equivalent to approximately 10^8 CFU/mL. After adjustment, dilutions in 0.85% saline solution (wt/vol) were made to obtain an inoculum with approximately 10^0 CFU/mL, 10^3 CFU/mL, or 10^5 CFU/mL in the growth and enterotoxin detection assays.

2.2. Experimental design

The culture medium used was BHI broth, prepared according to the manufacturer's instructions. The BHI formulations were defined using a central composite design (Table 1) with five levels of pH (5.3; 5.5; 6.0; 6.5; 6.7) and five levels of NaCl (0.8; 1.0; 1.5; 2.0; 2.2%). For pH adjustments, solutions of hydrochloric acid (HCl) and sodium hydroxide (NaOH) at a concentration of 1 mol/L were used. Saline concentrations were obtained by adding appropriate

amounts of NaCl to the medium (which has a NaCl concentration of 0.5% wt/vol as part of its composition).

Table 1. Experimental design with coded and uncoded values of pH and NaCl of the BHI formulations used for the *S. aureus* growth and SEA production assays.

Samples	Variables	
	pH	NaCl
1	5.5 (-1)	1 (-1)
2	6.5 (+1)	1 (-1)
3	5.5 (-1)	2 (+1)
4	6.5 (+1)	2 (+1)
5	5.3 (-1.41)	1.5 (0)
6	6.7 (+1.41)	1.5 (0)
7	6.0 (0)	0.8 (-1.41)
8	6.0 (0)	2.2 (+1.41)
9	6.0 (0)	1.5 (0)
10	6.0 (0)	1.5 (0)
11	6.0 (0)	1.5 (0)

After adjusting the pH and NaCl with the solutions mentioned above, the culture media were sterilized at 121 °C for 15 minutes. For each combination of factors (Table 1), three *S. aureus* concentration were used: 10 CFU/mL, 10^3 CFU/mL, or 10^5 CFU/mL. To assess the *S. aureus* count and enterotoxin production capacity, tubes containing BHI medium adjusted to the specified pH and NaCl concentrations, with the three *S. aureus* concentrations, were incubated at different temperatures (10, 15 and 25 °C) for up to 240 h. The tested values are within the range of conditions to which *S. aureus* may be exposed during the processing and storage of Minas frescal cheese. Every 24 h, aliquots were collected from each experiment for toxin detection and simultaneous microbial concentration evaluation. For *S. aureus* enumeration, each sample was 10-fold diluted up to 1:100,000 in a 0.85% (w/v) saline solution, plated on BHI agar in triplicate using the microdroplet technique, and incubated at 35 °C for 18 h. Colonies were counted, and the results were expressed in log CFU/mL. The experiment was done in three repeats in triplicate.

2.3. Enterotoxin detection

Samples at 24 h, 72 h, 144 h, and 240 h were subjected to an Enzyme-Linked Fluorescent Assay (ELFA) for the detection of staphylococcal enterotoxin A from *S. aureus* ATCC 13565. Samples in culture medium were centrifuged at 5,000 × *g* for 15 min, and 500

µL aliquots of the resulting supernatant were placed in the well of the VIDAS® Staphylococcal Enterotoxins (SET2) Kit and subjected to examination using the ELFA reader at 450 nm, MiniVIDAS (MiniVIDAS bioMérieux, Inc. France). As for the milk and cheese samples used for validation, they were extracted according to the Biomérieux® extraction protocol for dairy products. Samples with TV (Test Value) titers < 0.13 were considered negative for the presence of enterotoxin, while those with VT titers > 0.13 were considered positive. VT is the ratio between the relative fluorescence of the standard and the relative fluorescence of the sample.

2.4. Data analysis and mathematical modeling

2.4.1. Growth model

S. aureus growth responses were recorded every 24 h for up to 240 h. The response surface model was used to predict the effect of temperature, pH, NaCl concentration, *S. aureus* concentration, and time (independent variables) on the growth of *S. aureus* (dependent variable) (Equation 1).

$$Y = \beta 0 + \beta_1 time + \beta_2 T + \beta_3 pH + \beta_4 NaCl + \beta_5 inoculum + \beta_6 time^2 + \beta_7 T^2 + \beta_8 pH^2 + \beta_9 NaCl^2 \beta_{10} inoculum^2 + \beta_{11} time*T + \beta_8 time*pH + \beta_9 time*NaCl + \beta_{10} time*inoculum + \beta_{10} T*pH + \beta_{11} T*NaCl + \beta_{12} T*inoculum + \beta_{13} pH*NaCl + \beta_{14} pH*inoculum \quad (1)$$

Where: Y is the *S. aureus* 13565 growth response; $\beta_0 - \beta_{14}$ are adjustable coefficients; T, temperature; inoculum, the concentration initial value of the *S. aureus*; pH, pH value; NaCl, the concentration value (%) of sodium chloride.

The model fit was assessed using the determination coefficient (R^2), bias factor, and accuracy factor.

2.4.2. Enterotoxin model

The results were monitored by collecting samples every 24 h for up to 240 h to determine production or not SEA production. The matrices generated by combinations of the studied factors (temperature, pH, NaCl concentration, *S. aureus* concentration and time) and the enterotoxin production responses obtained by the ELFA method in MiniVidas (coded as 0 and 1) were fitted to the probabilistic logistic regression model ($P(x) = \frac{e^{|\beta 0 + \sum \beta i x i|}}{1 + e^{|\beta 0 + \sum \beta i x i|}}$) using SPSS Statistics 25.0 software. Temperature, pH, NaCl, *S. aureus* concentration and time represents

independent variables, and the probability of enterotoxin production represents the dependent variable. Thus, responses were classified as enterotoxin production (1) and no-enterotoxin production (0). Samples with VT equal to or greater than 0.13 were classified as enterotoxin producers (1) and those below 0.13 as no-enterotoxin producers (0). The logit model necessary for the linearization of the model used is represented by Equation 2.

$$Logit\ (P) = Ln\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 time + \beta_2 T + \beta_3 pH + \beta_4 NaCl + \beta_5 inoculum + \beta_6 time^2 + \beta_7 T^2 + \beta_8 pH^2 + \beta_9 NaCl^2\ \beta_{10} inoculum^2 + \beta_{11} time*T + \beta_8 time*pH + \beta_9 time*NaCl + \beta_{10} time*inoculum + \beta_{10} T*pH + \beta_{11} T*NaCl + \beta_{12} T*inoculum + \beta_{13} pH*NaCl + \beta_{14} pH*inoculum \quad (2)$$

Where: $g(x)$ = probability (0-1) of enterotoxin production/no-production by *S. aureus* 13565; $\beta_0 - \beta_{14}$ are adjustable coefficients; T, temperature; pH, pH value; NaCl, the concentration value (%) of sodium chloride; inoculum, the concentration value of the *S. aureus*.

The performance of the obtained model was evaluated using the Nagerlkerke R^2 test, Hosmer-Lemeshow test and percentage of agreement. The probabilities of SEA production by *S. aureus* under the studied conditions were also calculated using the equation 2.

2.4.3. Experimental validation of the models

The model was validated for their predictive performance against different combinations not tested during model construction, both in a culture medium and in a food matrix. For food matrix validation, milk and cheese were used, with pH, temperature and NaCl values within the studied range.

For culture medium validation, eight untested combinations were employed, which were not used during the data collection for model development (pH = 5.7, T = 12 °C, NaCl = 1.2%; pH = 5.7, T = 20 °C, NaCl = 1.2%; pH = 6.3, T = 12 °C, NaCl = 1.2%; pH = 6.3, T = 20 °C, NaCl = 1.2%; pH = 5.7, T = 13 °C, NaCl = 1.8%; pH = 5.7, T = 20 °C, NaCl = 1.8%; pH = 6.7, T = 12 °C, NaCl = 1.8%; pH = 6.7, T = 20 °C, NaCl = 1.8%), *S. aureus* at concentrations of 10^0 CFU/mL, 10^3 CFU/mL e 10^5 CFU/mL for each combination and incubation for 144 h.

For food validation, ultrapasteurized (UHT) milk and Minas frescal cheese were used. Five hundred mL volumes of UHT milk were placed in sterile 1,000-mL bottles, inoculated with *S. aureus* at concentrations of 10^0 CFU/mL, 10^3 CFU/mL e 10^5 CFU/mL, pH fixed at 6.6, and incubated at temperatures of 12 °C and 20 °C for 144 h. The cheeses were manufactured at

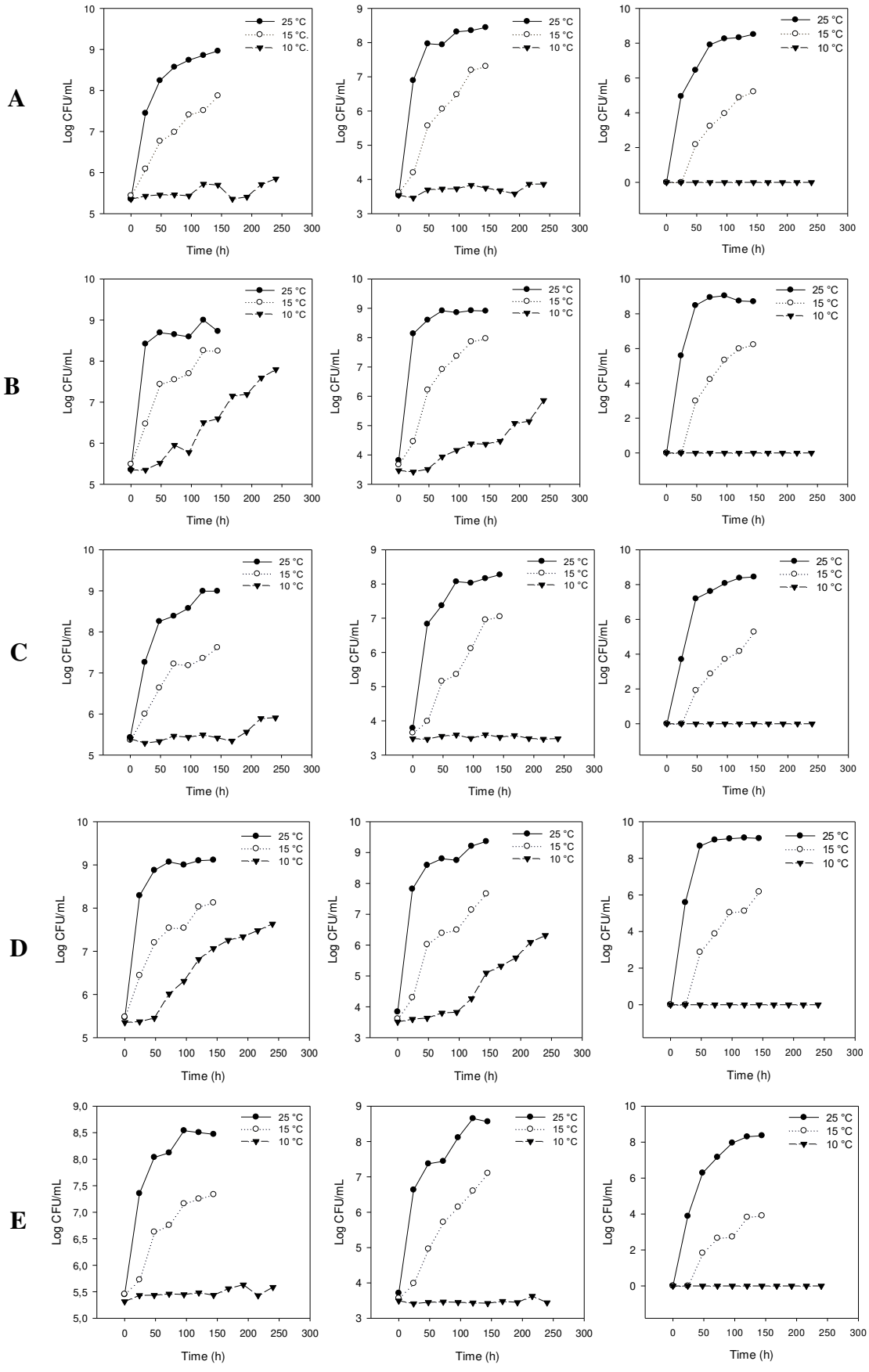
a laboratory scale according to the methodology described by Van Tassell, Ibarra-Sánchez, Takhar, Amaya-Llano, & Miller (2015) with adaptations: 500 mL of pasteurized milk were transferred to sterile 1,000-mL bottles, heated in water bath at 35 °C and inoculated with *S. aureus* at concentrations of 10^0 CFU/mL, 10^3 CFU/mL e 10^5 CFU/mL; then, the milk was added with calcium chloride and commercial rennet, and distributed into 50 mL portions in sterile tubes, and maintained at 35 °C for 45 minutes for coagulation; after cutting, the tubes were incubated again for 30 minutes at 40 °C, centrifuged, and part of the whey removed and replaced with 2.5% (w/v) NaCl for a final concentration of 1.1% (w/v) NaCl; the tubes were agitated, additionally incubated for 20 minutes at 35 °C, centrifuged, and the whey completely removed. The pH of the cheeses was determined and ranged from 6.0 to 6.5. The cheeses were incubated at temperatures of 12 °C and 20 °C for 144 h. Every 24 h, aliquots of the milk and cheeses incubated were collected for toxin detection and simultaneous evaluation of microbial concentration. For *S. aureus* enumeration, each sample was 10-fold diluted up to 1:100,000 in a 0.85% (w/v) saline solution, plated on BHI agar in triplicate using the microdroplet technique, and incubated at 35 °C for 18 h. Colonies were counted, and the results were expressed in log CFU/mL.

For the growth model validation, the bias factor and the accuracy factor were used to compare the values observed in the validation experiments in BHI, milk and cheese with the values predicted by the built model. For the SEA model validation, the probability values obtained in the generated model were compared with those obtained in validation. In the data analyses, predicted probability values above 0.5 were classified as enterotoxin production (1), while those below 0.5 were classified as no-enterotoxin production (0). Based on these results, the percentages of agreement were determined to evaluate the difference in the probability of SEA production between predicted and observed data.

3. RESULTS AND DISCUSSION

3.1. Growth model

Figure 1 shows the growth of *S. aureus* ATCC 13565 under different temperatures, pH, NaCl concentration, initial inoculum concentration, and time. The growth of *S. aureus* was monitored for 144 h for samples at 15 °C and 25 °C, and for 240 h for samples at 10 °C, as at this temperature, they had not yet reached the logarithmic (log) growth phase, during which the production of enterotoxin A occurs.


A
B
C
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E
25 °C
15 °C
10 °C
Log CFU/mL
Time (h)

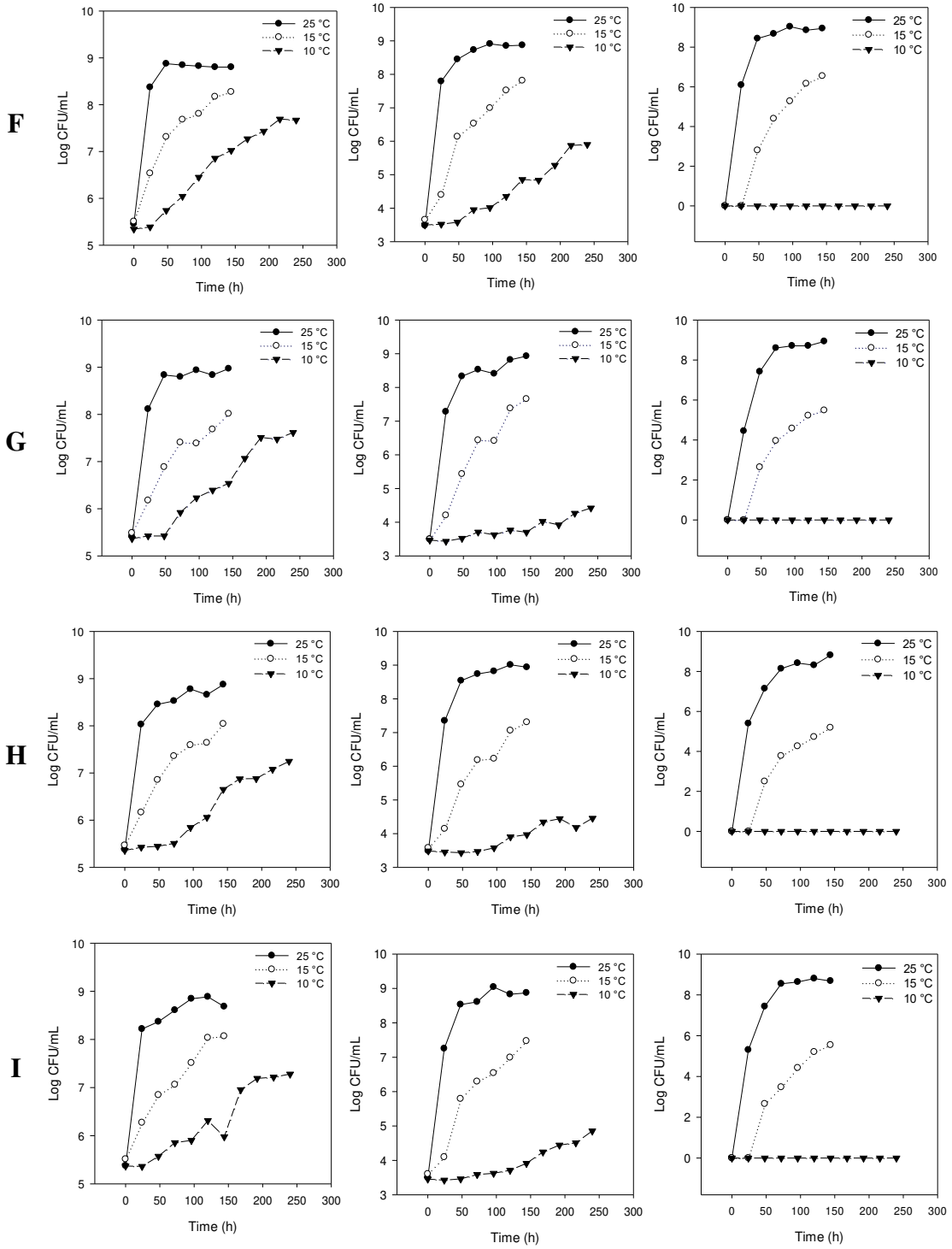


Figure 1. Growth of *S. aureus* ATCC 13565 in BHI at 240 h; three different *S. aureus* concentrations and temperatures. A) pH 5.5 and 1% NaCl; B) pH 6.5 and 1% NaCl; C) pH 5.5 and 2% NaCl; D) pH 6.5. and 2% NaCl; E) pH 5.3 and 1.5% NaCl; F) pH 6.7 and 1.5% NaCl; G) pH 6.0 and 0.8% NaCl; H) pH 6.0 and 2.2% NaCl, and I) pH 6 and 1.5% NaCl.

Among the evaluated conditions (Table 1), the maximum population found varied from 8.27 log CFU/mL to 9.36 log CFU/mL at 25 °C and from 3.90 log CFU/mL to 8.27 log CFU/mL at 15 °C. At 10 °C, with an inoculum concentration of 10^0 CFU/mL remained below the

detection limit (<1.0 CFU/mL) throughout the incubation period, while for the other studied inoculum concentrations, a maximum population of 7.79 log CFU/mL was observed.

When starting with the initial concentration of 10^5 CFU/mL and 10^3 CFU/mL, the *S. aureus* strain studied reached the logarithmic growth phase in all evaluated conditions at 15 °C and 25 °C. However, at 10 °C and an initial concentration of 10^3 CFU/mL the bacteria only reached the logarithmic phase under optimal pH conditions (6.0-6.7), regardless of the NaCl concentration and, remained in the lag phase at initial concentration of 10^0 CFU/mL. This demonstrates the influence of the initial concentration, temperature, and pH on the growth of *S. aureus*. Similar behavior was observed by other authors (Alomar et al., 2008; Valero et al., 2009). The lower the temperature and the initial contamination level, the longer the lag phase and the longer it takes to reach the logarithmic phase, where the production of SEA occurs (Chen, Song, Xu, & Zhang, 2018; Daughtry, Davey, & King, 1997; Tremaine et al., 1993).

In a previous study by our research group (Silva et al., 2021), *Combase* was used to assess the influence of pH, temperature, and NaCl concentration on the growth kinetics (maximum growth rate and lag time) of *S. aureus*. Based on the findings from that study, a response surface model was developed to evaluate the growth responses of *S. aureus* (log CFU/mL), with the variables of initial contamination levels and time included. According to the model, only the time, temperature and inoculum concentration linear, significantly influenced ($p < 0.05$) the growth of *S. aureus*. Quadratic effects of time, temperature and inoculum concentration, as well interactions between time and the factors temperature, pH and inoculum, and temperature with pH and inoculum concentration were also significant (Table 2). The factor pH individually did not show a significant effect, but it was retained in the model since its interaction was significant. Thus, the following second-order polynomial equation 3 was obtained:

$$Y = -8.1849 + 0.0137*time + 0.7016*Temperature - 0.1128*pH + 2.1574*Inoculum - 0.0002*time^2 - 0.0147*Temperature^2 - 0.0387*Inoculum^2 + 0.0017*time*Temperature + 0.0032*time*pH - 0.0037*time*Inoculum + 0.0226*Temperature*pH - 0.0573*Temperature*Inoculum \quad (3)$$

Where: Y = *S. aureus* 13565 growth response; T, temperature; pH, pH value; NaCl, the concentration value (%) of sodium chloride; inoculum, the concentration initial of the *S. aureus*.

The factors bias, accuracy and R^2 are commonly used for assessing the fit of response surface models (Heo, Lee, Baek, & Ha, 2009; Shrestha, Erdmann, & Smith, 2022; Wang & Oh, 2012). A R^2 close to 1.0 indicates an accurate prediction of the developed model. Bias factor and accuracy factor are estimates of the mean differences between predicted and observed responses (Baranyi et al., 1999). The closer to 1.0, better the fit of the model to the experimental data, i.e., the predicted values are closer to the observed values. Values of bias factor less than 1.0 indicates that the model is fail-safe, the model on the other hand, a bias factor higher than 1.0 is fail dangerous. The value 1 indicates a total agreement between predicted and observed data (Ross, 1996). The accuracy factor describes the variations of predicted values around the means. In the current model, a value of 1.09 was found, indicating that the predicted data differs 9% from the observations. Therefore, the obtained model demonstrated a good fit to the data in all assessed parameters, and thus, it can be used to predict the growth of *S. aureus* under conditions encountered during the production and storage of Minas frescal cheese.

For experimental validation, accuracy and bias factors were also calculated to assess the difference between the predicted model and observed data. Bias factor values of 1.12, 0.85, and 0.80, as well as accuracy factor values of 1.22, 1.19, and 1.08, were observed for the responses in BHI, milk, and cheese, respectively (Supplementary Table 1). Ross (1999) considers that the bias factor is variable depending on whether the model describes data for spoilage or pathogenic microorganisms. The author also suggests that for the accuracy factor, each independent variable included in the model 10% of error. Thus, based on the observed data, an accuracy factor of up to 1.5 can be considered acceptable for the current study with five variables. This indicates that the difference between the predicted validation data is not significant and confirms the reliability of the model to describe the growth of *S. aureus* under the production and storage conditions of Minas Frescal cheese.

Table 2. Parameters obtained and the respective adjustment indices of the prediction model of *S. aureus* ATCC 13465 growth.

Parameters	Coefficient	Standard error	*p*-value
Constant	-8.1849	0.9527	0.0000
Time	0.0137	0.0062	0.0270
Temperature	0.7016	0.0553	0.0000
pH	-0.1128	0.0223	0.4581
Inoculum	2.1574	0.0466	0.0000
$Time^2$	-0.0002	0.0000	0.0000
$Temperature^2$	-0.0147	0.0008	0.0000
$Inoculum^2$	-0.0387	0.0071	0.0000
Time*Temperature	0.0017	0.0000	0.0000
Time*pH	0.0032	0.0010	0.0012
Time*Inoculum	-0.0037	0. 0002	0.0000
Temperature*pH	0.0226	0.0076	0.0028
Temperature*Inoculum	-0.0573	0.0016	0.0000
Statistics			
R^2	0.90		
Bias factor	0.99		
Accuracy factor	1.09		

3.2. Enterotoxin model

The conditions found in Minas Frescal cheese were replicated to assess the influence of factors on the production of SEA by *S. aureus* ATCC 13565. The responses to combinations of pH and NaCl (Table 1), initial inoculum concentration (10^0 CFU/mL, 10^3 CFU/mL, and 10^5 CFU/mL), and storage at different temperatures (10 °C, 15 °C, and 25 °C) for 240 h (24 h, 72 h, 144 h, and 240 h) are described in Table 3.

Table 3. Enterotoxin production/No-enterotoxin production responses of *S. aureus* ATCC 13565 at environmental conditions.

Time (h)	Temperature (°C)	Enterotoxin	No-enterotoxin
0	10	0	33
	15	0	33
	25	0	33
24	10	0	33
	15	15	18
	25	33	0
72	10	2	31
	15	27	6
	25	33	0
144	10	11	22
	15	33	0
	25	33	0
240	10	14	19
	15	33	0
	25	33	0

In the first 24 h, *S. aureus* produced SEA in all samples at 25 °C, while at 15 °C, production ocurred in all conditions at 144 h. The temperature of 10 °C prevented the SEA production for up to 72 h and, and after 240 h of storage, 68% of the samples still had not produced SEA. The average shelf life of Minas frescal cheese is 30 days, and it should be mantained at temperatures of up to 8 °C. Silva et al. (2021) found Minas frescal cheese stored at abusive temperatures between 9 °C and 12 °C on market shelves. According to the findings of this study, if there is an initial contamination level between 10^3 CFU/mL and 10^5 CFU/mL in the product, stored at 10 °C, *S. aureus* can multiply and produce SEA after 72 h of storage. Therefore, it is essential to control the initial contamination level and keep the cheese under refrigeration temperatures of up to 8 °C to ensure its safety during its shelf life (Brasil, 1997).

The use of *S. aureus* growth models in culture media and foods of animal origin is well established (Valero et al., 2009; De Araújo et al., 2017; Elahi and Fujikawa, 2019). However, the application of probability models to predict the growth and production of enterotoxins by *S. aureus* under variable conditions in cheeses of importance in Brazil needs further exploration. Based on the conditions found in Minas frescal cheese from retail sale (Silva et al., 2021), a

logistic regression model was built to predict the influence of these factors on the production of SEA by *S. aureus*. The estimated parameters with standard error to the model are shown in Table 4. The parameters that significantly influenced the production of enterotoxins ($p \leq 0.05$) were selected by the backward stepwise method to create the model, resulting in Equation 4 below:

$$Logit\ (P) = Ln\left(\frac{P}{1-P}\right) = 70.503 - 0.2347 * time + 2.9965 * T - 34.1153 * pH + 1.0328 * inoculum - 0.0696 * T^2 + 2.7729 * pH^2 + 0.0146 * time * T + 0.0106 * time * inoculum - 0.0485 * T * inoculum \quad (4)$$

Where: $Logit\ (P)$ = probability (0-1) of enterotoxin production/no-production by *S. aureus* 13565; T, temperature; pH, pH value; NaCl, the concentration value (%) of sodium chloride; inoculum, the concentration value of the *S. aureus*.

The production of SEA by *S. aureus* was influenced by time, temperature, pH, inoculum concentration and their interactions ($p < 0.05$), but not by NaCl concentration. This result could be attributed to the NaCl range used in this study. Nunes & Caldas (2017) and Elahi & Fujikawa (2019), when using broader NaCl ranges (0.64 to 4.6% and 0.5% to 20%, respectively), observed a greater influence of salt on growth and enterotoxin production responses by *S. aureus*. However, as the present study aimed to investigate real situations observed in cheese, these values were within the range of NaCl found (Silva et al., 2021).

In a parametric model, the Wald test can be used to define the importance of each independent variable on the dependent variable (Wang & Guo, 2001). Thus, the combinations of time and temperature or initial concentration had the most significance influence on SEA, followed by the linear factors time and temperature (Table 4), consistent with the results found by (Hedberg, Palazzi-Churas, Radke, Selman, & Tauxe (2008) and Babic et al. (2019) and inconsistent with results found by Ding et al. (2016).

According to the model obtained, higher pH and longer time, to greater chances of *S. aureus* producing SEA. However, the influence of pH was more pronounced at 15 °C and an initial inoculum concentration of 10^3 CFU/mL, where SEA production was observed whitin 24 h only in samples with pH 6.5 and 6.7. Other factors, such as time, temperature and inoculum concentration, demonstrated a more significant impact on SEA production, corroborating the results of the Wald test, where the combination of time and pH were the factors that had the least impact on the responses. In the study conducted by Ding et al (2016), pH only affected

SEA production when combined with temperature and water activity. According to Genigeorgis (1989), the ideal pH for the enterotoxin production is between 7 and 8.

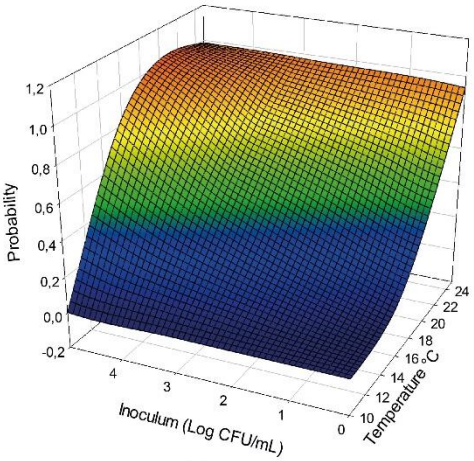
The logistic regression model has shown good performance in predicting microbial behavior in food (Huang, Jia, & Hwang, 2022; Koseki, Mizuno, & Yamamoto 2007; Morassi et al., 2022). The model obtained demonstrated good statistical fit to the data using Nagelkerke R2, Hosmer and Lemeshow test and % predicted correctly. The Nagelkerke R2 exceeded 0.90 and 94.40% of the data were correctly predicted. The Hosmer and Lemeshow test, which assesses whether there is a significant difference between the data, a $p > 0.05$ was found, demonstrating no significant difference between the obtained and predicted results (Table 3). This indicates that the model is adequate to describe the production of SEA by *S. aureus* in response to the evaluated factors.

Table 4. Parameters obtained and the respective adjustment indices of the prediction model of staphylococcal enterotoxin A production/no production.

Parameters	Coefficient	Standard error	Wald test	*p*-value
Constant	70.5030	21.7511	10.5064	0.0012
Time	-0.2347	0.0344	46.6304	0.0000
Temperature	2.9965	0.4656	41.4263	0.0000
pH	-34.1153	7.3013	21.8321	0.0000
Inoculum	1.0328	0.3844	7.21890	0.0072
Temperature^2	-0.0696	0.0113	38.0992	0.0000
pH^2	2.7729	0.6089	20.7365	0.0000
Time*Temperature	0.0146	0.0012	144.8725	0.0000
Time*pH	0.0106	0.0043	6.1362	0.0113
Time*Inoculum	0.0188	0.0022	70.8937	0.0000
Temperature*Inoculum	-0.0485	0.0189	6.6032	0.0102
Statistics				
Nagelkerke R^2 Statistic	0.91			
% predicted correctly	94.40			
Hosmer-Lemeshow	4.101 (df = 8, p = 0.848)			

The predicted probabilities of SEA production by the model ranged from 0% under conditions at 10 °C to 100% at 15 °C and 25 °C during the 240 h of incubation. For 25 °C, in all conditions, probabilities above 90% were observed at 24 h. The lowest probabilities (2% to 3%) for 15 °C were noted at initial concentrations of 10^0 CFU/mL and 10^3 CFU/mL, pH 6, 1.5% and 2.2% of NaCl, while at 10 °C, all conditions with initial concentrations of 10^0 CFU/mL showed a zero probability at 24 h. These results may be atributted to the fact that at 24 h, the bacteria are still in the lag phase at low temperatures and in the log phase at 25 °C. Probabilities above 95% at 10 °C were observed from the 144 h, 10^3 CFU/mL of *S. aureus*, pH 6.5 and 6.7, and 1% and 1.5% of NaCl. With increasing temperature and initial *S. aureus* concentration, there was an increase in the probability of SEA production (Figure 2).

A


Probability
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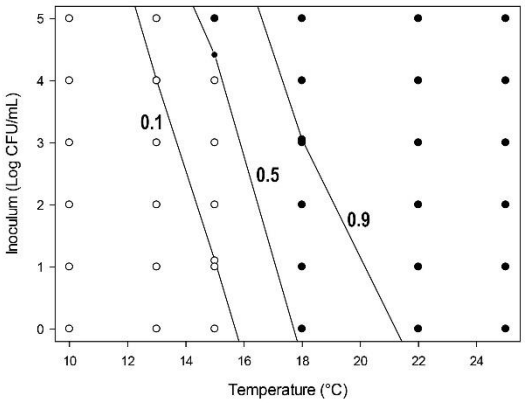
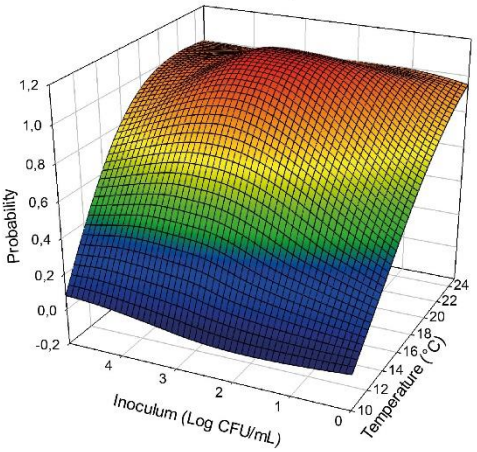
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
Inoculum (Log CFU/mL)
4
3
2
1
0
Temperature °C
10
12
14
16
18
20
22
24


Inoculum (Log CFU/mL)
0
1
2
3
4
5
0.1
0.5
0.9
10
12
14
16
18
20
22
24
Temperature (°C)

B


Probability
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1,0
0,8
0,6
0,4
0,2
0,0
-0,2
Inoculum (Log CFU/mL)
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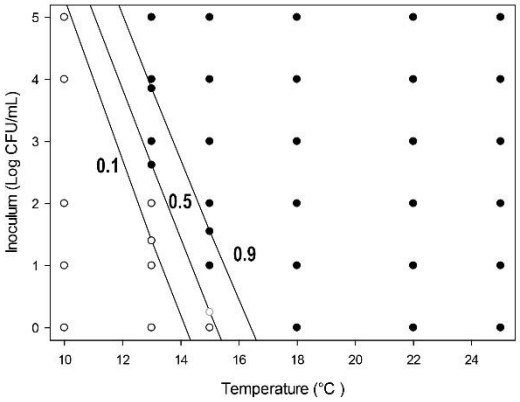
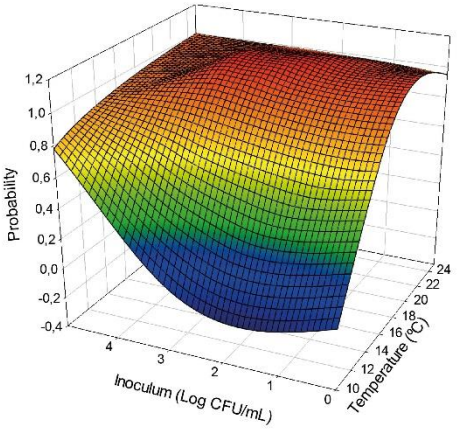
3
2
1
0
Temperature (°C)
10
12
14
16
18
20
22
24


Inoculum (Log CFU/mL)
0
1
2
3
4
5
0.1
0.5
0.9
10
12
14
16
18
20
22
24
Temperature (°C)

C


Probability
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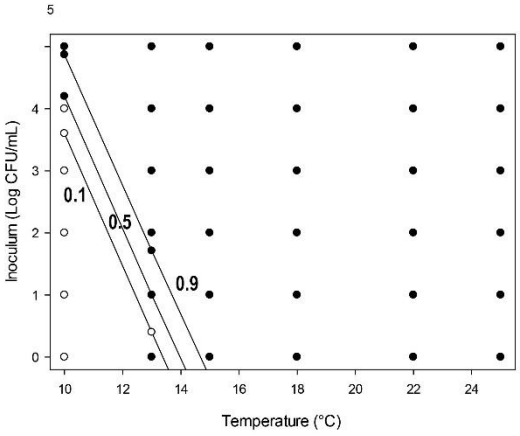
0,8
0,6
0,4
0,2
0,0
-0,2
-0,4
Inoculum (Log CFU/mL)
4
3
2
1
0
Temperature (°C)
10
12
14
16
18
20
22
24


5
Inoculum (Log CFU/mL)
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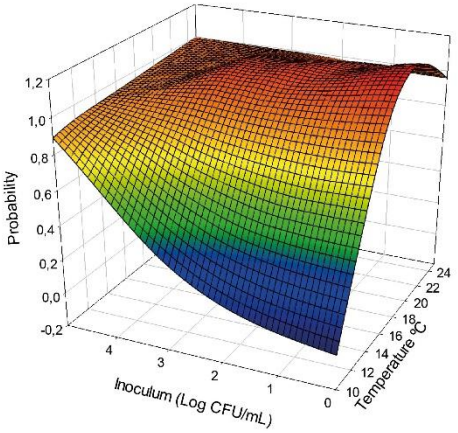
2
3
4
0.1
0.5
0.9
10
12
14
16
18
20
22
24
Temperature (°C)

D


Probability
1,2
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
Inoculum (Log CFU/mL)
4
3
2
1
0
Temperature °C
10
12
14
16
18
20
22
24

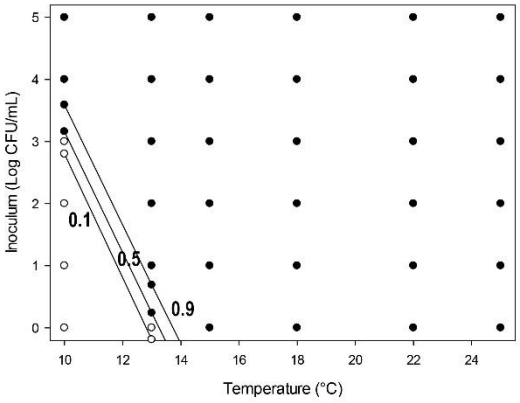

Inoculum (Log CFU/mL)
0
1
2
3
4
5
0.1
0.5
0.9
10
12
14
16
18
20
22
24
Temperature (°C)

Figure 2. Probability responses of enterotoxin (●) production/no-enterotoxin production (o) of *S. aureus* ATCC 13565 as a function of pH 5.5, inoculum concentration and storage temperatures at A) 24 h, B) 72 h, C) 144 h, D) 240 h. To the right the first line represents 0.1. the second 0.5. and the third 0.9 of probability.

Probabilities above 0.5 were considered as SEA production, while those below 0.5 indicated no-SEA production (Figure 2). An increase in probability can be observed, with a consequent expansion in the region of production (●) and a reduction in the region of no-production of SEA (o), can be observed as time and temperature progress. For scenarios at 10 °C and 10^0 CFU/mL of *S. aureus*, there was no production of SEA. Minas frescal cheese, especially when informally produced, is often at room temperature or sold without refrigeration. In this study, 25 °C was the ideal temperature for SEA production, and 10 °C exhibited the most inhibitory effect among the factors evaluated. Previously studies have documented staphylococcal enterotoxins production at refrigeration temperatures of 10 °C by Tsutsuura, Shimamura, & Murata, 2013) and Elahi and Fujikawa (2019). Therefore, it is essential to store the cheeses at adequate refrigeration temperatures.

Different concentration levels of *S. aureus* were tested to simulate low and high initial contamination in Minas frescal cheese. Figure 2 shows that the higher the initial inoculum concentration, the smaller the region of no-enterotoxin production ($P < 0.5$) and the greater the probability of enterotoxin-production. According to the Food and Drug Administration (FDA), enterotoxin production begins at 10^5 CFU/mL. In this study, for most conditions, SEA could only be detected when the *S. aureus* concentration reached this level, as observed by Mohammadi & Hanifian (2015) and Cai et al. (2023). In the model conducted by Hu, Lin, Chen, & Yan (2018) in cooked chicken, SEA was produced only when the concentration of *S. aureus* reached 10^6 CFU/mL. Therefore, the production of enterotoxins is not only associated with the inoculum (Al-Nabulsi et al., 2020). Other factors, such as temperature, the food matrix, and competing cultures, influence the enterotoxins production. In this study, the Wald test showed that other factors, such as time, temperature and their modifications, had a greater influence on the SEA responses.

Experimental validation of predictive models involves testing different conditions within the studied range during model development to test the applicability of these models. This can be performed in culture media and/or in a food matrix. In the present study, different combinations of the conditions in BHI, Minas frescal cheese and UHT milk were used to validate the model. The probability responses for SEA production observed during 144 h of storage were compared with predicted responses by the developed model (Supplementary Table

2). Of the 72 conditions tested in BHI, 88% were correctly classified by the model. The model overestimated SEA production in 7% (fail-safe) and underestimated it in 5% (fail-dangerous). This result is expected for models developed in culture media, as they contain nutrients that provide ideal conditions for the development of microorganisms, resulting in models with fail-safe variations (Te Giffel & Zwietering, 1999).

The behavior of microorganisms in culture media under the influence of environmental factors may differ in food matrices. Studies conducted with logistic regression models were validated in culture media (Elahi & Fujikawa, 2019; Moraes et al., 2018; Tabanelli et al., 2014). However, models developed in culture media were considered inaccurate when tested in food (Te Giffel & Zwietering, 1999). Therefore, validation in food is important to confirm the applicability of the model to real situations. In this study, validation in UHT milk and Minas fresh cheese showed agreement with the observed data of 78% and 83%, respectively. These results confirm the applicability of the developed model to predict SEA production responses by *S. aureus* in Minas frescal cheese.

4. Conclusions

This study was conducted to investigate the growth and probability of SEA production by *S. aureus* against combinations of temperatures, pH, NaCl concentrations, initial concentrations of *S. aureus*, and time. The studied factors influenced the growth and SEA production responses, except for NaCl. At a temperature of 25 °C, *S. aureus* produced SEA in all studied conditions within the first 24 h of storage, while a temperature of 10 °C delayed the growth of *S. aureus* and prevented the production of SEA at a low initial contamination level (10^0 CFU/mL). The influence of the inoculum concentration demonstrated the importance of controlling and including the initial contamination level in the models. The statistical validations demonstrated the good performance of the growth and SEA production models in describing the experimental data. The validations in milk and cheese confirmed the effectiveness of the model in predicting the responses. Therefore, these models can be used to predict the growth and SEA production by *S. aureus* in Minas frescal cheese. New models should be proposed to assess the influence of these factors on the concentration of SEA produced by *S. aureus*.

Acknowledgments

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Supplementary Table 1. Validation responses in BHI, Minas frescal cheese and UHT milk of growth of *S. aureus* ATCC 13565 at environmental conditions.

Time	Temperature	pH	NaCl	Inoculum	Pred.*	Obs.**
BHI						
24	20	5.7	1.2	5	7.02	7.00
24	20	5.7	1.8	5	7.02	7.07
24	20	6.3	1.2	5	7.27	8.02
24	20	6.3	1.8	5	7.27	7.74
24	12	5.7	1.2	5	6.03	5.79
24	12	5.7	1.8	5	6.03	5.78
24	12	6.3	1.2	5	6.17	6.02
24	12	6.3	1.8	5	6.17	5.81
24	20	5.7	1.2	3	5.93	5.43
24	20	5.7	1.8	3	5.93	5.35
24	20	6.3	1.2	3	6.18	6.23
24	20	6.3	1.8	3	6.18	5.86
24	12	5.7	1.2	3	4.28	3.8
24	12	5.7	1.8	3	4.28	3.69
24	12	6.3	1.2	3	4.42	4.01
24	12	6.3	1.8	3	4.42	3.81
24	20	5.7	1.2	0	4.30	2.57
24	20	5.7	1.8	0	4.30	2.49
24	20	6.3	1.2	0	4.55	3.27
24	20	6.3	1.8	0	4.55	3.02
24	12	5.7	1.2	0	1.65	<1.00
24	12	5.7	1.8	0	1.65	<1.00
24	12	6.3	1.2	0	1.79	<1.00
24	12	6.3	1.8	0	1.79	<1.00
72	20	5.7	1.2	5	8.39	8.43
72	20	5.7	1.8	5	8.39	8.28
72	20	6.3	1.2	5	8.73	8.79
72	20	6.3	1.8	5	8.73	8.89
72	12	5.7	1.2	5	6.75	6.69
72	12	5.7	1.8	5	6.75	6.44
72	12	6.3	1.2	5	6.98	7.17
72	12	6.3	1.8	5	6.98	7.06
72	20	5.7	1.2	3	7.56	6.47
72	20	5.7	1.8	3	7.56	6.70
72	20	6.3	1.2	3	7.9	8.35
72	20	6.3	1.8	3	7.9	7.77
72	12	5.7	1.2	3	5.25	5.07
72	12	5.7	1.8	3	5.25	4.87
72	12	6.3	1.2	3	5.49	5.67
72	12	6.3	1.8	3	5.49	5.62
72	20	5.7	1.2	0	6.31	4.59
72	20	5.7	1.8	0	6.31	4.97
72	20	6.3	1.2	0	6.66	6.36
72	20	6.3	1.8	0	6.66	5.30
72	12	5.7	1.2	0	3.01	2.37
72	12	5.7	1.8	0	3.01	2.07
72	12	6.3	1.2	0	3.25	2.50
72	12	6.3	1.8	0	3.25	2.56
144	20	5.7	1.2	5	8.71	8.36
144	20	5.7	1.8	5	8.71	8.37
144	20	6.3	1.2	5	9.19	0.03
144	20	6.3	1.8	5	9.19	8.98
144	12	5.7	1.2	5	6.09	6.95
144	12	5.7	1.8	5	6.09	6.93
144	12	6.3	1.2	5	6.47	7.40

144	12	6.3	1.8	5	6.47	8.36
144	20	5.7	1.2	3	8.27	8.36
144	20	5.7	1.8	3	8.27	8.41
144	20	6.3	1.2	3	8.75	8.36
144	20	6.3	1.8	3	8.75	8.42
144	12	5.7	1.2	3	4.99	6.08
144	12	5.7	1.8	3	4.99	6.59
144	12	6.3	1.2	3	5.36	7.09
144	12	6.3	1.8	3	5.36	6.68
144	20	5.7	1.2	0	7.61	5.55
144	20	5.7	1.8	0	7.61	5.14
144	20	6.3	1.2	0	8.09	8.40
144	20	6.3	1.8	0	8.09	7.49
144	12	5.7	1.2	0	3.33	3.91
144	12	5.7	1.8	0	3.33	3.73
144	12	6.3	1.2	0	3.70	4.92
144	12	6.3	1.8	0	3.70	5.0
Cheese						
24	20	6.5	1.1	5	8.35	6.87
24	20	6.4	1.1	3	7.20	6.94
24	20	6.2	1.1	0	5.46	6.08
24	12	6.2	1.1	5	7.10	7.36
24	12	6.1	1.1	3	5.31	6.55
24	12	6.0	1.1	0	2.64	3.24
72	20	6.5	1.1	5	10.34	7.75
72	20	6.4	1.1	3	9.43	8.2
72	20	6.2	1.1	0	8.03	8.78
72	12	6.2	1.1	5	8.37	7.16
72	12	6.1	1.1	3	6.81	7.16
72	12	6.0	1.1	0	4.51	6.74
144	20	6.5	1.1	5	6.36	7.96
144	20	6.4	1.1	3	8.83	8.05
144	20	6.2	1.1	0	5.66	8.26
144	12	6.2	1.1	5	4.05	7.3
144	12	6.1	1.1	3	2.93	7.09
144	12	6.0	1.1	0	1.26	7.81
Milk						
24	20	6.6		5	8.40	7.29
24	20	6.6		3	7.32	6.42
24	20	6.6		0	5.68	4.16
24	12	6.6		5	7.26	5.86
24	12	6.6		3	5.50	4.27
24	12	6.6		0	2.87	<1.00
72	20	6.6		5	10.42	8.11
72	20	6.6		3	9.59	7.63
72	20	6.6		0	8.35	7.06
72	12	6.6		5	8.62	7.26
72	12	6.6		3	7.12	6.06
72	12	6.6		0	4.88	5.47
144	20	6.6		5	6.39	8.15
144	20	6.6		3	5.95	8.11
144	20	6.6		0	5.29	8.04
144	12	6.6		5	3.61	7.48
144	12	6.6		3	2.51	6.94
144	12	6.6		0	0.85	6.57

Supplementary Table 2. Validation responses in BHI, Minas frescal cheese and UHT milk of the enterotoxin production by *S. aureus* ATCC 13565 at environmental conditions.

Time	Temperature	pH	NaCl	Inoculum	Pred.*	Obs.**
BHI						
24	20	5.7	1.2	5	0.98	1
24	20	5.7	1.8	5	0.98	1
24	20	6.3	1.2	5	0.97	1
24	20	6.3	1.8	5	0.97	1
24	12	5.7	1.2	5	0.04	0
24	12	5.7	1.8	5	0.04	0
24	12	6.3	1.2	5	0.03	0
24	12	6.3	1.8	5	0.03	0
24	20	5.7	1.2	3	0.94	1
24	20	5.7	1.8	3	0.94	1
24	20	6.3	1.2	3	0.91	1
24	20	6.3	1.8	3	0.91	1
24	12	5.7	1.2	3	0.01	0
24	12	5.7	1.8	3	0.01	0
24	12	6.3	1.2	3	0.00	0
24	12	6.3	1.8	3	0.00	0
24	20	5.7	1.2	0	0.76	0
24	20	5.7	1.8	0	0.76	0
24	20	6.3	1.2	0	0.69	0
24	20	6.3	1.8	0	0.69	0
24	12	5.7	1.2	0	0.00	0
24	12	5.7	1.8	0	0.00	0
24	12	6.3	1.2	0	0.00	0
24	12	6.3	1.8	0	0.00	0
72	20	5.7	1.2	5	1.00	1
72	20	5.7	1.8	5	1.00	1
72	20	6.3	1.2	5	1.00	1
72	20	6.3	1.8	5	1.00	1
72	12	5.7	1.2	5	0.84	1
72	12	5.7	1.8	5	0.84	1
72	12	6.3	1.2	5	0.84	1
72	12	6.3	1.8	5	0.84	1
72	20	5.7	1.2	3	1.00	1
72	20	5.7	1.8	3	1.00	1
72	20	6.3	1.2	3	1.00	1
72	20	6.3	1.8	3	1.00	1
72	12	5.7	1.2	3	0.12	1
72	12	5.7	1.8	3	0.12	0
72	12	6.3	1.2	3	0.12	0
72	12	6.3	1.8	3	0.12	1
72	20	5.7	1.2	0	1.00	1
72	20	5.7	1.8	0	1.00	1
72	20	6.3	1.2	0	1.00	1
72	20	6.3	1.8	0	1.00	1
72	12	5.7	1.2	0	0.00	0
72	12	5.7	1.8	0	0.00	0
72	12	6.3	1.2	0	0.00	0
72	12	6.3	1.8	0	0.00	0
144	20	5.7	1.2	5	1.00	1
144	20	5.7	1.8	5	1.00	1
144	20	6.3	1.2	5	1.00	1
144	20	6.3	1.8	5	1.00	1
144	12	5.7	1.2	5	1.00	1
144	12	5.7	1.8	5	1.00	1
144	12	6.3	1.2	5	1.00	1

144	12	6.3	1.8	5	1.00	1
144	20	5.7	1.2	3	1.00	1
144	20	5.7	1.8	3	1.00	1
144	20	6.3	1.2	3	1.00	1
144	20	6.3	1.8	3	1.00	1
144	12	5.7	1.2	3	0.94	1
144	12	5.7	1.8	3	0.94	0
144	12	6.3	1.2	3	0.96	1
144	12	6.3	1.8	3	0.96	1
144	20	5.7	1.2	0	1.00	1
144	20	5.7	1.8	0	1.00	1
144	20	6.3	1.2	0	1.00	1
144	20	6.3	1.8	0	1.00	1
144	12	5.7	1.2	0	0.00	1
144	12	5.7	1.8	0	0.00	0
144	12	6.3	1.2	0	0.00	1
144	12	6.3	1.8	0	0.00	0
Cheese						
24	20	6.5	1.1	5	0.98	1
24	20	6.4	1.1	3	0.92	1
24	20	6.2	1.1	0	0.67	0
24	12	6.2	1.1	5	0.03	0
24	12	6.1	1.1	3	0.00	0
24	12	6.0	1.1	0	0.00	0
72	20	6.5	1.1	5	1.00	1
72	20	6.4	1.1	3	1.00	1
72	20	6.2	1.1	0	1.00	1
72	12	6.2	1.1	5	0.82	1
72	12	6.1	1.1	3	0.10	1
72	12	6.0	1.1	0	0.00	0
144	20	6.5	1.1	5	1.00	1
144	20	6.4	1.1	3	1.00	1
144	20	6.2	1.1	0	1.00	1
144	12	6.2	1.1	5	1.00	1
144	12	6.1	1.1	3	0.94	1
144	12	6.0	1.1	0	0.00	1
Milk						
24	20	6.6		5	0.98	1
24	20	6.6		3	0.95	1
24	20	6.6		0	0.80	0
24	12	6.6		5	0.05	0
24	12	6.6		3	0.01	0
24	12	6.6		0	0.00	0
72	20	6.6		5	1.00	1
72	20	6.6		3	1.00	1
72	20	6.6		0	1.00	0
72	12	6.6		5	0.92	1
72	12	6.6		3	0.22	0
72	12	6.6		0	0.00	0
144	20	6.6		5	1.00	1
144	20	6.6		3	1.00	1
144	20	6.6		0	1.00	0
144	12	6.6		5	1.00	1
144	12	6.6		3	0.98	0
144	12	6.6		0	0.00	0

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CONCLUSÕES GERAIS

Neste trabalho, altas contagens de *Staphylococcus* spp foram encontradas, dentre estes, *S. aureus* foram identificados, indicando más condições de higiene na produção de queijo Minas frescal. Além disso, os fatores temperatura, pH e concentração de NaCl foram analisados para servirem de entrada em modelos de crescimento e produção de SEA por *S. aureus*. Foi constatado que 20% das amostras estavam armazenadas inadequadamente em temperaturas acima do preconizado pela legislação. A partir dessas informações, modelos secundários e um modelo terciário foi criado para predizerem diferentes respostas de multiplicação e produção de SEA por *S. aureus* submetidos a situações reais de queijo Minas frescal. Todos os modelos foram adequados para descreverem os dados obtidos experimentalmente, demonstrando que estes podem ser utilizados para descreverem respostas de multiplicação e produção de SEA por *S. aureus* em queijo Minas frescal. Portanto, este trabalho demonstra a importância e flexibilidade de modelos preditivos em estudarem o comportamento microbiano e sua utilidade para a segurança dos alimentos.