

Universidade Federal da Grande Dourados
Faculdade de Ciências e Tecnologia – FACET
Programa de Pós-Graduação em Ciências e Tecnologia Ambiental

Manoel Armando Delgado Junior

**Desenvolvimento e caracterização de alimentos funcionais
enriquecidos com endósporos de *Alkalihalobacillus clausii***

Dourados - MS

2026

Manoel Armando Delgado Junior

**Desenvolvimento e caracterização de alimentos funcionais
enriquecidos com endósporos de *Alkalihalobacillus clausii***

Tese apresentada ao Programa de Pós-graduação em Ciências e Tecnologia Ambiental da Universidade Federal da Grande Dourados, como requisito parcial para obtenção do título de Doutor em Ciências e Tecnologia Ambiental, área de concentração em Ciência Ambiental.

Orientadora Prof.^a Dra. Kelly Cristina da Silva Brabes

Coorientadora Prof.^a Dra. Danielle Marques Vilela

Dourados - MS
2026

Dados Internacionais de Catalogação na Publicação (CIP).

D352d Delgado Junior, Manoel Armando

Desenvolvimento e caracterização de alimentos funcionais enriquecidos com endósporos de *Alkalihalobacillus clausii* [recurso eletrônico] / Manoel Armando Delgado Junior. -- 2026.

Arquivo em formato pdf.

Orientadora: Kelly Cristina da Silva Brabes.

Coorientadora: Danielle Marques Vilela.

Tese (Doutorado em Ciência e Tecnologia Ambiental)-Universidade Federal da Grande Dourados, 2026.

Disponível no Repositório Institucional da UFGD em:

<https://portal.ufgd.edu.br/setor/biblioteca/repositorio>

1. Chocolate. 2. Bala de goma. 3. Probiótico. 4. *Bacillus clausii*. I. Brabes, Kelly Cristina Da Silva. II. Vilela, Danielle Marques. III. Título.

Ficha catalográfica elaborada automaticamente de acordo com os dados fornecidos pelo(a) autor(a).

©Direitos reservados. Permitido a reprodução parcial desde que citada a fonte.



TERMO DE APROVAÇÃO

Após apresentação, arguição e apreciação pela banca examinadora, foi emitido o parecer **Aprovado**, para a tese intitulada: **Estudo do desenvolvimento e caracterização de alimentos funcionais enriquecidos com endósporo de *Alkalihalobacillus clausii***, de autoria de **Manoel Armando Delgado Junior**, apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia Ambiental da Universidade Federal da Grande Dourados.



Documento assinado digitalmente
KELLY CRISTINA DA SILVA BRABES
Data: 19/10/2026 09:17:54-0300
Verifique em <https://validar.iti.gov.br>

Prof. Dr^a. Kelly Cristina da Silva Brabes
Presidente da Banca Examinadora



Documento assinado digitalmente
MARICY RAQUEL LINDENBAH BONFA
Data: 16/10/2026 17:37:45-0300
Verifique em <https://validar.iti.gov.br>

Prof. Dr^a. Maricy Raquel Lindenbah Bonfá
Membro Examinador (UFGD)



Documento assinado digitalmente
FABIO JULIANO NEGRAO
Data: 16/10/2026 17:18:13-0300
Verifique em <https://validar.iti.gov.br>

Prof. Dr. Fabio Juliano Negrão
Membro Examinador (UFGD)



Documento assinado digitalmente
PRISCILLA NARCISO JUSTI CAMARGO
Data: 19/10/2026 09:09:35-0300
Verifique em <https://validar.iti.gov.br>

Prof. Dr^a. Priscilla Narciso Justi Camargo
Membro Examinador (UFGD)



Documento assinado digitalmente
MARIA FERNANDA DE CASTRO BURBARELLI
Data: 17/10/2026 12:16:28-0300
Verifique em <https://validar.iti.gov.br>

Prof. Dr^a. Maria Fernanda de Castro Burbarelli
Membro Examinador (Faculdade Anhanguera)

Dourados/MS, 09 de outubro de 2026.

Dedico esta tese a quem me ensinou a sonhar e a realizar. Aos meus pais, pelo amor incondicional e apoio constante.

Agradecimentos

A Deus, pela presença constante em minha vida, por me fortalecer nos momentos de dificuldade e por tornar possível cada conquista alcançada.

Aos meus pais, que sempre foram meu exemplo de vida. Pelo amor incondicional, pelo carinho, pela compreensão e por todo o esforço dedicado ao meu crescimento pessoal e acadêmico. Sou imensamente grato por tudo que fizeram e continuam fazendo por mim.

À minha orientadora, Professora Doutora Kelly Cristina da Silva Brabes, pela atenção, paciência, confiança, incentivo, ensinamentos e amizade, fundamentais para a realização deste trabalho.

À minha coorientadora, Professora Doutora Danielle Marques Vilela, pela disponibilidade, orientação e contribuições valiosas durante esta trajetória acadêmica.

Ao meu esposo, Gilmar Alves de Holanda, pela parceria, apoio e incentivo ao longo dessa caminhada. Obrigado por estar sempre ao meu lado, oferecendo amor e força. Te amo!

Às minhas estagiárias e colegas de laboratório, Elizete Afonso e Claudini Kumakura, por terem me auxiliado nessa pesquisa e trajetória.

Às minhas colegas e amigas de laboratório, Débora Brait, Lujan Sanabria e Alexandra Vila Maior, pela ajuda nos experimentos, pela parceria, amizade e pelos ensinamentos compartilhados, que tornaram esta jornada mais leve e enriquecedora.

Às técnicas Priscilla Narciso Justi, Lígia Boarin Alcalde e Carolina Aparecida Antunes Amadeu pela ajuda e pela transmissão de conhecimentos nas análises físico-químicas.

E, por fim, a todos que, de alguma forma, contribuíram para a realização deste trabalho, minha sincera gratidão.

Descobrir consiste em olhar para o que todo mundo está vendo e pensar uma coisa diferente.

Roger Von Oech

Resumo

A indústria de alimentos funcionais está em rápido crescimento, impulsionada pelo interesse por produtos associados à promoção da saúde, sobretudo aqueles contendo probióticos. Probióticos são microrganismos vivos que, quando administrados em quantidades adequadas, podem conferir benefícios à saúde do hospedeiro. Nesse contexto, microrganismos formadores de endósporos, como *Alkalihalobacillus clausii*, apresentam características de interesse tecnológico, incluindo resistência térmica, estabilidade durante o processamento e maior tolerância às condições do trato gastrointestinal. Este trabalho teve como objetivo desenvolver e caracterizar chocolate e bala de goma enriquecidos com endósporos de *A. clausii*, vitaminas e sais minerais. As formulações foram elaboradas em parceria com uma farmácia de manipulação, e a viabilidade dos endósporos foi avaliada quinzenalmente durante quatro meses. As matrizes foram caracterizadas quanto às propriedades físico-químicas, pH, acidez titulável, cor, textura, comportamento térmico, qualidade microbiológica e manutenção da viabilidade após exposição a condições simuladas do trato gastrointestinal. Os resultados demonstraram a viabilidade tecnológica da incorporação dos endósporos de *A. clausii* nas duas matrizes, com manutenção da viabilidade microbiana durante 120 dias de armazenamento e após a exposição às condições simuladas do trato gastrointestinal. As análises físico-químicas, de textura e de cor indicaram que a incorporação dos endósporos, vitaminas e sais minerais não comprometeu significativamente as propriedades tecnológicas avaliadas. As formulações também apresentaram qualidade microbiológica adequada e valores energéticos semelhantes entre os tratamentos. Conclui-se que o chocolate e a bala de goma desenvolvidos apresentam viabilidade tecnológica para a incorporação de endósporos de *A. clausii*, constituindo alternativas não lácteas para o desenvolvimento de alimentos funcionais contendo probióticos e apresentando potencial para futuras aplicações em escala industrial.

Palavras-chave: Chocolate, Bala de Goma, Probiótico. *Bacillus clausii*.

Abstract

The functional food industry is experiencing rapid growth, driven by increasing interest in products associated with health promotion, particularly those containing probiotics. Probiotics are live microorganisms that, when administered in adequate amounts, may confer health benefits to the host. In this context, endospore-forming microorganisms, such as *Alkalihalobacillus clausii*, exhibit technological characteristics of interest, including thermal resistance, stability during processing, and greater tolerance to gastrointestinal conditions. This study aimed to develop and characterize chocolate and gummy candy enriched with *A. clausii* endospores, vitamins, and minerals. The formulations were developed in partnership with a compounding pharmacy, and endospore viability was evaluated every 15 days over a four-month period. The matrices were characterized in terms of physicochemical properties, pH, titratable acidity, color, texture, thermal behavior, microbiological quality, and maintenance of viability following exposure to simulated gastrointestinal conditions. The results demonstrated the technological feasibility of incorporating *A. clausii* endospores into both matrices, with maintenance of microbial viability during 120 days of storage and following exposure to simulated gastrointestinal conditions. The physicochemical, texture, and color analyses indicated that the incorporation of endospores, vitamins, and minerals did not significantly compromise the evaluated technological properties. The formulations also exhibited adequate microbiological quality and similar energy values among treatments. It is concluded that the chocolate and gummy candy developed exhibit technological feasibility for the incorporation of *A. clausii* endospores, constituting non-dairy alternatives for the development of probiotic-containing functional foods and showing potential for future industrial-scale applications.

Keywords: Chocolate, Gummy Candy, Probiotic, *Bacillus clausii*.

Lista de Tabelas

Table 1. Viability of *A. clausii* (log CFU/g) in 5.5 g of chocolate during 120 days of storage.....30

Table 2. Physicochemical parameters of the different chocolate formulations.....32

Table 3. pH and titratable acidity of chocolate formulations.....34

Table 4. Colorimetric analysis (L*, a*, b*) of chocolate formulations.....35

Table 5. Viable counts of *A. clausii* endospores (log CFU/g) after exposure to simulated *in vitro* gastrointestinal tract (GIT) conditions.....39

Table 1 – Viability of *A. clausii*, expressed as log CFU/g, over 4 months of storage.....53

Table 2 – Physicochemical parameters of the different gummy candy formulations.....54

Table 3 – pH values and titratable acidity of gummy candies.....56

Table 4 – Colorimetric parameters (L, a*, b*) of the gummy candy formulations.....57

Table 5 – *A. clausii* endospore counts, expressed as log CFU/g, after exposure to *in vitro* GIT conditions.....60

Sumário

INTRODUÇÃO	11
Capítulo 1: Referencial Teórico	13
1.1 Alimentos Funcionais.....	13
1.2 Probiótico aplicado em alimentos	14
1.3 Chocolate probiótico	16
1.4 Bala de goma probiótica.....	17
1.5 <i>Alkalihalobacillus clausii</i>	18
Capítulo 02 – Artigo Científico 1	23
Development of a Lactose-Free Probiotic Chocolate Supplemented with <i>Alkalihalobacillus clausii</i> Endospores	25
1. Introduction	25
2. Materials and Methods	26
2.1 Production of Probiotic Chocolate.....	26
2.2 Viability of <i>A. clausii</i>	28
2.3 Physicochemical Characterization of Chocolate	28
2.4 pH and Titratable Acidity	28
2.5 Color Measurement.....	28
2.6 Shear Force Analysis.....	29
2.7 Calorimetric Analysis	29
2.8 Microbiological Quality Analysis.....	29
2.9 Simulated Gastrointestinal Tract Conditions.....	30
2.10 Statistical Analysis.....	30
3. Results and Discussion	30
Viability of <i>A. clausii</i>	30
Physicochemical Characterization of Chocolate	31
pH and Titratable Acidity	34
Color Analysis.....	35
Shear Force Analysis.....	36
Calorimetric Analysis	37
Microbiological Quality	38
<i>In Vitro</i> Simulated Gastrointestinal Tract	38
4. Conclusion.....	40

Referências.....	42
Capítulo 03 – Artigo Científico 2	45
LWT.....	45
Innovation in Gummy Candies: Supplementation with <i>Alkalihalobacillus clausii</i> Endospores for Probiotic Potential.....	46
1. Introduction	46
2. Materials and Methods	47
2.1. Production of Probiotic Gummy Candies	47
2.2. Viability of <i>A. clausii</i>	49
2.3. Physicochemical Characterization of the Gummy Candies	49
2.4. Determination of pH and Titratable Acidity	50
2.5. Colorimetric Analysis	50
2.6. Shear Analysis	50
2.7. Calorimetric Analysis	50
2.8. Microbiological Quality Analysis.....	51
2.9. In Vitro Simulated Gastrointestinal Tract.....	51
2.10. Statistical Analysis.....	52
3. Results and Discussion	52
Viability of <i>A. clausii</i>	52
Physicochemical Characterization.....	54
Determination of pH and Titratable Acidity	56
Colorimetric Analysis	57
Shear Analysis	58
Calorimetric Analysis	59
Microbiological Quality Analysis.....	60
In Vitro Simulated Gastrointestinal Tract.....	60
4. Conclusion.....	61
Referências.....	63
Conclusão	66

INTRODUÇÃO

Os alimentos funcionais apresentam diferentes definições; entretanto, sabe-se que, além de fornecer nutrientes básicos para o corpo, são capazes de oferecer benefícios adicionais à saúde quando consumidos regularmente como parte de uma dieta equilibrada (Gomes *et al.*, 2024). Dentro desse amplo universo de alimentos funcionais, os probióticos se destacam como um dos grupos mais estudados, devido ao impacto positivo que exercem na microbiota intestinal e, conseqüentemente, na saúde geral do organismo, sendo frequentemente investigados por seu potencial de auxiliar na superação de diversos desafios atrelados à saúde (Paula *et al.*, 2025).

De acordo com a Organização Mundial da Saúde (OMS) e a Organização das Nações Unidas para a Alimentação e a Agricultura (FAO), os probióticos são definidos como “microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro” (FAO/OMS, 2001).

Atualmente, a maioria dos produtos que contêm cepas bacterianas probióticas é constituída por produtos lácteos fermentados e devido à crescente prevalência de intolerância à lactose na população, o desenvolvimento de novos produtos enriquecidos com probióticos está se tornando especialmente relevante, pois pode se tornar uma alternativa atraente para consumidores que limitam o consumo de laticínios devido a condições de saúde individuais (Tuigunov *et al.*, 2025). Nesse contexto, o desenvolvimento de alimentos não lácteos, como chocolate e bala de goma, apresenta-se como uma alternativa promissora para a incorporação de probióticos.

O chocolate, além do fornecimento de energia, parece estar relacionado principalmente ao seu potencial de proporcionar prazer sensorial e emoções positivas às pessoas (Cao *et al.*, 2025), além de diversos efeitos benéficos para a saúde, associados aos compostos presentes no cacau (Nascimento *et al.*, 2025), o pH mais alto, o teor de lipídios, que pode atuar como tampão e auxiliar na proteção de microrganismos probióticos contra condições adversas do processo de fabricação e do trânsito gastrointestinal (Achi *et al.*, 2024).

As balas de goma são um produto de confeitaria amplamente apreciado, atraindo um público diversificado, desde crianças até adultos, devido à sua textura mastigável (Maringka; Putra; Lo, 2024) e são veículos promissores para probióticos, pois são fáceis de consumir e podem ser formuladas com uma ampla gama de ingredientes ativos (Nascimento *et al.*, 2025).

No entanto, criar alimentos funcionais que preservem a viabilidade e a eficácia dos probióticos durante o processamento e o armazenamento é um desafio, já que a atividade da

água, a temperatura de armazenamento, a umidade e o tempo podem impactar significativamente a viabilidade dos probióticos ao longo da vida útil de um produto (Payne *et al.*, 2025). Dessa forma, o uso de endósporos como probióticos é vantajoso, pois são extremamente estáveis e podem sobreviver indefinidamente na ausência de água e nutrientes e na presença de produtos químicos tóxicos, enzimas líticas e condições extremas de temperatura e pH e sua estabilidade e resistência podem garantir uma vida útil extremamente longa para o produto (Corona *et al.*, 2025).

Dentre os probióticos formadores de endósporos, *Alkalihalobacillus clausii* (antigo *Bacillus clausii*), destaca-se por ser uma bactéria Gram-positiva, não patogênica, capaz de colonizar o intestino mesmo após tratamento contínuo com antibióticos (Pirozzi *et al.*, 2023), pois possui resistência completa a alguns macrolídeos (eritromicina, azitromicina, claritromicina, espiramicina), lincosamidas (clindamicina, lincomicina) e ao metronidazol, portanto, essa cepa tem sido usada rotineiramente por mais de 60 anos para restaurar a microbiota intestinal durante tratamentos com antibióticos (Wong-Chew *et al.*, 2024).

Diante disso, este trabalho tem como objetivo desenvolver e caracterizar chocolate e bala de goma enriquecidos com endósporos de *A. clausii*, vitaminas e sais minerais, bem como avaliar a viabilidade dos endósporos ao longo de quatro meses de armazenamento. Além dos parâmetros físico-químicos, o pH, a acidez titulável, a cor, a textura, a calorimetria, a qualidade microbiológica e a resistência dos endósporos às condições simuladas do trato gastrointestinal.

Capítulo 1: Referencial Teórico

1.1 Alimentos Funcionais

Na última década, apesar da crescente popularidade de alimentos orgânicos e naturais, os alimentos funcionais despertaram um interesse significativo do público (Vignesh *et al.*, 2024), devido ao aumento de ocorrência de doenças crônicas e ao crescente interesse da população por uma alimentação mais saudável (Herdiana, 2023). Nesse contexto, os alimentos funcionais têm conquistado um destaque cada vez maior, não apenas no consumo cotidiano, mas também como objeto central de diversas pesquisas científicas e iniciativas da indústria alimentícia (Paula *et al.*, 2025).

Alimentos funcionais são definidos como aqueles que conferem benefícios à saúde, bem como ao bem-estar físico e mental, que se estendem além do fornecimento de nutrientes essenciais (Khani *et al.*, 2025). Esses alimentos não se limitam a fornecer apenas as propriedades nutricionais básicas, destacam-se por proporcionarem benefícios adicionais à saúde, desempenhando um papel relevante na prevenção de doenças e na promoção de uma melhor qualidade de vida (Paula *et al.*, 2025).

Em 1980, o Japão introduziu o conceito de alimentos funcionais, posteriormente associado à categoria de Alimentos para Uso Especificado em Saúde (FOSHU, do inglês *Foods for Specified Health Uses*). Regulamentados pelo Ministério da Saúde, Trabalho e Bem-Estar do Japão, esses produtos são autorizados a apresentar alegações específicas de benefícios à saúde decorrentes de seu consumo (Ma; Fu; Lee, 2025).

De acordo com a Comissão Europeia, Functional Food Science in Europe (FuFoSE), um alimento pode ser considerado funcional se demonstrar influência benéfica em uma ou mais funções-alvo do organismo além de seu valor nutricional. Esses benefícios incluem melhorias na saúde e no bem-estar e/ou redução do risco de doenças (Aili *et al.*, 2024).

Já no Brasil, alimentos funcionais são definidos como todo alimento ou ingrediente que, além das funções nutricionais básicas, produz efeitos metabólicos e/ou fisiológicos e/ou efeitos benéficos à saúde, devendo ser seguro para consumo sem supervisão médica por meio da Portaria nº 398, de 30 de abril de 1999, da Secretaria de Vigilância Sanitária do Ministério da Saúde do Brasil (Brasil, 1999).

A Agência Nacional de Vigilância Sanitária (ANVISA), por meio da RDC nº 02, de 07 de janeiro de 2002, estabelece que as alegações de propriedades funcionais e de saúde estão

relacionadas com produtos que contenham ácidos graxos, carotenoides, fibras, fitoesteróis, polióis, probióticos e proteína de soja.

Juntamente com a definição de alimentos funcionais, os principais critérios de inclusão para uma determinada alegação funcional de um ingrediente ou alimento em um rótulo alimentar são a segurança alimentar, o livre acesso, sem necessidade de prescrição médica (ou aconselhamento médico), e a evidência de benefícios à saúde quando consumidos regularmente em uma dieta balanceada (Granato *et al.*, 2020).

No Brasil, a Comissão Técnico-Científica de Assessoramento em Alimentos Funcionais e Novos Alimentos (CTCAF), por meio da Resolução da Diretoria Colegiada (RDC) nº 02 de 07 de janeiro de 2002, que aprova o regulamento técnico de substâncias bioativas e probióticas isoladas com alegação de propriedade funcional ou de saúde (Brasil, 2002) estabelece que os alimentos de competência da ANVISA que veiculem essas alegações devem ser enquadrados e registrados na categoria de alimentos com alegações de propriedades funcionais ou de saúde conforme a Resolução nº. 19, de 30 de abril de 1999 (Brasil, 1999).

A indústria de alimentos funcionais está em rápido desenvolvimento em todo o mundo, e o desenvolvimento e a utilização de alimentos funcionais estão recebendo cada vez mais atenção (Su *et al.*, 2022). Isso denota o crescente interesse pelos consumidores de alimentos saudáveis, com destaque para o potencial dos alimentos probióticos, devido ao seu efeito do perfil metabólico significativo para a manutenção da saúde intestinal e na prevenção de doenças (Gomes *et al.*, 2024).

1.2 Probiótico aplicado em alimentos

Um dos componentes que um alimento funcional pode incluir são os probióticos (Colmenares-Cuevas *et al.*, 2024), derivados das palavras gregas “para a vida”, referem-se a cepas nativas de saúde, definidas como “microrganismos vivos que, se administrados em doses suficientes, podem regular as funções biológicas do hospedeiro e ter um efeito benéfico na saúde” (Cheng *et al.*, 2025).

De acordo com a OMS (Organização Mundial da Saúde) e a FAO (Organização das Nações Unidas para a Alimentação e a Agricultura), os probióticos são “microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro” (FAO/OMS, 2001).

Os probióticos são frequentemente considerados uma alternativa segura e de baixo custo para tratar uma variedade de doenças crônicas e melhorar a saúde humana em geral (Colmenares-Cuevas *et al.*, 2024), em particular, oferecem inúmeros benefícios, incluindo proteção contra microrganismos patogênicos, hipertensão, inflamação e diabetes (Peruzzolo *et al.*, 2025).

Além disso, os probióticos são reconhecidos por sua capacidade de modular a imunidade do hospedeiro e proteger contra diversas patologias infecciosas e não infecciosas (Colmenares-Cuevas *et al.*, 2024) e sua capacidade única de aderir às células epiteliais intestinais auxilia no equilíbrio da microbiota intestinal e na digestão, além de oferecer potenciais propriedades anticancerígenas (Peruzzolo *et al.*, 2025).

No entanto, para proporcionar esses benefícios, os probióticos devem sobreviver às diferentes etapas de processamento, condições de armazenamento e durante a passagem pelo trato gastrointestinal, como a exposição ao ácido gástrico, sais biliares e enzimas digestivas, em quantidades suficientes para colonizar o intestino e exercer seus efeitos benéficos, mesmo que temporariamente (Cozentino *et al.*, 2022; Peruzzolo *et al.*, 2025).

No Brasil, a Agência Nacional de Vigilância Sanitária (ANVISA) estabelece que a quantidade mínima viável deve ser de 8,0 a 9,0 log de UFC/ingestão na recomendação diária do produto pronto para consumo, podendo ser aceitos valores menores caso sua eficácia seja comprovada (Zoldan *et al.*, 2023).

O interesse crescente pelo uso de probióticos impulsionou a pesquisa e o desenvolvimento de produtos inovadores enriquecidos com probióticos (Peruzzolo *et al.*, 2025); dessa forma, são usados em uma ampla gama de indústrias, desde produtos tradicionais até produtos de ponta, para melhorar o equilíbrio dos microrganismos intestinais, aumentar a função imunológica e até mesmo ajudar a aliviar os sintomas de certas doenças (Cheng *et al.*, 2025).

Além dos alimentos fermentados tradicionais, produtos não fermentados com probióticos adicionados, como barras energéticas, sucos e cereais matinais, já estão disponíveis comercialmente. O desenvolvimento desses produtos não apenas atende à demanda do consumidor por alimentos saudáveis, mas também impulsiona a inovação na indústria alimentícia (Cheng *et al.*, 2025).

Existem dois métodos principais para incorporar probióticos em alimentos: eles podem ser cultivados no produto final por meio de fermentação ou adicionados diretamente ao produto alimentício antes do processamento. A incorporação de probióticos adicionando-os diretamente aos alimentos é desafiadora, pois esses probióticos devem enfrentar condições adversas durante a produção e após o consumo. Eles precisam sobreviver não apenas no trato gastrointestinal, mas também em vários processos de produção e condições de armazenamento (Payne *et al.*, 2024).

Dessa forma, o desenvolvimento de alimentos probióticos não lácteos apresenta desafios relacionados à manutenção da viabilidade dos microrganismos, especialmente em produtos armazenados sem refrigeração. Essa viabilidade pode ser influenciada por condições físico-químicas e ambientais, como temperatura, pH, disponibilidade de oxigênio e água, exposição à luz e disponibilidade de nutrientes, além de fatores relacionados à matriz alimentar, como a presença de microrganismos concorrentes e compostos antimicrobianos. Essas condições podem afetar tanto os atributos sensoriais do produto final (aroma, textura e sabor) quanto a resistência dos probióticos às condições ácidas e à bile durante a passagem pelo trato gastrointestinal, influenciando sua funcionalidade (Arratia-Quijada *et al.*, 2024).

1.3 Chocolate probiótico

O chocolate é um alimento popular altamente desejável, apesar do amargor e da adstringência notáveis, especialmente em chocolates com alto teor de cacau (Loi *et al.*, 2025) e de acordo com RDC nº 264, de 22 de setembro de 2005, entende-se por chocolate o produto obtido a partir da mistura de derivados de cacau (*Theobroma cacao* L.), massa (ou pasta ou liquor) de cacau, cacau em pó e ou manteiga de cacau, com outros ingredientes, contendo, no mínimo, 25 % (g/100 g) de sólidos totais de cacau (Brasil, 2005).

A popularidade desse alimento, além do fornecimento de energia, parece estar relacionada principalmente ao seu potencial de proporcionar prazer sensorial e emoções positivas às pessoas, desta forma, tem alto potencial para uso no desenvolvimento de alimentos funcionais no futuro (Cao *et al.*, 2025).

O chocolate possui diversos efeitos benéficos para a saúde, associados aos compostos presentes no cacau com propriedades antioxidantes, antiproliferativas, anti-inflamatórias e anticancerígenas, além de diversos fitoquímicos, incluindo polifenóis (Nascimento *et al.*, 2025).

Por exemplo, o cacau é rico em flavonoides, que são compostos que reduzem o risco de doenças cardiovasculares, diminuindo a pressão arterial e melhorando a funcionalidade dos vasos sanguíneos e seus produtos são boas fontes de antioxidantes naturais (Raji *et al.*, 2025).

Dentre as principais propriedades benéficas do chocolate, destaca-se sua capacidade antioxidante, a partir da presença de compostos fenólicos em altos níveis no cacau, que podem desempenhar um importante papel no retardo do estresse oxidativo em probióticos levando à maior viabilidade e ao prolongamento da vida útil do produto (Zoldan *et al.*, 2023).

Além do pH mais alto, o teor de lipídios/gorduras do chocolate atua como tampão e auxilia na proteção de microrganismos probióticos contra condições adversas no processo de fabricação e no trânsito gastrointestinal (Achi *et al.*, 2024). Ademais, vários parâmetros, incluindo a atividade da água, a disponibilidade de oxigênio e a temperatura, demonstraram desempenhar papel no desenvolvimento de chocolate com probiótico (Islam, Masum; Harun-ur-Rashid; 2022).

Diante disso, a introdução de chocolate enriquecido com probióticos na dieta corresponde às tendências modernas em alimentação saudável e medicina preventiva, já que a maioria dos produtos que contêm cepas bacterianas probióticas são produtos lácteos fermentados, e devido à crescente prevalência de intolerância à lactose na população, a criação de chocolate contendo culturas probióticas pode se tornar uma alternativa atraente para consumidores que limitam o consumo de laticínios devido às condições de saúde individuais (Tuigunov *et al.*, 2025).

1.4 Bala de goma probiótica

As balas de goma são um produto de confeitaria amplamente apreciado, atraindo um público diversificado, desde crianças até adultos, devido à sua textura mastigável (Maringka; Putra; Lo, 2024) e a uma variedade de formas, aromas e cores apresentáveis (Cebin *et al.*, 2024).

As balas de goma podem ser consideradas um complexo gelatinoso que consiste em agentes gelificantes, açúcares, água e diferentes componentes menores. Essa formulação peculiar as torna matrizes ideais para a inclusão e retenção de compostos de alto valor agregado (Boninsegna *et al.*, 2024).

As balas de goma têm um lugar significativo no setor de confeitaria e são um grupo de produtos importante em termos de transporte de componentes funcionais, pois são frequentemente consumidas por adultos e especialmente crianças (Kahraman *et al.*, 2023),

contudo o conceito de balas funcionais seja relativamente novo e ainda em desenvolvimento, a existência de confeitos de açúcar para combater tosse, dor de garganta, mau hálito e náusea é conhecida há muito tempo (Cebin *et al.*, 2024).

Formulações convencionais de balas e confeitos apresentam baixos valores nutricionais, no entanto, as balas de goma são veículos promissores para diversos nutrientes, pois são fáceis de consumir e podem ser formuladas com uma ampla gama de ingredientes ativos, que podem auxiliar na saúde imunológica (vitaminas e minerais), na saúde cardíaca (ômega-3) e na saúde intestinal como os probióticos (Nascimento *et al.*, 2023).

1.5 *Alkalihalobacillus clausii*

Bacillus clausii, agora denominado *Alkalihalobacillus clausii*, é uma bactéria Gram-positiva, formadora de esporos, não patogênica, capaz de colonizar o intestino mesmo após tratamento contínuo com antibióticos, sendo extremamente estável e resistente, o esporo pode sobreviver ao ambiente ácido do estômago e então atingir o intestino (Pirozzi *et al.*, 2023), como já comprovado por Delgado Junior, Vilela e Brabes (2024) ao avaliarem a viabilidade de esporos de *Bacillus clausii* em simulação do trato gastrointestinal humano, notou-se que os esporos foram capazes de sobreviver as condições semelhantes às do TGI sem alterações significativas nas análises.

Há mais de 55 anos, disponível no mercado e se distingue pela presença de quatro cepas probióticas, incluindo O/C, SIN, N/R e T, sendo essas cepas combinadas em um produto probiótico que exhibe altos níveis de resistência a poliantibióticos, garantindo sua eficácia mesmo quando usadas em conjunto com tratamento com antibióticos (Dang *et al.*, 2024).

Desde 1999, têm sido frequentemente comercializadas sob o nome comercial de *Enterogermina* como suplementos de venda livre. Cepas foram extraídas principalmente de um único grupo resistente à penicilina, denominado *B. subtilis* ATCC 9799, e foram posteriormente reintroduzidas como *B. clausii* e atualmente como *Alkalihalobacillus clausii* (Sadrimovahed, Ulusoy; 2024).

Dentre os benefícios fisiológicos observados, a bactéria auxilia na regeneração da mucosa intestinal e na correção de carências vitamínicas (Sadrimovahed; Ulusoy, 2024). Essa ação é viabilizada pela produção de vitamina B2 (riboflavina), fortalecimento da barreira epitelial (via adesão, secreção de mucina e menor permeabilidade) e síntese de peptídeos

antimicrobianos como a clausina, cujas propriedades imunomoduladoras mitigam os danos da antibioticoterapia (Abreu *et al.*, 2025).

Sobretudo, o benefício de maior relevância dessa bactéria reside na sua resistência aos antibióticos. Trata-se de uma propriedade única que não pode ser adquirida, acumulada ou transferida horizontalmente para outros microrganismos do ambiente intestinal (Sadrimovahed; Ulusoy, 2024).

Em comparação com outras espécies probióticas, *A. clausii* tem uma vantagem devido à sua capacidade de formar endósporos resistentes a estressores ambientais, como calor, pH gástrico ácido e bile, sendo que essas características permitem que sobrevivam melhor nas condições adversas do trato digestivo e cheguem aos intestinos de forma intacta, onde podem proporcionar benefícios à saúde (Dang *et al.*, 2024).

As formulações probióticas à base de endósporos apresentam uma longa vida útil e não requerem refrigeração durante o armazenamento. Além disso, os endósporos apresentaram maior estabilidade durante o processamento e armazenamento de alimentos funcionais, como doces, sucos e conservas de frutas, salsichas e massas em comparação com outros probióticos, como *Lactobacillus* spp (Khokhlova *et al.*, 2023).

Referências

- ABREU, A. T. *et al.* Effectiveness of *Bacillus clausii* (O/C, N/R, SIN, T) in the Prevention of Antibiotic-Associated Diarrhea and Gastrointestinal Symptoms: A Systematic Review. **Antibiotics**, v. 14, n. 5. 2025.
- ACHI, S. C. *et al.* Dark chocolate: delivery medium for probiotic *Bifidobacterium breve* NCIM 5671. **Journal of Food Science and Technology**, v. 61. 2024.
- AILI, Q. *et al.* Composing functional food from agro-forest wastes: Selectively extracting bioactive compounds using supercritical fluid extraction. **Food Chemistry**, v. 455. 2024.
- ARRATIA-QUIJADA, J. *et al.* Nano-encapsulation of probiotics: Need and critical considerations to design new non-dairy probiotic products. **Journal of Functional Foods**, v. 116. 2024.
- BONINSEGNA, M. A. *et al.* Sustainable use of coffee roasting by-products: development of high-value-added gummy candies. **Journal of Food Measurement & Characterization**, v. 18, n. 11. 2024
- BRASIL. Agência Nacional de Vigilância Sanitária (ANVISA). **Portaria nº 398, de 30 de abril de 1999**. Aprova o Regulamento Técnico que estabelece as diretrizes básicas para análise e comprovação de propriedades funcionais e/ou de saúde alegadas em rotulagem de alimentos. Diário Oficial da União: seção 1, Brasília, DF, 13 de maio de 1999.
- BRASIL. Agência Nacional de Vigilância Sanitária (ANVISA). **Resolução RDC nº 264, de 22 de setembro de 2005**. Aprova o Regulamento Técnico para produtos de cereais, amidos, farinhas e farelos. Diário Oficial da União: seção 1, Brasília, DF, 23 set. 2005.
- BRASIL. Agência Nacional de Vigilância Sanitária (ANVISA). **Resolução nº 19, de 30 de abril de 1999**. Aprova o Regulamento Técnico de procedimentos para registro de novos alimentos e/ou novos ingredientes. Diário Oficial da União: seção 1, Brasília, DF, 3 de maio de 1999.
- BRASIL. Agência Nacional de Vigilância Sanitária (ANVISA). **Resolução RDC nº 2, de 7 de janeiro de 2002**. Aprova o Regulamento Técnico de Substâncias Bioativas e Probióticos Isolados com Alegação de Propriedades Funcional e/ou de Saúde. Diário Oficial da União: seção 1, Brasília, DF, 9 jan. 2002.
- BRASIL. Agência Nacional de Vigilância Sanitária. **Resolução RDC nº 241, de 26 de julho de 2018**. Dispõe sobre os requisitos para comprovação da segurança e dos benefícios à saúde dos probióticos para uso em alimentos. Diário Oficial da União: seção 1, Brasília, DF, 27 jul. 2018.
- CAO, H. *et al.* Porous starch-probiotics encapsulation for 3D-printed chocolate. **Journal of Food Engineering**, v. 387. 2025.
- CEBIN, V. A. *et al.* Physicochemical and Sensory Stability Evaluation of Gummy Candies Fortified with Mountain Germander Extract and Prebiotics. **Polymers**, v. 16, n. 2. 2024.
- CHENG, J. *et al.* Advances in polysaccharide-based probiotic delivery systems: A review. **Carbohydrate Polymer Technologies and Applications**, v. 10. 2025.

- COLMENARES-CUEVAS, S. I. *et al.* Development and study of the functional properties of marshmallow enriched with bee (*Apis mellifera*) honey and encapsulated probiotics (*Lactobacillus rhamnosus*). **Frontiers in nutrition**, v. 11. 2024.
- CORONA, R. *et al.* Probiotic Spores of *Shouchella clausii* SF174 and Displayed Bromelain Show Beneficial Additive Potential. **International Journal of Molecular Sciences**, v. 26, n. 3. 2025.
- COZENTINO, I. S. C. *et al.* Development of a potentially functional chocolate spread containing probiotics and structured triglycerides. **LWT**, v. 154. 2022.
- DANG, H. T. *et al.* Promising clinical and immunological efficacy of *Bacillus clausii* spore probiotics for supportive treatment of persistent diarrhea in children. **Scientific Reports**, v. 14, n. 1. 2024.
- DELGADO JUNIOR, M. A.; VILELA, D. M.; BRABES, K. C. S. Avaliação da viabilidade de esporos de *Bacillus clausii* em simulação do trato gastrointestinal humano. **Revista Brasileira Multidisciplinar**, v. 27, n. 3. 2024.
- FAO/WHO (2001). **Report Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria**. Disponível em: < <http://www.fao.org/3/a-a0512e.pdf> >.
- GOMES, E. S. *et al.* Alimentos funcionais integrados com prebióticos e probióticos: uma revisão. **Observatório de la Economía Latinoamericana**, v. 22, n. 5. 2024.
- GRANATO, D. *et al.* Functional Foods: Product Development, Technological Trends, Efficacy Testing, and Safety. **Annual Review of Food Science and Technology**, v. 11, n. 1. 2020.
- HERDIANA, Y. Functional food in relation to Gastroesophageal Reflux Disease (GERD). **Nutrients**, v. 15, n. 16. 2023.
- ISLAM, M. Z.; MASUM, A. K. M.; HARUN-UR-RASHID, M. Milk chocolate matrix as a carrier of novel *Lactobacillus acidophilus* LDMB-01: Physicochemical analysis, probiotic storage stability and in vitro gastrointestinal digestion. **Journal of Agriculture and Food Research**, v. 7. 2022.
- KAHRAMAN, B. *et al.* Production and characterization of probiotic jelly candy containing *Bacillus* species. **Journal of Food Measurement and Characterization**, v. 17, n. 6. 2023.
- KHANI, N. *et al.* The role of functional foods based on probiotics in improving fertility: A review. **Journal of Functional Foods**, v. 129. 2025.
- KHOKHLOVA, E. *et al.* Immunomodulatory and Antioxidant Properties of a Novel Potential Probiotic *Bacillus clausii* CSI08. **Microorganisms**, v. 11, n. 2. 2023.
- LOI, C. *et al.* Olfaction modulates taste attributes in different types of chocolate. **Food Quality and Preference**, v. 132. 2025.
- MA, Z. F.; FU, C.; LEE, Y. Y. The Modulatory Role of Bioactive Compounds in Functional Foods on Inflammation and Metabolic Pathways in Chronic Diseases. **Foods**, v. 14, n. 5. 2025.

- MARINGKA, C. T; PUTRA, A. B. N; LO, D. Development of gummy candy with polydextrose, isomalto-oligosaccharides, fructo-oligosaccharides, and xylitol as sugar replacers. **International Journal of Gastronomy and Food Science**, v. 35. 2024.
- NASCIMENTO, M. B. *et al.* Functional and technological potential of by-products from the cocoa (*Theobroma cacao* L.) production chain. **Food Chemistry**, v. 493. 2025.
- PAULA, K. B. L. *et al.* Métodos de produção e regulamentação do kefir. **Revista Eletrônica Acervo Saúde**, v. 25. 2025.
- PAYNE, J. *et al.* Impact of relative humidity on *Bacillus* probiotic viability during storage in baked products. **Applied Food Research**, v. 5, n. 1. 2025.
- PAYNE, J. *et al.* The Potential of *Bacillus* Species as Probiotics in the Food Industry: A Review. **Foods**, v. 13, n. 15. 2024.
- PERUZZOLO, M. *et al.* Probiotics: health benefits, microencapsulation, and viability, combination with natural compounds, and applications in foods. **Food Bioscience**, v. 66. 2025.
- PIROZZI, C. *et al.* *Alkalihalobacillus clausii* (formerly *Bacillus clausii*) spores lessen antibiotic-induced intestinal injury and reshape gut microbiota composition in mice. **Biomedicine & Pharmacotherapy**, v. 163. 2023.
- RAJI, A. O. *et al.* Cocoa-fortified warankashi: Nutritional enhancement, antioxidant properties and storage stability. **Food Nutrition**, v. 1, n.1. 2025.
- SADRIMOVAHED, M; ULUSOY, B. H. *Bacillus clausii*: A Review into Story of Its Probiotic Success and Potential Food Applications. **Fermentation**, v. 10, n. 10. 2024.
- SU, Q. *et al.* Nano Functional Food: Opportunities, Development, and Future Perspectives. **International Journal of Molecular Sciences**, v. 24, n. 1. 2022.
- TUIGUNOV, D. *et al.* Functionalization of Chocolate: Current Trends and Approaches to Health-Oriented Nutrition. **Processes**, v. 13, n. 5. 2025.
- VIGNESH, A. *et al.* A review on the influence of nutraceuticals and functional foods on health. **Food chemistry advances**, v.5. 2024.
- WONG-CHEW, R. M. *et al.* Potential Complementary Effect of Zinc and *Alkalihalobacillus clausii* on Gut Health and Immunity: A Narrative Review. **Nutrients**, v. 16, n. 6. 2024.
- ZOLDAN, J. *et al.* Evaluation of viability to simulated gastrointestinal tract passage of probiotic strains and pioneer bioaccessibility analyses of antioxidants in chocolate. **Food Bioscience**, v. 52. 2023.

Capítulo 02 – Artigo Científico 1

Applied Food Research

Development of a lactose-free probiotic chocolate supplemented with *Alkalihalobacillus clausii* endospores

--Manuscript Draft--

Manuscript Number:	AFRES-D-26-02222R1
Full Title:	Development of a lactose-free probiotic chocolate supplemented with <i>Alkalihalobacillus clausii</i> endospores.
Article Type:	Full Length Article
Keywords:	functional foods; Food technology; <i>Bacillus clausii</i>
Corresponding Author:	Manoel Armando Delgado Junior, Mestre Great Dourados Federal University BRAZIL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Great Dourados Federal University
Corresponding Author's Secondary Institution:	
First Author:	Manoel Armando Delgado Junior, Mestre
First Author Secondary Information:	
Order of Authors:	Manoel Armando Delgado Junior, Mestre
	Elizete Alves Proença Afonso, Granduanda
	Claudini Ferreira Kumakura, Granduanda
	Danielle Marques Vilela, Doutora
	Kelly Cristina da Silva Brabes, Doutora
Order of Authors Secondary Information:	

Abstract:	<p>The development of probiotic chocolate aligns with current trends in functional foods and preventive nutrition. Chocolate is considered a promising vehicle for probiotics, as its high lipid content and relatively high pH offer protection against processing stress and gastrointestinal conditions. This study aimed to develop chocolate supplemented with <i>Alkalihalobacillus clausii</i> endospores, vitamins, and minerals, and to evaluate its probiotic viability, physicochemical characteristics, microbiological quality, texture, color, energy value, and resistance under simulated gastrointestinal conditions. Endospore viability was monitored every 15 days over a 120-day storage period. The supplemented chocolates maintained high probiotic viability throughout storage and under simulated gastrointestinal conditions. The incorporation of endospores, vitamins, and minerals did not affect the product's physicochemical, colorimetric, textural, microbiological, or energy characteristics. These results demonstrate that chocolate is a safe and effective vehicle for <i>A. clausii</i> endospores, preserving probiotic functionality while maintaining the product's technological quality.</p>
------------------	--

Development of a Lactose-Free Probiotic Chocolate Supplemented with *Alkalihalobacillus clausii* Endospores

The development of probiotic chocolate is aligned with current trends in functional foods and preventive nutrition. Chocolate is considered a promising carrier for probiotics because its high lipid content and relatively high pH may provide protection against processing stresses and gastrointestinal conditions. This study aimed to develop lactose-free chocolate supplemented with *Alkalihalobacillus clausii* endospores, vitamins, and minerals and to evaluate its probiotic viability, physicochemical characteristics, microbiological quality, texture, color, energy value, and behavior under simulated gastrointestinal conditions. Three formulations were developed: a chocolate control, chocolate supplemented with *A. clausii* endospores, and chocolate containing endospores combined with vitamins D3 and K2 and magnesium malate. Endospore viability was monitored every 15 days over 120 days of storage. The supplemented chocolates maintained high endospore viability throughout storage and under simulated gastrointestinal conditions. The incorporation of endospores, vitamins, and minerals did not adversely affect the physicochemical, colorimetric, textural, microbiological, or energetic characteristics of the product. These findings support the technological feasibility of using lactose-free chocolate as a food matrix for maintaining *A. clausii* endospore viability during storage and under simulated gastrointestinal conditions, while preserving the technological quality of the product.

Keywords: Functional foods; Food technology; *Bacillus clausii*.

1. Introduction

The development of functional foods reflects the growing consumer interest in healthy foods, highlighting the potential of probiotic foods due to their effects on the metabolic profile, which are significant for maintaining intestinal health and preventing diseases (Gomes *et al.*, 2024).

The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) define probiotics as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2001).

The incorporation of probiotic-enriched chocolate into the diet aligns with current trends in healthy nutrition and preventive medicine, since most products containing probiotic bacterial strains are fermented dairy products. Given the increasing prevalence of lactose intolerance in the population, the development of chocolate containing probiotic cultures may represent an attractive alternative for consumers who limit dairy consumption due to individual health conditions (Tuigunov *et al.*, 2025).

Furthermore, chocolate has a relatively high pH and a lipid/fat content that acts as a buffering agent, helping to protect probiotic microorganisms against adverse conditions

during both food processing and gastrointestinal transit (Achi *et al.*, 2024). Consequently, the potential use of chocolate as a probiotic carrier has become increasingly recognized (Islam; Masum; Harun-Ur-Rashid, 2022).

In this context, spore-forming bacteria have emerged as promising probiotic candidates because they are highly stable and can survive indefinitely in the absence of water and nutrients, while also resisting toxic chemicals, lytic enzymes, and extreme temperature and pH conditions. Therefore, the use of bacterial endospores as probiotics takes advantage of their remarkable stability and resistance, ensuring both an extended product shelf life and safe passage through the gastrointestinal tract (Corona *et al.*, 2025).

Among spore-forming probiotic bacteria, *Alkalihalobacillus clausii* (*A. clausii*) stands out as an aerobic, Gram-positive, rod-shaped, non-pathogenic bacterium capable of withstanding gastrointestinal conditions (Delgado Junior *et al.*, 2024), as well as resisting thermal treatments applied during pharmaceutical processing and the manufacture of functional foods (Sadrimovahed & Ulusoy, 2024).

In addition to probiotic incorporation, the fortification of foods with vitamins and mineral salts has been widely explored in the development of functional foods as an effective strategy to improve nutritional value and prevent micronutrient deficiencies (Ashraf, 2025). Nutrients such as vitamin D3, vitamin K2, and magnesium are directly involved in the regulation of bone metabolism, immune function, and essential enzymatic processes, and have frequently been associated with the prevention of chronic diseases and the strengthening of the immune system (Atieh *et al.*, 2025; Ashraf, 2025).

The present study aimed to develop a lactose-free chocolate containing *Alkalihalobacillus clausii* endospores and to evaluate probiotic viability during 120 days of storage and under simulated gastrointestinal conditions. Additionally, the influence of incorporating vitamins D3 and K2 and magnesium malate on probiotic viability was evaluated, as well as the physicochemical, microbiological, colorimetric, textural, and calorimetric characteristics of the formulations.

2. Materials and Methods

2.1 Production of Probiotic Chocolate

The chocolate was produced in partnership with a compounding pharmacy located in Dourados, Mato Grosso do Sul, Brazil.

In this study, three chocolate formulations were developed (Figure 1). Formulation 1 (F1) consisted of chocolate only and was used as the control. Formulation 2 (F2) consisted of chocolate enriched with *A. clausii* endospores. Formulation 3 (F3) consisted of chocolate supplemented with *A. clausii* endospores, vitamin D3, vitamin K2, and magnesium malate. The endospore concentrations were defined based on the amount of *Bacillus clausii* present in the commercial product Enterogermina®, which was used as a reference for probiotic incorporation.

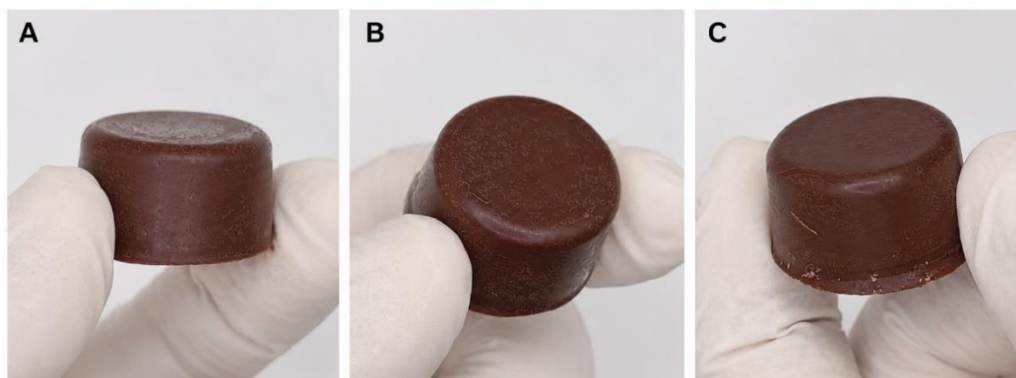
For the preparation of F1, the chocolate was melted, and approximately 5.5 g was distributed into individual molds. The molds were then refrigerated for 30 min and subsequently hermetically sealed.

F2 was prepared following the same procedure as F1. After the chocolate had cooled to approximately 45 °C, *A. clausii* endospores were added at a concentration of 2×10^9 CFU (9.3 log CFU). The mixture was homogenized, transferred to the molds, refrigerated, and subsequently hermetically sealed.

F3 was prepared similarly to F2. After the chocolate had cooled to approximately 45 °C, 1×10^9 CFU of *A. clausii* endospores, 400 IU of vitamin D3 (1:1000 dilution), 50 µg of vitamin K2 (1:10 dilution), and 50 mg of magnesium malate were added. The mixture was then homogenized, distributed into the molds, refrigerated for 30 min, and hermetically sealed.

The samples were stored under refrigeration at 4 °C until the physicochemical and microbiological analyses were performed.

Figure 1. Visual appearance of the developed functional chocolate formulations.



A – Control formulation (F1), without probiotic supplementation; B – F2 formulation, supplemented with *Alkalihalobacillus clausii* endospores; and C – F3 formulation, supplemented with *A. clausii* endospores, vitamin D3, vitamin K2, and magnesium malate.

2.2 Viability of *A. clausii*

The viability of *Alkalihalobacillus clausii* was evaluated on the first day of storage and subsequently every 15 days over a period of four months. Serial decimal dilutions were prepared, and viable cells were enumerated using the spread plate method on Mueller–Hinton agar. The plates were incubated at 37 °C for 24 h. After incubation, colonies were counted, and the results were expressed as colony-forming units per gram of chocolate (CFU/g), according to Goktas and Agan (2025).

2.3 Physicochemical Characterization of Chocolate

The physicochemical characterization included the determination of water activity, moisture, ash, lipid, protein, and total carbohydrate contents according to the analytical methods described by the Adolfo Lutz Institute (IAL, 4th edition, 2008).

2.4 pH and Titratable Acidity

The pH was measured using a calibrated digital pH meter (Tecnal, model TEC-7), previously standardized with pH 4.0 and 7.0 buffer solutions. Total titratable acidity was determined by volumetric titration using 0.1 mol/L NaOH, and the results were expressed as percentage (%) according to AOAC (2019).

2.5 Color Measurement

Color analysis was performed directly on the chocolate surface using a calibrated digital colorimeter (Konica Minolta Sensing, model CR-400). Color parameters were

determined according to the CIELAB color system, including lightness (L^*), redness (a^*), and yellowness (b^*). Three measurements were taken at different points on the surface of each sample, and the results were expressed as mean values. The total color difference (ΔE^*) was calculated using the control chocolate as the reference according to the following equation.

$$\Delta E^* = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2}$$

2.6 Shear Force Analysis

The shear force of the chocolate samples was determined using a TA.XT2i Texture Analyzer (Stable Micro Systems, Godalming, UK) equipped with a guillotine blade set (HDP/BSG). The test was performed in compression mode using a pre-test speed of 1.0 mm/s, a test speed of 2.0 mm/s, and a post-test speed of 10.0 mm/s. Penetration distance was set at 35 mm, with automatic sample contact detection at a trigger force of 0.04903 N. The instrument was configured to return automatically to the initial position after each measurement, with automatic tare correction and advanced settings enabled. The maximum force required to cut the chocolate was recorded and expressed in Newtons (N).

2.7 Calorimetric Analysis

Calorimetric analysis was performed using a bomb calorimeter (IKA®, PARR 6200 model). Samples were previously dried in a forced-air oven at 55 °C for 72 h, homogenized, and approximately 1 g was placed into the calorimetric crucible. The combustion chamber was pressurized with oxygen to approximately 30 atm and immersed in a calorimetric vessel containing distilled water. Combustion was initiated automatically, and the increase in water temperature was digitally recorded, allowing the instrument to calculate the energy released during combustion. Results were expressed as kilocalories per gram (kcal/g). The calorimeter was calibrated using benzoic acid as the standard, and corrections for ignition wire combustion and other components were applied according to the manufacturer's recommendations.

2.8 Microbiological Quality Analysis

Microbiological quality was assessed by determining the absence of *Salmonella* spp., quantifying *Staphylococcus aureus*, enumerating total and thermotolerant coliforms, and quantifying molds and yeasts according to the methodologies recommended by the American Public Health Association (APHA) and the International Organization for Standardization (ISO), as described by Silva *et al.* (2017).

2.9 Simulated Gastrointestinal Tract Conditions

Simulated gastrointestinal tract (GIT) conditions were evaluated at pH 2.0 and 3.0. Tolerance to acidic conditions, pepsin, pancreatin, and bile salts was assessed according to Delgado Junior *et al.* (2024), with adaptations for the food matrix.

2.10 Statistical Analysis

All analyses were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation. Data were subjected to analysis of variance (ANOVA) using a completely randomized design in R software (version 4.5.2). When significant differences were detected, Tukey's multiple comparison test was applied at a significance level of 5% ($p < 0.05$).

3. Results and Discussion

Viability of *A. clausii*

The viability of *Alkalihalobacillus clausii* in chocolate remained stable throughout 120 days of storage, maintaining counts close to 9 log CFU/g with only minor fluctuations within the experimental error, as shown in Table 1. Statistical analysis using analysis of variance (ANOVA) followed by Tukey's post hoc test revealed no significant differences among storage times ($p > 0.05$), indicating that probiotic viability was maintained throughout the experimental period.

Table 1. Viability of *A. clausii* (log CFU/g) in 5.5 g of chocolate during 120 days of storage

Days	F1	F2	F3
1	0 ^a	9.31 $\pm$ 0.21 ^a	8.92 $\pm$ 0.24 ^a
15	0 ^a	9.52 $\pm$ 0.31 ^a	9.08 $\pm$ 0.31 ^a
30	0 ^a	8.90 $\pm$ 0.45 ^a	8.83 $\pm$ 0.31 ^a

45	0 ^a	9.32 ± 0.16 ^a	8.91 ± 0.18 ^a
60	0 ^a	9.18 ± 0.57 ^a	9.04 ± 0.47 ^a
75	0 ^a	9.32 ± 0.21 ^a	8.71 ± 0.66 ^a
90	0 ^a	8.90 ± 0.54 ^a	9.09 ± 0.42 ^a
105	0 ^a	9.51 ± 0.18 ^a	8.82 ± 0.41 ^a
120	0 ^a	9.22 ± 0.47 ^a	8.94 ± 0.51 ^a

Values are expressed as mean ± standard deviation. Means followed by the same lowercase letter within the same column do not differ significantly according to Tukey's test ($p \leq 0.05$). F1: control formulation; F2: formulation supplemented with *A. clausii* endospores; F3: formulation supplemented with *A. clausii* endospores, vitamins, and minerals.

The high stability observed can be attributed to the ability of *A. clausii* to form endospores, which are highly resistant structures capable of withstanding adverse environmental conditions, including fluctuations in temperature, pH, and water availability (Sadrimovahed & Ulusoy, 2024). This characteristic enhances microbial resistance during food processing and storage, making spore-forming microorganisms particularly attractive for applications in functional foods.

Furthermore, the chocolate matrix itself may have contributed to maintaining microbial viability because of its high lipid content, which acts as a protective barrier for probiotic microorganisms by reducing cellular degradation and improving stability during storage (Achi *et al.*, 2024). These properties reinforce the suitability of chocolate as a promising carrier for probiotic delivery.

According to the FAO/WHO (2002), probiotic microorganisms must be consumed in adequate amounts, generally ranging from 6 to 9 log CFU per daily serving, to confer health benefits to the host. In the present study, probiotic viability ranged from 8.8 to 9.5 log CFU/g throughout storage, demonstrating that the developed chocolate formulations not only met but exceeded the minimum recommended probiotic levels established by the FAO/WHO.

Physicochemical Characterization of Chocolate

The physicochemical parameters evaluated, including water activity, moisture, total solids, ash, protein, lipid, and carbohydrate contents, are presented in Table 2.

Water activity (a_w) is an important parameter associated with the availability of free water in foods, ranging from 0 to 1. Values close to 1.0 favor microbial growth, whereas foods with a_w values below 0.60 are considered less susceptible to microbial

proliferation. Chocolate is regarded as a low-risk food from a microbiological standpoint because of its low water activity (<0.60) (Marchioretto *et al.*, 2024). In the present study, water activity values ranged from 0.417 to 0.421, indicating that the incorporation of *A. clausii* endospores and micronutrients did not significantly affect water availability, thereby preserving the typical characteristics of the chocolate matrix.

Table 2. Physicochemical parameters of the different chocolate formulations

Parameter	F1	F2	F3
Water activity	0.417 ± 0.00^a	0.417 ± 0.00^a	0.421 ± 0.01^a
Moisture (%)	0.65 ± 0.02^b	0.64 ± 0.06^b	1.03 ± 0.00^a
Total solids (%)	99.35 ± 0.00^a	99.36 ± 0.06^a	98.97 ± 0.20^b
Ash (%)	1.69 ± 0.01^b	1.72 ± 0.04^b	2.18 ± 0.01^a
Protein (%)	6.63 ± 0.81^a	6.90 ± 0.02^a	6.63 ± 0.81^a
Lipids (%)	32.73 ± 0.13^a	32.33 ± 0.35^a	32.44 ± 0.49^a
Carbohydrates (%)	40.42 ± 0.11^a	40.14 ± 0.03^b	40.33 ± 0.18^a

Values are expressed as mean $\pm$ standard deviation. Means followed by the same lowercase letter within the same row do not differ significantly according to Tukey's test ($p > 0.05$). F1: control formulation; F2: formulation supplemented with *A. clausii* endospores; F3: formulation supplemented with *A. clausii* endospores, vitamins, and minerals.

Moisture content directly influences the physical properties of chocolate, including its melting behavior, hardness, flowability, and appearance. In addition, excessive moisture may promote particle agglomeration and impair product quality (Azhar and Basri, 2025). Moisture values ranged from 0.64% to 1.03%, with a slight increase observed in Formulation 3. This increase may be attributed to the addition of bioactive compounds, such as vitamins and mineral salts, which exhibit hygroscopic properties and may enhance water retention within the food matrix. Nevertheless, the observed values remained low and within the expected range for chocolate products, indicating that product stability was not compromised.

Likewise, total solids showed only minor variation among formulations, ranging from 98.97% to 99.36%. These values are consistent with those expected for chocolate products, which are characterized by low moisture content and high concentrations of cocoa solids, sugar, fat, and milk solids (Konar *et al.*, 2024).

Ash content, which reflects the total mineral content of the product, ranged from 1.69% in Formulation 1 to 1.72% in Formulation 2, indicating that supplementation with *A. clausii* endospores alone did not significantly alter mineral content. In contrast,

Formulation 3 exhibited a significantly higher ash content (2.18%), which can be directly attributed to the addition of vitamin D3, vitamin K2, and magnesium malate.

Protein content showed minimal variation among formulations, ranging from 6.63% to 6.90%, demonstrating that the incorporation of probiotic endospores did not significantly affect the protein fraction of the chocolate. Likewise, supplementation with vitamins and minerals did not influence protein content.

Lipid content remained remarkably consistent among formulations, ranging from 32.33% to 32.73%. According to the literature, chocolate typically contains between 30% and 40% lipids (Tuigunov *et al.*, 2025). Therefore, the results confirm that the addition of probiotic endospores and micronutrients preserved the characteristic lipid composition of chocolate.

Carbohydrates in chocolate consist primarily of sugars and generally account for 38–45% of the total product mass (Tuigunov *et al.*, 2025). In the present study, Formulations 1 and 3 exhibited very similar carbohydrate contents, approximately 40%, indicating that the modifications introduced did not substantially affect the carbohydrate fraction. In contrast, Formulation 2 showed a lower carbohydrate content (40.14%), suggesting a slight dilution effect resulting from the incorporation of probiotic endospores.

The sum of the quantified components in the proximate composition did not reach 100%, which can be explained by the presence of chocolate constituents that were not included in the analyses performed. Components such as dietary fiber and other minor constituents not quantified in the determinations of moisture, ash, protein, lipids, and carbohydrates may account for the observed difference.

Overall, the results demonstrate that the incorporation of *A. clausii* endospores and micronutrients did not cause substantial changes in the physicochemical characteristics of the chocolate. All evaluated parameters remained consistent with values reported in the literature, reinforcing the suitability of chocolate as an appropriate food matrix for the development of functional foods.

pH and Titratable Acidity

The pH and titratable acidity values of the different chocolate formulations are presented in Table 3. Significant differences were observed among the formulations, indicating that the incorporation of probiotic endospores and micronutrients influenced the acid–base characteristics of the chocolate matrix.

Table 3. pH and titratable acidity of chocolate formulations

Parameter	F1	F2	F3
pH	6.22 ± 0.10 ^b	6.13 ± 0.01 ^c	6.80 ± 0.00 ^a
Titratable acidity (%)	0.54 ± 0.07 ^a	0.42 ± 0.00 ^a	0.24 ± 0.04 ^b

Values are expressed as mean ± standard deviation. Means followed by the same lowercase letter within the same row do not differ significantly according to Tukey's test ($p \leq 0.05$). F1: control formulation; F2: formulation supplemented with *A. clausii* endospores; F3: formulation supplemented with *A. clausii* endospores, vitamins, and minerals.

Formulation 1 exhibited a pH of 6.22, indicating low acidity and a value close to neutrality, together with a titratable acidity of $0.54 \pm 0.07\%$. This result was expected, as chocolate generally exhibits a pH close to neutrality (Achi *et al.*, 2024).

In Formulation 2, enriched with *Alkalihalobacillus clausii* endospores, a slight decrease in pH (6.13 ± 0.01) was observed, accompanied by a lower titratable acidity ($0.42 \pm 0.00\%$). This behavior may be associated with the incorporation of endospores and residual metabolic components, which may have slightly altered the acid–base balance of the product.

In contrast, Formulation 3, supplemented with *A. clausii* endospores, vitamin D3, vitamin K2, and magnesium malate, exhibited the highest pH value (6.80 ± 0.00) together with a marked reduction in titratable acidity ($0.24 \pm 0.04\%$). This behavior may be attributed to the presence of mineral compounds, particularly magnesium malate, which may act as a buffering agent, reducing acidity and shifting the pH toward neutrality.

Overall, these findings indicate that micronutrient supplementation can influence the acid–base balance of the chocolate matrix, whereas the incorporation of probiotic endospores alone produces only minor changes in these parameters.

Color Analysis

The instrumental color evaluation of the different chocolate formulations was performed using the CIELAB color system, and the results are presented in Table 4.

Table 4. Colorimetric analysis (L^* , a^* , b^*) of chocolate formulations

Parameter	F1	F2	F3
L^*	32.4 ± 1.56^a	32.25 ± 2.26^a	33.4 ± 1.89^a
a^*	6.55 ± 1.60^a	6.22 ± 1.04^a	7.13 ± 0.95^a
b^*	2.38 ± 1.78^a	1.71 ± 0.67^a	2.9 ± 1.01^a
ΔE^*	-----	0.76	1.08

Values are expressed as mean $\pm$ standard deviation. Means followed by the same lowercase letter within the same row do not differ significantly according to Tukey's test ($p \leq 0.05$). L : lightness; a : green (–) to red (+) axis; b : blue (–) to yellow (+) axis; ΔE^* : total color difference relative to the control formulation (F1). F1: control formulation; F2: formulation supplemented with *A. clausii* endospores; F3: formulation supplemented with *A. clausii* endospores, vitamins, and minerals.

In the CIELAB system, the L^* parameter represents lightness, ranging from 0 (black) to 100 (white), whereas a^* represents the green (negative values) to red (positive values) axis, and b^* represents the blue (negative values) to yellow (positive values) axis.

The L^* values ranged from 32.25 to 33.40, characterizing all formulations as dark chocolates, which is consistent with products containing a high cocoa content. The a^* values ranged from 6.22 to 7.13, indicating a predominance of reddish tones, whereas the b^* values were relatively low (1.71–2.90), suggesting only a slight contribution of yellow hues.

The total color difference (ΔE^*) indicated only minor visual differences between the supplemented formulations and the control. Formulation 2, containing only *A. clausii* endospores, exhibited a ΔE^* value of 0.76, whereas Formulation 3, supplemented with endospores, vitamins, and minerals, presented a ΔE^* value of 1.08 relative to the control. These findings demonstrate that the incorporation of the functional ingredients caused only minimal changes in color, without compromising the characteristic appearance of chocolate.

According to Marchioretto *et al.* (2024), color is one of the most important quality attributes of foods because it is the first characteristic perceived by consumers and is directly associated with product acceptance. Changes in color may also indicate

299 deficiencies during processing or storage, thereby compromising the visual quality of
300 chocolate.

301 Accordingly, the addition of *A. clausii* endospores (Formulation 2) and the
302 combination of probiotics with vitamins and minerals (Formulation 3) did not
303 significantly affect the color parameters compared with the control formulation. This
304 finding suggests that the added ingredients did not interfere with the visual characteristics
305 of the product, probably because of the low concentrations used and the absence of
306 pigments capable of altering the appearance of the chocolate matrix.

307 Overall, the results indicate that functional enrichment did not compromise the
308 visual quality of the chocolate. Therefore, probiotic- and micronutrient-enriched
309 chocolates can be developed without adversely affecting their characteristic appearance,
310 which is an important factor for consumer acceptance and further supports the potential
311 of chocolate as an attractive carrier for probiotics and bioactive nutrients.

312 **Shear Force Analysis**

313 Texture is one of the most important quality attributes of chocolate, particularly
314 hardness, which is directly associated with consumer perception and product quality.
315 Hardness can be defined as the force required to fracture or deform chocolate during
316 mastication and is strongly influenced by the formulation composition and the crystalline
317 structure of cocoa butter (Silva *et al.*, 2024).

318 Formulation 1 exhibited the highest shear force (85.14 N), indicating a firmer
319 structure than the enriched formulations. This result may be attributed to the original
320 chocolate composition, in which the absence of additional ingredients favors a more
321 homogeneous structural organization, particularly regarding the distribution of solid
322 particles and the lipid phase formed by cocoa butter.

323 Following the incorporation of *Alkalihalobacillus clausii* endospores,
324 Formulation 2 exhibited a reduction in shear force (79.24 N). This decrease may be
325 associated with the presence of the incorporated particles, which could interfere with the
326 structural organization of the chocolate matrix and influence interactions among its
327 components during solidification. Since no substantial differences were observed in the
328 proximate composition between Formulations 1 and 2, the reduction in shear force is

more likely related to the physical effect of incorporating the functional ingredient than to compositional changes.

The lowest shear force was observed in Formulation 3 (69.37 N), which also exhibited the highest moisture content (1.03%) and the lowest total solids content (98.97%). The increased moisture content may have contributed to a less rigid structure, allowing greater deformation during the shear test. Furthermore, the higher mineral content, reflected by the increased ash content (2.18%), may also have influenced the structural organization of the chocolate matrix.

Texture is a fundamental quality parameter in food technology because it plays a key role in consumer acceptance and overall sensory perception, directly influencing eating satisfaction and product preference (Rune *et al.*, 2025).

Overall, these findings demonstrate that the incorporation of functional ingredients influenced the textural properties of the chocolate by reducing its mechanical resistance, particularly in the formulation supplemented with vitamins and minerals. Nevertheless, all formulations maintained the structural characteristics expected for chocolate products.

Calorimetric Analysis

The calorimetric analysis revealed very similar energy values among the chocolate formulations. Formulation 1 presented an energy value of 6.10 kcal/g, whereas Formulations 2 and 3 exhibited values of 6.15 and 6.04 kcal/g, respectively.

Considering the 5.5 g portion used for molding the chocolate samples, these values corresponded to approximately 33.55 kcal, 33.83 kcal, and 33.22 kcal for Formulations 1, 2, and 3, respectively. These results indicate that supplementation with probiotic endospores, vitamins, and minerals did not significantly affect the caloric value of the product, since the amounts of these ingredients were too small to contribute substantially to the total energy content.

Consequently, the caloric value of the chocolate remained primarily determined by its major macronutrients, especially lipids and carbohydrates, which constitute the largest proportion of the product.

Overall, the results demonstrate that functional enrichment does not significantly alter the energy value of chocolate. This is an important characteristic for the development

of functional foods, as it enables the incorporation of bioactive compounds without substantially modifying the nutritional profile of the final product.

Microbiological Quality

Microbiological analyses demonstrated the absence of pathogenic microorganisms in all chocolate formulations, confirming the adequacy of the processing conditions and the good manufacturing and storage practices adopted during product development.

Accordingly, the results comply with the microbiological standards established by Brazilian legislation, specifically RDC N°. 331/2019 and Normative Instruction N°. 60/2019, which establish microbiological criteria for indicator and pathogenic microorganisms in foods.

The absence of microbiological contamination may be mainly attributed to the intrinsic characteristics of chocolate, including its low water activity, high solid content, and elevated lipid concentration, all of which restrict the growth of spoilage and pathogenic microorganisms (Achi *et al.*, 2024). Furthermore, appropriate processing, handling, and storage conditions also contributed to maintaining the microbiological quality and safety of the product.

***In Vitro* Simulated Gastrointestinal Tract**

According to Dimitrellou *et al.* (2025), the food matrix plays a crucial role in maintaining probiotic viability. In addition to remaining viable throughout inoculation, production, processing, and storage, probiotic microorganisms must also survive the adverse conditions of the gastrointestinal tract to reach the intestine in sufficient numbers to exert their beneficial health effects.

Following the evaluation of the viability of *Alkalihalobacillus clausii* endospores in the chocolate formulations, their survival under simulated gastrointestinal tract (GIT) conditions was assessed in vitro. Exposure to the simulated conditions demonstrated high stability of the endospores in both supplemented formulations throughout the 4 h incubation period, indicating that they remained viable under acidic conditions and in the presence of digestive enzymes (pepsin and pancreatin) and bile salts, as shown in Table 5.

Overall, viable counts remained between 8.8 and 9.4 log CFU/g, demonstrating the remarkable resistance of *A. clausii* endospores to simulated gastrointestinal conditions. These findings reinforce the intrinsic ability of spore-forming microorganisms to withstand harsh environments, including low pH and exposure to digestive enzymes (Delgado Junior *et al.*, 2024).

Table 5. Viable counts of *A. clausii* endospores (log CFU/g) after exposure to simulated *in vitro* gastrointestinal tract (GIT) conditions

Formulation 2				
Time	pH 2	Pepsin	Pancreatin	Bile salts
0h	9.23 ± 0.27 ^a	9.10 ± 0.32 ^a	9.02 ± 0.37 ^a	9.11 ± 0.27 ^a
1h	9.31 ± 0.26 ^a	9.41 ± 0.49 ^a	9.27 ± 0.27 ^a	9.21 ± 0.33 ^a
2h	9.08 ± 0.38 ^a	9.04 ± 0.37 ^a	9.14 ± 0.18 ^a	9.42 ± 0.16 ^a
3h	9.17 ± 0.21 ^a	9.31 ± 0.24 ^a	9.41 ± 0.31 ^a	8.99 ± 0.12 ^a
4h	9.14 ± 0.23 ^a	-----	9.32 ± 0.29 ^a	9.27 ± 0.27 ^a
Formulação 3				
Time	pH 2	Pepsin	Pancreatin	Bile salts
0h	8.81 ± 0.15 ^a	8.81 ± 0.27 ^a	8.77 ± 0.22 ^a	9.06 ± 0.18 ^a
1h	9.01 ± 0.12 ^a	9.10 ± 0.19 ^a	8.98 ± 0.22 ^a	8.87 ± 0.20 ^a
2h	9.11 ± 0.28 ^a	8.99 ± 0.21 ^a	8.83 ± 0.38 ^a	9.28 ± 0.37 ^a
3h	9.07 ± 0.21 ^a	9.01 ± 0.38 ^a	9.01 ± 0.29 ^a	8.91 ± 0.29 ^a
4h	8.97 ± 0.20 ^a	-----	8.85 ± 0.43 ^a	8.76 ± 0.25 ^a

Values are expressed as mean ± standard deviation. Means followed by the same lowercase letter within the same column do not differ significantly according to Tukey's test ($p > 0.05$). F1: control formulation; F2: formulation supplemented with *A. clausii* endospores; F3: formulation supplemented with *A. clausii* endospores, vitamins, and minerals.

Similar results were reported by Delgado Junior *et al.* (2024), who evaluated the viability of *Bacillus clausii* endospores obtained from the commercial probiotic product Enterogermina® and observed high survival rates following exposure to simulated gastrointestinal tract conditions.

According to the FAO/WHO (2002), probiotic microorganisms should reach the intestine at concentrations of at least 6–7 log CFU to exert beneficial effects on host health. In the present study, viable counts remained well above this threshold throughout the *in vitro* gastrointestinal simulation, indicating that the developed chocolate formulations have considerable potential to serve as effective carriers of probiotic microorganisms.

These findings were expected because, according to Singh and Gaur (2025), cocoa butter forms a hydrophobic barrier that further protects probiotic microorganisms against the acidic conditions of gastric fluids. In addition, flavonoids and polyphenols naturally present in cocoa provide antioxidant protection, further enhancing probiotic survival during both food processing and gastrointestinal transit.

Taken together, these results demonstrate that chocolate is an excellent delivery matrix for *A. clausii* endospores, preserving probiotic viability during storage while providing effective protection under simulated gastrointestinal conditions. Therefore, the formulations developed in this study represent promising functional foods capable of delivering high numbers of viable probiotic microorganisms to the intestinal tract, where they may exert their beneficial physiological effects.

4. Conclusion

This study demonstrated the technological feasibility of incorporating *Alkalihalobacillus clausii* endospores into chocolate, with maintenance of microbial viability during 120 days of storage. The formulations containing endospores presented counts ranging from approximately 8.8 to 9.5 log CFU/g throughout the evaluated period, with no significant differences among storage times, indicating endospore stability in the chocolate matrix under the experimental conditions evaluated.

Exposure to simulated gastrointestinal conditions in vitro also resulted in the maintenance of high *A. clausii* counts, ranging from approximately 8.8 to 9.4 log CFU/g, following exposure to acidic conditions, pepsin, pancreatin, and bile salts. These results demonstrate the maintenance of endospore viability under the simulated gastrointestinal conditions employed in this study.

The incorporation of endospores and supplementation with vitamins D3 and K2 and magnesium malate produced specific changes in some physicochemical parameters, such as moisture, ash, pH, acidity, and shear force, but did not compromise the overall technological characteristics of the matrix. The color parameters showed only minor variations among the formulations, while the energy values remained similar. In addition, the formulations presented satisfactory microbiological quality under the conditions evaluated, with no detection of the indicator and pathogenic microorganisms analyzed.

Taken together, the results indicate that chocolate presents technological feasibility as a matrix for the incorporation of *A. clausii* endospores, allowing their maintenance during storage and survival under the simulated gastrointestinal conditions evaluated, without substantial compromise of the main technological characteristics assessed. However, the results obtained do not allow conclusions to be drawn regarding health effects, micronutrient bioavailability, or the clinical efficacy of the formulations.

Although the results demonstrate the technological feasibility of the developed formulations, some complementary steps are still necessary for a more comprehensive evaluation of the product. The absence of sensory analyses and consumer acceptance testing did not allow the perception of the formulations regarding their sensory characteristics to be assessed. Likewise, the evaluation of *A. clausii* survival was conducted exclusively under simulated gastrointestinal conditions in vitro, and in vivo studies are therefore necessary to investigate the behavior of the endospores after consumption. For the supplemented formulations, the bioavailability of vitamins D3 and K2 and magnesium was also not determined, while aspects related to economic feasibility and industrial-scale production were not addressed in this study. These issues represent important steps for the continued technological development of the formulations.

Therefore, future studies should include sensory and consumer acceptance evaluations, in vivo models or clinical studies to investigate the behavior of the endospores after consumption, micronutrient bioavailability analyses, and assessments of cost, scalability, and stability

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

CRedit Author Statement

Manoel Armando Delgado Junior: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft.

Elizete Alves Proença Afonso: Methodology, Investigation, Data curation, Validation, Writing – review and editing.

Claudini Ferreira Kumakura: Methodology, Investigation, Validation, Writing – review and editing.

Danielle Marques Vilela: Conceptualization, Supervision, Validation, Writing – review and editing.

Kelly Cristina da Silva Brabes: Conceptualization, Supervision, Project administration, Resources, Writing – review and editing.

Declaration of AI

During the preparation of this manuscript, the authors used ChatGPT (OpenAI), Grammarly, and Consensus to assist with English language editing, grammar correction, improvement of writing clarity, literature exploration, and reference formatting. All suggestions generated by these tools were critically reviewed, verified, and edited by the authors. The authors take full responsibility for the content of the final manuscript, including the interpretation of results, conclusions, and scientific accuracy.

Declaration of Conflict of Interest

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

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Referências

Achi, S. C., Chetana, R., Asha, M. R., Raphel, S., & Halami, P. M. (2024). *Dark chocolate: Delivery medium for probiotic Bifidobacterium breve* NCIM 5671. *Journal of Food Science and Technology*, 61(7), 1411–1415. <https://doi.org/10.1007/s13197-024-05958-6>

AOAC International. (2019). *AOAC guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals*. AOAC International.

Ashraf, S. A. (2025). Food fortification as a sustainable global strategy to mitigate micronutrient deficiencies and improve public health. *Discover Food*, 5(1), Article 201. <https://doi.org/10.1007/s44187-025-00512-5>

Atieh, O., Daher, J., Durieux, J., Abboud, M., Labbato, D., Baissary, J., Koberssy, Z., Ailstock, K., Cummings, M., Funderburg, N., & McComsey, G. A. (2025). Vitamins K2 and D3 improve long COVID, fungal translocation, and inflammation: Randomized controlled trial. *Nutrients*, 17(2), Article 304. <https://doi.org/10.3390/nu17020304>

Azhar, F. N., & Basri, H. (2025). Physicochemical, antioxidant and sensory evaluation of dark chocolate fortified with Sacha Inchi powder roasted at different condition.

- 505 *Enhanced Knowledge in Sciences and Technology*, 5(1), 239–248.
 506 <https://doi.org/10.30880/ekst.2024.05.01.029>
- 507 Brazil. National Health Surveillance Agency. (2019). *Collegiate Board Resolution*
 508 *(RDC) No. 331, of December 23, 2019: Establishes the microbiological standards for*
 509 *foods and their application*. [https://www.gov.br/anvisa/pt-br/assuntos/noticias-](https://www.gov.br/anvisa/pt-br/assuntos/noticias-anvisa/2020/alimentos-normas-estabelecem-padroes-microbiologicos)
 510 [anvisa/2020/alimentos-normas-estabelecem-padroes-microbiologicos](https://www.gov.br/anvisa/pt-br/assuntos/noticias-anvisa/2020/alimentos-normas-estabelecem-padroes-microbiologicos)
- 511 Brazil. National Health Surveillance Agency. (2019). *Normative Instruction No. 60, of*
 512 *December 23, 2019: Establishes the lists of microbiological standards for foods ready*
 513 *for consumer sale*. [https://site.sindicarnes-sp.org.br/wp2/wp-](https://site.sindicarnes-sp.org.br/wp2/wp-content/uploads/2020/05/IN6020191223.pdf)
 514 [content/uploads/2020/05/IN6020191223.pdf](https://site.sindicarnes-sp.org.br/wp2/wp-content/uploads/2020/05/IN6020191223.pdf)
- 515 Camelo-Silva, C., Mota e Souza, B., Vicente, R., Arend, G. D., Sanches, M. A. R.,
 516 Barreto, P. L. M., Ambrosi, A., Verruck, S., & Di Luccio, M. (2024). Polyfunctional
 517 sugar-free white chocolate fortified with *Lacticaseibacillus rhamnosus* GG co-
 518 encapsulated with beet residue extract (*Beta vulgaris* L.). *Food Research International*,
 519 179, Article 114016. <https://doi.org/10.1016/j.foodres.2024.114016>
- 520 Corona, R., Bontà, V., Baccigalupi, L., & Ricca, E. (2025). Probiotic spores of
 521 *Shouchella clausii* SF174 and displayed bromelain show beneficial additive potential.
 522 *International Journal of Molecular Sciences*, 26(3), Article 942.
 523 <https://doi.org/10.3390/ijms26030942>
- 524 Delgado Junior, M. A., Vilela, D. M., & Brabes, K. C. da S. (2024). Evaluation of the
 525 viability of *Bacillus clausii* spores in human gastrointestinal tract simulation. *Revista*
 526 *Brasileira Multidisciplinar*, 27(3), 103–110. [https://doi.org/10.25061/2527-](https://doi.org/10.25061/2527-2675/ReBraM/2024.v27i3.1973)
 527 [2675/ReBraM/2024.v27i3.1973](https://doi.org/10.25061/2527-2675/ReBraM/2024.v27i3.1973)
- 528 Dimitrellou, D., Sakadani, E., & Kandylis, P. (2025). Enhancing probiotic viability in
 529 yogurt: The role of apple fibers in supporting *Lacticaseibacillus casei* ATCC 393
 530 during storage and gastrointestinal transit. *Foods*, 14(3), Article 376.
 531 <https://doi.org/10.3390/foods14030376>
- 532 Food and Agriculture Organization of the United Nations, & World Health
 533 Organization. (2001). *Report of the Joint FAO/WHO Expert Consultation on Evaluation*
 534 *of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with*
 535 *Live Lactic Acid Bacteria*. Food and Agriculture Organization of the United Nations.
- 536 Food and Agriculture Organization of the United Nations, & World Health
 537 Organization. (2002). *Guidelines for the evaluation of probiotics in food: Report of a*
 538 *Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of*
 539 *Probiotics in Food*. Food and Agriculture Organization of the United Nations.
- 540 Goktas, H., & Agan, C. (2025). Production of sucrose-free and probiotic chocolate
 541 pudding using xylitol in combination with *Lacticaseibacillus rhamnosus* GG and
 542 *Saccharomyces boulardii*. *Journal of Food Measurement & Characterization*, 19(1),
 543 725–735. <https://doi.org/10.1007/s11694-024-03003-w>

- Gomes, E. da S., Schipfer, C. W. T., Oliveira, V. de A. F. de, Freitas, M. M. G. de, Muxfeldt, L. C., Maestro, A. D., Frez, F. L. V., & Santos Júnior, O. de O. (2024). Alimentos funcionais integrados com prebióticos e probióticos: Uma revisão. *Observatório de la Economía Latinoamericana*, 22(5), Article e4911. <https://doi.org/10.55905/oelv22n5-198>
- Instituto Adolfo Lutz. (2008). *Analytical standards of the Adolfo Lutz Institute: Chemical and physical methods for food analysis* (4th ed., 1st digital ed.). Instituto Adolfo Lutz.
- Islam, M. Z., Masum, A. K. M., & Harun-ur-Rashid, M. (2022). Milk chocolate matrix as a carrier of novel *Lactobacillus acidophilus* LDMB-01: Physicochemical analysis, probiotic storage stability and *in vitro* gastrointestinal digestion. *Journal of Agriculture and Food Research*, 7, Article 100263. <https://doi.org/10.1016/j.jafr.2021.100263>
- Konar, N., Palabiyik, I., Karimidastjerd, A., & Said Toker, O. (2024). Chocolate microstructure: A comprehensive review. *Food Research International*, 196, Article 115091. <https://doi.org/10.1016/j.foodres.2024.115091>
- Marchioretto, C., Luccas, V., Gorup, L. F., Borges Gomes, R. A., Simionatto, E., de Oliveira, K. M. P., de Araújo, R. P., Altemio, Â. D. C., Porzani, G. B., Martelli, S. M., & de Arruda, E. J. (2024). Nutritional value and acceptability of chocolate with high cocoa content and green banana biomass. *Lebensmittel-Wissenschaft & Technologie [Food Science and Technology]*, 191, Article 115667. <https://doi.org/10.1016/j.lwt.2023.115667>
- Rune, C. J. B., Clausen, M. P., & Giacalone, D. (2026). Sensory evaluation of plant-based cheese: A systematic review with a focus on texture and mouthfeel. *Critical Reviews in Food Science and Nutrition*, 66(4), 754–779. <https://doi.org/10.1080/10408398.2025.2531220>
- Sadrimovahed, M., & Ulusoy, B. H. (2024). *Bacillus clausii*: A review into story of its probiotic success and potential food applications. *Fermentation*, 10(10), Article 522. <https://doi.org/10.3390/fermentation10100522>
- Silva, N. da, Junqueira, V. C. A., Silveira, N. F. de A., Taniwaki, M. H., Gomes, R. A. R., & Okazaki, M. M. (2017). *Manual de métodos de análise microbiológica de alimentos e água* (5th ed.). Blucher.
- Singh, S., & Gaur, S. (2025). Novel formulations of cinnamon- and orange-flavored synbiotic corn chocolates with enhanced functional properties and probiotic survival rates. *ACS Food Science & Technology*, 5(4), 1268–1279. <https://doi.org/10.1021/acsfoodscitech.4c00741>
- Tuigunov, D., Smagul, G., Sinyavskiy, Y., Omarov, Y., & Barmak, S. (2025). Functionalization of chocolate: Current trends and approaches to health-oriented nutrition. *Processes*, 13(5), Article 1431. <https://doi.org/10.3390/pr13051431>.

Capítulo 03 – Artigo Científico 2

LWT

Innovation in Gummy Candies: Supplementation with *Alkalihalobacillus clausii* Endospores for Probiotic Potential

--Manuscript Draft--

Manuscript Number:	LWT-D-26-05306
Article Type:	Research paper
Keywords:	Functional Food; <i>Bacillus clausii</i> ; Food Technology
Corresponding Author:	Manoel Armando Delgado Junior Federal University of Grande Dourados
First Author:	Manoel Armando Delgado Junior
Order of Authors:	Manoel Armando Delgado Junior
	Elizete Alves Proença Afonso
	Claudini Ferreira Kumakura
	Danielle Marques Vilela
	Kelly Cristina da Silva Brabes
Abstract:	<p>The confectionery industry offers a wide diversity of products, including gummy candies, whose reformulation has been encouraged by the growing demand for functional foods. This study aimed to develop gummy candies supplemented with <i>Alkalihalobacillus clausii</i> endospores, vitamins, and minerals and evaluate their stability during storage and under simulated gastrointestinal conditions. The formulations were developed in partnership with a compounding pharmacy. Endospore viability was monitored every 15 days for 120 days, together with physicochemical, pH, titratable acidity, color, texture, calorimetry, microbiological, and simulated gastrointestinal analyses. Formulations containing <i>A. clausii</i> maintained high viability, with counts above 8.8 log CFU/g throughout storage. Physicochemical differences were observed among formulations, particularly in moisture and total solids, whereas water activity remained similar. Color showed small variations, while titratable acidity decreased progressively. Texture remained consistent with the soft characteristics of gummy candies, and microbiological quality was satisfactory. Under simulated gastrointestinal conditions, <i>A. clausii</i> endospores demonstrated high resistance, maintaining high counts throughout the evaluated stages. These findings demonstrate the technological potential of gummy candies as a non-dairy matrix for incorporating <i>A. clausii</i> endospores, with maintenance of microbial viability during storage and resistance to simulated gastrointestinal conditions. Further studies should investigate sensory acceptance and the effects of the food matrix on microorganism functionality.</p>

Innovation in Gummy Candies: Supplementation with *Alkalihalobacillus clausii* Endospores for Probiotic Potential

Abstract

The confectionery industry offers a wide diversity of products, including gummy candies, whose reformulation has been encouraged by the growing demand for functional foods. This study aimed to develop gummy candies supplemented with *Alkalihalobacillus clausii* endospores, vitamins, and minerals and evaluate their stability during storage and under simulated gastrointestinal conditions. The formulations were developed in partnership with a compounding pharmacy. Endospore viability was monitored every 15 days for 120 days, together with physicochemical, pH, titratable acidity, color, texture, calorimetry, microbiological, and simulated gastrointestinal analyses. Formulations containing *A. clausii* maintained high viability, with counts above 8.8 log CFU/g throughout storage. Physicochemical differences were observed among formulations, particularly in moisture and total solids, whereas water activity remained similar. Color showed small variations, while titratable acidity decreased progressively. Texture remained consistent with the soft characteristics of gummy candies, and microbiological quality was satisfactory. Under simulated gastrointestinal conditions, *A. clausii* endospores demonstrated high resistance, maintaining high counts throughout the evaluated stages. These findings demonstrate the technological potential of gummy candies as a non-dairy matrix for incorporating *A. clausii* endospores, with maintenance of microbial viability during storage and resistance to simulated gastrointestinal conditions. Further studies should investigate sensory acceptance and the effects of the food matrix on microorganism functionality.

Keywords: Functional Food, *Bacillus clausii*, Food Technology.

1. Introduction

The confectionery industry offers a wide range of products, with an almost unlimited variety. Soft confectionery products stand out as one of the most widely consumed groups. However, consumers are increasingly moving away from traditional foods, creating a growing demand for soft confectionery products with functional properties and, consequently, driving research aimed at reformulating these products (Gunes *et al.*, 2025).

Among functional ingredients, probiotics play an important role in food systems and have also gained popularity in the confectionery industry (Kahraman *et al.*, 2023), as probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2001).

The incorporation of probiotic bacteria into confectionery products, such as sweets, represents a promising opportunity, allowing a broader range of consumers, including younger individuals, to access this dietary component through an appealing food format. However, research evaluating the effectiveness of products such as gummy candies in delivering live microorganisms to the human intestine remains limited (Perotti *et al.*, 2024).

Nevertheless, ensuring the survival of probiotics throughout processing and storage is essential. In addition, the shelf life of products must be extended to ensure that they contain an adequate number of viable microorganisms until the time of consumption (Kahraman *et al.*, 2023). As an alternative to non-dairy products, the food industry is increasingly turning to endospore-forming bacteria (Perotti *et al.*, 2024), such as *Alkalihalobacillus clausii* (formerly *Bacillus clausii*).

Alkalihalobacillus clausii is an aerobic, Gram-positive, rod-shaped, alkali-tolerant, and non-pathogenic bacterium that forms resilient endospores capable of withstanding harsh environmental conditions and exhibiting greater tolerance to bile salts and gastric HCl, as well as greater resistance to heat treatments during pharmaceutical processing or the manufacture of functional foods (Sadrimovahed & Ulusoy, 2024).

The incorporation of *A. clausii* endospores into gummy candies represents a potential application of endospore-forming microorganisms in an accessible confectionery matrix. However, the compositional and processing conditions of this type of product may influence microorganism stability. Although there is growing interest in the development of functional foods containing probiotics, studies on the use of *A. clausii* endospores in gummy candies remain limited, particularly regarding the maintenance of viability during storage and the behavior of microorganisms under simulated gastrointestinal conditions.

Therefore, the present study aimed to develop a gummy candy supplemented with *A. clausii* endospores and to evaluate their viability during 120 days of storage and under simulated gastrointestinal tract conditions. The physicochemical, microbiological, colorimetric, textural, and calorimetric characteristics of the formulations were also determined to assess possible changes in product properties associated with the incorporation of the endospores.

2. Materials and Methods

2.1. Production of Probiotic Gummy Candies

The gummy candies were developed in partnership with a compounding pharmacy located in Dourados, Mato Grosso do Sul, Brazil. Three formulations were prepared, with a final weight of approximately 5.5 g per unit (Figure 1). Formulation 1 (F1) corresponded

to the control, consisting of the base gummy candy containing flavoring and coloring agents, without the addition of endospores. Formulation 2 (F2) was supplemented with *A. clausii* endospores, whereas formulation 3 (F3) was enriched with *A. clausii* endospores, vitamin D3, vitamin K2, and magnesium malate.

The gummy candy base formulation consisted of gelatin (11%), hydrolyzed collagen (10%), citric acid (2%), sorbitol (24%), glycerin (5%), sucralose (0.1%), and potassium sorbate (0.15%), in addition to flavoring and coloring agents in a sufficient quantity.

For the preparation of F1, the ingredients were mixed under heating and constant stirring until complete dissolution of the gelatin and hydrolyzed collagen, resulting in a homogeneous mixture. Strawberry flavoring and red coloring were then added. The mixture was transferred to suitable molds, refrigerated for 30 min for gelation, and subsequently hermetically sealed.

For the production of F2, the base formulation was prepared according to the procedure described for F1 and cooled to approximately 45 °C, a temperature considered suitable for preserving microorganism viability. Subsequently, a concentration of 2×10^9 colony-forming units (CFU) of *A. clausii* (9.3 log CFU) was added. The mixture was homogenized, transferred to molds, refrigerated until solidification, and subsequently hermetically sealed.

F3 was prepared following the same procedure as F2 and contained 1×10^9 CFU of *A. clausii* (9 log CFU), 400 IU of vitamin D3 (1:1000 dilution), 50 µg of vitamin K2 (1:10 dilution), and 50 mg of magnesium malate. After homogenization, the mixture was transferred to molds, refrigerated for gelation, and hermetically sealed.

The endospore concentration used in the formulations was established based on the concentration of *Alkalihalobacillus clausii* present in the commercial product Enterogermina®, which was used as a reference for defining the dose of the microorganism incorporated into the food matrix. Based on this reference, concentrations of 2×10^9 CFU for F2 and 1×10^9 CFU for F3 were established, considering the specific composition of each formulation. Thus, F2 was developed to evaluate the viability of *A. clausii* incorporated into the gummy candy matrix, whereas F3 was formulated with a

reduced concentration of the microorganism in combination with vitamin D3, vitamin K2, and magnesium malate, allowing the stability of the microorganism to be evaluated in a functional matrix containing additional ingredients.

The samples were stored under refrigeration (4 °C) until the physicochemical and microbiological analyses were performed.



Fig. 1. Visual and morphological characteristics of the developed gummy candies.

2.2. Viability of *A. clausii*

The viability of *A. clausii* was determined on the first day of storage and subsequently every 15 days over four months of storage. Each gummy candy was transferred to 49.5 mL of sterile peptone water, and the container was maintained in a water bath at 45 °C until complete dissolution of the sample. Serial dilutions were then prepared and inoculated onto Mueller-Hinton agar using the spread plate method, followed by incubation at 37 °C for 24 h. After incubation, colonies were counted, and the results were expressed as colony-forming units per gram of gummy candy (CFU/g) (Meira *et al.*, 2024).

2.3. Physicochemical Characterization of the Gummy Candies

For the physicochemical characterization of the gummy candies, water activity, moisture, ash, lipid, protein, and total carbohydrate contents were determined according to the methods described in the Analytical Standards of the Adolfo Lutz Institute, 4th edition (2008).

2.4. Determination of pH and Titratable Acidity

The pH was evaluated using a digital pH meter (Tecnal, model TEC-7), previously calibrated with pH 4.0 and 7.0 buffer solutions. Total titratable acidity was determined by the volumetric method using a 0.1 mol/L sodium hydroxide (NaOH) solution. The results were expressed as a percentage, according to the method employed (AOAC, 2019).

2.5. Colorimetric Analysis

Color analyses were performed directly on the surface of the gummy candies using a digital colorimeter (Konica Minolta Sensing, model CR-400), previously calibrated with a standard white plate. Measurements were performed using the CIELAB system, evaluating the parameters of lightness (L^*), red color intensity (a^*), and yellow color intensity (b^*). Three measurements were performed at different points on the surface of each sample, and the results were expressed as mean values. The total color difference (ΔE^*) was calculated using formulation 1 as the reference, according to the following equation:

$$\Delta E^* = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2}$$

2.6. Shear Analysis

Shear analysis of the gummy candies was performed using a TA.XT2i texture analyzer (Stable Micro Systems, Godalming, England), equipped with a guillotine blade assembly (HDP/BSG). The test was conducted in compression mode, with a pre-test speed of 1.0 mm/s, test speed of 2.0 mm/s, and post-test speed of 10.0 mm/s. The penetration distance was set to 35 mm, with automatic sample contact detection at a trigger force of 0.04903 N. The system was configured to return to the initial position after each test, with automatic tare correction and advanced options enabled. The parameter evaluated was the maximum force required for sample shearing, expressed in Newtons (N).

2.7. Calorimetric Analysis

Calorimetric analysis was performed by combustion using a bomb calorimeter (IKA®, model PARR 6200). The samples were previously dried in an oven at 55 °C for

72 h, homogenized, and approximately 1 g was placed in the metal crucible of the bomb. The bomb was pressurized with oxygen at approximately 30 atm and placed in a calorimetric vessel containing distilled water. Combustion was automatically initiated, and the change in water temperature was digitally recorded, allowing the equipment to calculate the energy released during combustion. The results were expressed in calories per gram and kilocalories per kilogram (kcal/kg). The bomb was calibrated using standard benzoic acid, and corrections for the combustion of the ignition wire and other components were applied according to the manufacturer's recommendations.

2.8. Microbiological Quality Analysis

Microbiological quality was assessed by the absence of *Salmonella* spp., quantification of *Staphylococcus aureus*, enumeration of total and thermotolerant coliforms, and quantification of molds and yeasts. The analyses were performed according to microbiological procedures recommended by the American Public Health Association (APHA) and the International Organization for Standardization (ISO), as described by Silva *et al.* (2017).

2.9. In Vitro Simulated Gastrointestinal Tract

The tolerance of *Alkalihalobacillus clausii* endospores to simulated gastrointestinal tract (GIT) conditions was evaluated based on the methodology described by Delgado Junior *et al.* (2024), with adaptations for the gummy candy matrix. Initially, one gummy candy unit, corresponding to approximately 5.5 g, was transferred to 49.5 mL of sterile diluent, resulting in an initial 10^{-1} dilution. After homogenization, the suspension was used in the different assays to evaluate tolerance to simulated gastrointestinal conditions.

To evaluate acid tolerance, the suspension containing the endospores was subjected to a medium at pH 2.0 and maintained at 37 °C for 0, 1, 2, 3, and 4 h. After each exposure period, serial dilutions were prepared and plated on Mueller-Hinton agar, followed by incubation at 37 °C for 24 h. For the pepsin assay, the sample was subjected to Mueller-Hinton medium adjusted to pH 2.0 and containing pepsin at a concentration of 3 mg/mL. For the pancreatin assay, Mueller-Hinton medium adjusted to pH 8.0 and containing pancreatin at a concentration of 1 mg/mL was used. The samples were

incubated at 37 °C and evaluated at 0, 1, 2, and 3 h for the pepsin assay and at 0, 1, 2, 3, and 4 h for the pancreatin assay.

Tolerance to bile salts was determined by using the suspension in Mueller-Hinton medium adjusted to pH 8.0 and supplemented with 0.3% bile salts. The samples were incubated at 37 °C for 0, 1, 2, 3, and 4 h. After each exposure period, serial dilutions were prepared and plated on Mueller-Hinton agar, followed by incubation at 37 °C for 24 h. The viability of *A. clausii* after exposure to the different simulated conditions was determined by microbiological counting, and the results were expressed as CFU/g.

2.10. Statistical Analysis

All analyses in this study were performed in triplicate, and the respective means and standard deviations were calculated. The data were subjected to analysis of variance (ANOVA) in a completely randomized design using R software (version 4.5.2). When significant differences among means were identified, Tukey's test was applied for multiple comparisons, adopting a significance level of 5% ($p < 0.05$).

3. Results and Discussion

Viability of *A. clausii*

Maintaining microbial viability during storage is one of the main aspects to be considered in the development of foods containing microorganisms with probiotic potential, since microorganism survival until the time of consumption is essential for the product to provide an adequate number of viable cells (Liu *et al.*, 2025). In the Brazilian context, Resolution RDC No. 241/2018 establishes that probiotic claims in foods must be supported by scientific evidence demonstrating the presence of viable microorganisms in sufficient quantities to promote health benefits. In this context, international recommendations from FAO/WHO (2001) indicate that probiotic products should provide between 10^6 and 10^9 CFU per daily serving to ensure functional efficacy.

Based on the results presented in Table 1, the formulations maintained high counts of *A. clausii* endospores throughout the 120-day storage period, with no significant reduction in viability. These results demonstrate the stability of the microorganism in the gummy candy matrix during the evaluated period.

Table 1 – Viability of *A. clausii*, expressed as log CFU/g, over 4 months of storage.

Days	F1	F2	F3
1	0 ^a	9.27 ± 0.18 ^a	8.91 ± 0.52 ^a
15	0 ^a	9.42 ± 0.12 ^a	9.12 ± 0.27 ^a
30	0 ^a	9.10 ± 0.29 ^a	8.90 ± 0.19 ^a
45	0 ^a	9.21 ± 0.44 ^a	9.09 ± 0.21 ^a
60	0 ^a	8.98 ± 0.28 ^a	8.83 ± 0.34 ^a
75	0 ^a	9.13 ± 0.32 ^a	8.81 ± 0.08 ^a
90	0 ^a	9.51 ± 0.19 ^a	8.72 ± 0.17 ^a
105	0 ^a	9.20 ± 0.21 ^a	9.11 ± 0.29 ^a
120	0 ^a	9.12 ± 0.21 ^a	8.90 ± 0.38 ^a

Values are expressed as mean ± standard deviation. Means followed by the same letter within the same column do not differ significantly among storage periods within each formulation, according to Tukey's test ($p \leq 0.05$). **F1**: control formulation; **F2**: formulation containing endospores only; **F3**: formulation containing endospores, vitamins, and minerals.

The observed stability demonstrates that, under the evaluated conditions, both F2 and F3 maintained high endospore counts throughout the 120-day storage period. F2, containing the gummy candy matrix and endospores, showed stable viability throughout the storage period, as did F3, which additionally contained vitamin D3, vitamin K2, and magnesium malate. Thus, the results indicate that both formulations were capable of preserving *A. clausii* viability during refrigerated storage.

The high stability observed may be related to the sporulated nature of *A. clausii*, whose endospores exhibit high resistance to adverse environmental conditions, including acidity and heat treatments (Sadrimovahed & Ulusoy, 2024). This characteristic may favor the maintenance of microorganism viability during processing and storage in the food matrix.

The results obtained in the present study differ from those reported by Meira *et al.* (2024), who developed a gelatin-based candy containing *Bacillus coagulans* endospores at an initial concentration of approximately 9 log CFU/g. The authors observed a reduction in viability to values between 6.06 and 6.11 log CFU/g during the product's shelf life. Similarly, Costa *et al.* (2024) evaluated a pectin-based gummy candy containing *B. coagulans* GBI-30 6086, also added at an initial concentration of approximately 9 log CFU/g, and reported a mean viability of 6.57 log CFU/g after 30 days of storage. In contrast, in the present study, the formulations containing *A. clausii* maintained counts above 8.7 log CFU/g throughout the entire experimental period, with no statistically significant reduction in viability.

Thus, the maintenance of high *A. clausii* counts over 120 days indicates endospore stability in the gummy candy matrix under refrigeration and highlights its potential as a vehicle for this microorganism. However, microbiological viability alone does not allow the probiotic efficacy of the product to be established and should therefore be considered together with the other results obtained.

Physicochemical Characterization

The physicochemical parameters evaluated in the different gummy candy formulations, including water activity, moisture, total solids, ash, protein, lipids, and carbohydrates, are presented in Table 2.

The water activity (A_w) values of the formulations ranged from 0.912 to 0.921. Despite the small numerical variation observed among the formulations, no statistically significant differences were observed among F1, F2, and F3 ($p \leq 0.05$). These values are compatible with a gummy candy matrix containing ingredients such as gelatin, collagen, sorbitol, and glycerin, which can interact with water and influence the physicochemical properties of the product. The absence of significant differences among the formulations indicates that the incorporation of *A. clausii* endospores and micronutrients did not cause a relevant change in water availability in the matrix under the evaluated conditions.

Table 2 – Physicochemical parameters of the different gummy candy formulations.

Parameters	F1	F2	F3
Water activity	0.921 ± 0.00^a	0.912 ± 0.01^a	0.913 ± 0.00^a
Moisture (%)	51.01 ± 0.32^a	51.29 ± 0.31^a	50.40 ± 0.11^b
Total solids (%)	48.99 ± 0.32^b	48.71 ± 0.31^b	49.60 ± 0.11^a
Ash (%)	0.19 ± 0.012^b	0.19 ± 0.017^b	0.64 ± 0.00^a
Protein (%)	20.49 ± 0.21^a	21.48 ± 0.59^a	19.43 ± 0.55^b
Lipids (%)	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
Carbohydrates (%)	28.23 ± 0.31^b	27.68 ± 0.3^c	27.03 ± 0.6^a

Values are expressed as mean $\pm$ standard deviation. Means followed by the same letter within the same row do not differ significantly according to Tukey's test ($p \leq 0,05$). **F1**: control formulation; **F2**: formulation containing endospores only; **F3**: formulation containing endospores, vitamins, and minerals.

The moisture content ranged from 50.40% to 51.29%. These results are expected for gummy candies, since this type of matrix has a high moisture content and, consequently, higher A_w (Tarahi *et al.*, 2023). F1 presented 51.01%, F2 51.29%, and F3

50.40%, with a statistically significant difference observed between F3 and the other formulations ($p \leq 0.05$). As a consequence of the inverse relationship between moisture and total solids, total solids values ranged from 48.71% to 49.60%. F3 presented the highest total solids content (49.60%), differing significantly from F1 (48.99%) and F2 (48.71%), which did not differ from each other. The reduction in moisture content and increase in total solids observed in F3 may be related to changes in matrix composition resulting from the incorporation of vitamin D3, vitamin K2, and magnesium malate.

Regarding ash content, F1 and F2 presented similar values of 0.19%. The absence of significant differences suggests that the incorporation of endospores, under the conditions employed, did not cause a detectable change in the mineral fraction of the matrix. In contrast, F3 presented a significantly higher value (0.64%), which is compatible with the inclusion of magnesium malate and the other components added to the enriched formulation, which may contribute to the increase in the inorganic fraction determined by the ash analysis.

Regarding protein content, significant differences were observed among the formulations. F2 presented the highest value (21.48%), followed by F1 (20.49%), whereas F3 presented the lowest protein content (19.43%). This behavior may be associated with the composition of the formulation, which is rich in gelatin and collagen, the main protein sources in the matrix. The reduction in protein content in formulation 3 may be related to the addition of micronutrients and the probiotic, which diluted the protein fraction without compromising the nutritional value of the product.

Lipids were not detected in any of the formulations, which is consistent with the composition used to prepare the gummy candies, as it does not contain ingredients known to be rich in lipids. The absence of this macronutrient also contributes to characterizing the developed matrix as a low-lipid product.

Carbohydrate contents ranged from 27.03% to 28.23%, with statistically significant differences observed among all formulations. F1 presented 28.23%, F2 27.68%, and F3 27.03%. The results are directly associated with sorbitol and glycerin, which were used in significant amounts in the formulation and are considered polyol-type carbohydrates that function as bulking agents, sweeteners, and humectants (Dragomir *et al.*, 2025). Citric acid and sucralose, although present at lower concentrations, also

contribute to the final carbohydrate calculation when analyzed by the indirect method. However, the progressive reduction observed among the formulations may be related to the proportional replacement of formulation components by the incorporation of endospores and, particularly, the micronutrients present in F3.

Determination of pH and Titratable Acidity

The acidity of a gummy candy is a relevant parameter, and an appropriate balance among sweetness, aroma, and acidity contributes to its flavor and, consequently, to greater product acceptance (Meira *et al.*, 2024). In this context, the developed formulations presented pH values ranging from 4.14 to 4.72 (Table 3), characterizing them as acidic products, a condition compatible with the presence of citric acid in the formulation.

Table 3 – pH values and titratable acidity of gummy candies.

Parameters	F1	F2	F3
pH	4.63 ± 0.04 ^a	4.14 ± 0.01 ^a	4.72 ± 0.01 ^a
Titratable acidity (%)	3.71 ± 0.16 ^a	3.16 ± 0.16 ^b	1.96 ± 0.06 ^c

Values are expressed as mean ± standard deviation. Means followed by the same letter within the same row do not differ significantly according to Tukey's test ($p \leq 0.05$). **F1**: control formulation; **F2**: formulation containing endospores only; **F3**: formulation containing endospores, vitamins, and minerals.

Titratable acidity values differed significantly among the formulations ($p \leq 0.05$), showing a progressive reduction from F1 to F3, with values of $3.71 \pm 0.16\%$, $3.16 \pm 0.16\%$, and $1.96 \pm 0.06\%$, respectively. This behavior is mainly related to differences in formulation composition, particularly the presence of citric acid, used as an acidulant, and the other ingredients incorporated during enrichment.

The control formulation (F1) presented the highest titratable acidity ($3.71 \pm 0.16\%$), which is consistent with the presence of citric acid in the matrix, responsible for acidifying the product and contributing to its sensory characteristics. In F2, containing only the addition of *A. clausii* endospores, a significant reduction in titratable acidity to $3.16 \pm 0.16\%$ was observed. This difference may be related to the proportional change in matrix composition resulting from the incorporation of the endospores. However, the specific contribution of the microorganism to this behavior cannot be established independently, since titratable acidity results from the interaction among the different components present in the product.

F3 presented the lowest titratable acidity ($1.96 \pm 0.06\%$), differing significantly from the other formulations. This reduction may be associated with the combined incorporation of vitamin D3, vitamin K2, and magnesium malate, which modify the chemical composition of the matrix. The combined incorporation of these components may have altered the composition and acid–base balance of the matrix. Magnesium malate, which contains malate in its composition, may also be related to this change. However, this possibility should be interpreted with caution, since the individual effect of each component on titratable acidity was not evaluated in the present study.

Overall, the results demonstrate that the incorporation of the different components into the formulations caused significant changes in titratable acidity, with a progressive reduction from F1 to F3, whereas pH remained statistically similar among the formulations. These results indicate that modifications in the composition of the gummy candies mainly affected the fraction of titratable compounds in the matrix, without causing a statistically significant change in the active acidity of the product.

Colorimetric Analysis

Color is a fundamental attribute with regard to consumers' sensory expectations, serving as an initial selection parameter and at the time of purchase. In products such as gummy candies, visual appearance plays an important role in expectations regarding flavor and product quality (Meira *et al.*, 2024). Therefore, the results of the colorimetric analysis of the formulations are presented in Table 4.

Table 4 – Colorimetric parameters (L , a^* , b^*) of the gummy candy formulations.

Parameters	F1	F2	F3
L^*	33.37 ± 1.22^a	30.37 ± 1.45^a	29.32 ± 1.52^a
a^*	16.62 ± 1.11^a	17.51 ± 0.73^a	16.95 ± 1.26^a
b^*	3.74 ± 0.61^a	3.56 ± 0.40^a	3.80 ± 0.52^a
ΔE^*	—	3.13	4.06

Values are expressed as mean $\pm$ standard deviation. Means followed by the same letter within the same row do not differ significantly according to Tukey's test ($p \leq 0.05$). L^* : lightness; a^* : variation from green (–) to red (+); b^* : variation from blue (–) to yellow (+). ΔE^* : total color difference calculated relative to the control formulation (F1). **F1**: control formulation; **F2**: formulation containing endospores only; **F3**: formulation containing endospores, vitamins, and minerals.

The CIELAB color system expresses color through three main coordinates. The L^* parameter represents lightness, ranging from 0 (black) to 100 (white). The a^* and b^* parameters correspond to chromatic coordinates, in which positive a^* values indicate a

tendency toward red and negative values indicate green, whereas positive b^* values indicate a yellow hue and negative values indicate blue (ISO & CIE, 2019).

L^* values ranged from 29.32 to 33.37 among the formulations. Although F2 and F3 presented numerically lower values than the control formulation, indicating lower lightness, no statistically significant differences were observed among the formulations ($p > 0.05$). Thus, under the evaluated conditions, the incorporation of endospores and micronutrients did not significantly affect the lightness of the gummy candies.

Regarding the a^* coordinate, all values were positive, ranging from 16.62 to 17.51, indicating a predominance of the reddish component. No statistically significant differences were observed among the formulations ($p > 0.05$). This behavior is consistent with the presence of the red coloring agent used in the formulation. For the b^* coordinate, the positive values, ranging from 3.56 to 3.80, indicate a slight contribution of the yellow component, also with no significant differences among F1, F2, and F3 ($p > 0.05$).

The total color difference (ΔE^*) showed greater changes in the enriched formulations compared with the control formulation. The formulation containing only endospores presented a ΔE^* of 3.13, whereas the formulation containing endospores, vitamin D3, vitamin K2, and magnesium malate presented a ΔE^* of 4.06. The greater difference observed in F3 may be associated with the distinct composition of this formulation, particularly the presence of vitamins and minerals, which may influence interactions among the matrix components and the coloring agent, thereby affecting the final color of the product.

Overall, the results demonstrate that the incorporation of *A. clausii* endospores, either alone or in combination with vitamin D3, vitamin K2, and magnesium malate, did not cause statistically significant changes in the L^* , a^* , and b^* colorimetric parameters of the gummy candies. Although numerical differences in lightness and total color difference were observed, the formulations exhibited colorimetric behavior similar to that of the control formulation under the evaluated conditions.

Shear Analysis

Texture is a fundamental quality parameter in food technology and is widely used to evaluate consumer acceptability. It plays a significant role in the overall sensory

experience and satisfaction, directly influencing how foods are perceived (Rune *et al.*, 2026).

Accordingly, Gunes *et al.* (2025) classified the texture values of chewy candies as very soft (0.18–0.77 N), soft (4.08–15.43 N), hard (56.39 N), and very hard (171.09 N).

In the present study, the gummy candies presented texture values of 5.07 ± 0.57 N for the base gummy candy, 4.13 ± 0.16 N for formulation 1, and 4.27 ± 0.06 N for formulation 2, indicating that all formulations fall within the soft gummy candy category, a characteristic typical of this type of product.

The formulations containing probiotics and micronutrients showed a slight decrease in texture firmness compared with the base gummy candy. This effect may be explained by interactions between the additional ingredients and the structural network formed by gelatin and collagen, which may affect the gel structure and result in a slightly softer matrix.

Calorimetric Analysis

The formulations developed in this study presented caloric values of approximately 4.06 kcal/g for formulation 1, 4 kcal/g for formulation 2, and 3.84 kcal/g for formulation 3. Considering that each product unit had an approximate average mass of 5.5 g, the energy value per unit corresponded to approximately 22.33 kcal, 22 kcal, and 21.12 kcal, respectively.

The energy values of F1 and F2 were similar, whereas F3 showed a numerical reduction compared with the other formulations, which may be attributed to the addition of micronutrients. These components do not provide a significant contribution to the caloric content of the formulation but may lead to a slight decrease in energy density. Overall, the results demonstrate that functional supplementation of the gummy candies did not compromise the energy profile of the product.

Overall, the results demonstrate that the incorporation of functional components, including *A. clausii* endospores, vitamins, and minerals, was accompanied by similar energy values among the formulations, with a more evident difference in F3. Thus, the inclusion of these components in the gummy candy matrix did not result in marked

changes in the energy value of the product, which remained within a range of approximately 21–22 kcal per unit.

Microbiological Quality Analysis

The microbiological analyses showed negative results for *Salmonella*, *Escherichia coli*, molds and yeasts, and *Staphylococcus* in all evaluated formulations (F1, F2, and F3), indicating good manufacturing, handling, and storage practices.

In addition, the results complied with the microbiological standards established by Brazilian legislation, according to Resolution RDC No. 331/2019 and Normative Instruction No. 60/2019, which establish the microbiological criteria applicable to foods intended for human consumption.

In Vitro Simulated Gastrointestinal Tract

The results of the *in vitro* simulated gastrointestinal tract (GIT) assay demonstrated maintenance of *Alkalihalobacillus clausii* endospore viability under the different conditions evaluated, with counts above 8.6 log CFU/g, as presented in Table 5. No significant differences were observed between F2 and F3 at any stage, indicating that the addition of vitamin D3, vitamin K2, and magnesium malate did not compromise endospore recovery during the simulation.

Table 5 – *A. clausii* endospore counts, expressed as log CFU/g, after exposure to in vitro GIT conditions.

Formulation 2				
Time	pH 2	Pepsin	Pancreatin	Bile salts
0h	9.21 ± 0.27 ^a	9.11 ± 0.30 ^a	9.03 ± 0.34 ^a	9.19 ± 0.24 ^a
1h	8.80 ± 0.26 ^a	9.42 ± 0.47 ^a	9.23 ± 0.23 ^a	9.27 ± 0.33 ^a
2h	9.20 ± 0.38 ^a	9.27 ± 0.38 ^a	9.17 ± 0.18 ^a	8.95 ± 0.11 ^a
3h	8.91 ± 0.27 ^a	9.50 ± 0.23 ^a	9.08 ± 0.32 ^a	9.14 ± 0.19 ^a
4h	9.13 ± 0.21 ^a	—	9.35 ± 0.21 ^a	9.09 ± 0.21 ^a
Formulation 3				
Time	pH 2	Pepsin	Pancreatin	Bile salts
0h	8.81 ± 0.11 ^a	9.01 ± 0.21 ^a	8.71 ± 0.22 ^a	8.68 ± 0.16 ^a
1h	8.90 ± 0.18 ^a	9.12 ± 0.19 ^a	8.93 ± 0.23 ^a	8.97 ± 0.26 ^a
2h	8.67 ± 0.23 ^a	8.97 ± 0.23 ^a	8.80 ± 0.37 ^a	9.10 ± 0.37 ^a
3h	8.78 ± 0.22 ^a	8.86 ± 0.37 ^a	9.01 ± 0.20 ^a	8.88 ± 0.21 ^a
4h	8.93 ± 0.29 ^a	—	8.64 ± 0.44 ^a	9.06 ± 0.28 ^a

Values are expressed as mean ± standard deviation. Means followed by the same letter within the same column do not differ significantly according to Tukey's test ($p \leq 0.05$). **F1**: control formulation; **F2**: formulation containing endospores only; **F3**: formulation containing endospores, vitamins, and minerals.

The scientific literature indicates that, for probiotics to exert beneficial effects on the host, at least 10^6 (6 log) to 10^7 (7 log) viable CFU must reach the colon (Treven *et al.*, 2024). In this context, the counts observed in this study, which were above 8.6 log CFU/g throughout the *in vitro* digestive simulation, indicate high resistance of the endospores to adverse GIT conditions.

The high maintenance of viability may be related to the characteristic resistance of *A. clausii* endospores to adverse conditions, such as low pH and exposure to digestive enzymes and bile salts (Sadrimovahed & Ulusoy, 2024). A similar result was observed by Delgado Junior *et al.* (2024), who found that *Bacillus clausii* spores maintained their viability after exposure to *in vitro* simulated GIT conditions.

These results reinforce the potential of *A. clausii* endospores for application in functional foods, since the intrinsic resistance of these biological structures contributes to greater stability during digestion and a greater likelihood of intestinal colonization.

4. Conclusion

The results of this study demonstrated the technological feasibility of using gummy candies as a matrix for the incorporation of *Alkalihalobacillus clausii* endospores, with maintenance of microbial viability throughout the 120-day storage period evaluated. The formulations containing endospores maintained counts ranging from approximately 8.8 to 9.5 log CFU/g throughout the 120 days of storage. As no significant differences were observed among the evaluated storage periods, the results indicate that the viability of *A. clausii* was maintained in the gummy candy matrix throughout storage under the experimental conditions used.

From a technological perspective, the formulations exhibited physicochemical, textural, and color characteristics appropriate for the developed matrix, without compromising the main characteristics evaluated. The microbiological analyses also indicated satisfactory microbiological quality in the formulations analyzed. The presence of vitamins and minerals in F3 did not compromise endospore stability or the technological characteristics evaluated.

Therefore, the results indicate that gummy candies have potential as a matrix for the incorporation and delivery of *Alkalihalobacillus clausii* endospores, considering the

458 maintenance of viability during storage and under simulated *in vitro* gastrointestinal tract
459 conditions.

460 The limitations of the study include the absence of sensory and consumer
461 acceptance evaluations, the use of an exclusively *in vitro* gastrointestinal model, and the
462 lack of determination of the bioavailability of vitamins D3 and K2 and magnesium.
463 Aspects related to economic feasibility, scalability, and industrial production were also
464 not evaluated. Therefore, future studies should include sensory evaluations, *in vivo*
465 models or clinical studies, assessment of micronutrient bioavailability, and analyses of
466 stability, cost, and scalability to further evaluate the technological potential of the
467 developed formulations.

468 **Acknowledgements**

469 This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de
470 Nível Superior – Brasil (CAPES) – Finance Code 001.

471 **CRedit Author Statement**

472 Manoel Armando Delgado Junior: Conceptualization, Methodology, Investigation, Data
473 curation, Formal analysis, Visualization, Writing – original draft.

474 Elizete Alves Proença Afonso: Methodology, Investigation, Data curation, Validation,
475 Writing – review and editing.

476 Claudini Ferreira Kumakura: Methodology, Investigation, Validation, Writing – review
477 and editing.

478 Danielle Marques Vilela: Conceptualization, Supervision, Validation, Writing – review
479 and editing.

480 Kelly Cristina da Silva Brabes: Conceptualization, Supervision, Project administration,
481 Resources, Writing – review and editing.

482 **Declaration of AI**

During the preparation of this manuscript, the authors used ChatGPT (OpenAI), Grammarly, and Consensus to assist with English language editing, grammar correction, improvement of writing clarity, literature exploration, and reference formatting. All suggestions generated by these tools were critically reviewed, verified, and edited by the authors. The authors take full responsibility for the content of the final manuscript, including the interpretation of results, conclusions, and scientific accuracy.

Declaration of Conflict of Interest

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

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Referências

- AOAC. AOAC guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals. Association of Official Analytical Chemists, Gaithersburg, MD, USA. pp. 5–13 (2019).
- Brazil, Agência Nacional de Vigilância Sanitária. (2018). *Resolution of the Collegiate Board – RDC No. 241, of July 26, 2018: Establishes requirements for demonstrating the safety and health benefits of probiotics for use in foods*. Ministry of Health. https://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2018/rdc0241_26_07_2018.pdf
- Brazil, Agência Nacional de Vigilância Sanitária. (2019a). *Normative Instruction No. 60, of December 23, 2019: Establishes the lists of microbiological standards for foods*. Ministry of Health. https://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2019/int0060_23_12_2019.pdf
- Brazil, Agência Nacional de Vigilância Sanitária. (2019b). *Resolution of the Collegiate Board – RDC No. 331, of December 23, 2019: Provides for microbiological standards for foods and their application*. Ministry of Health. https://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2019/rdc0331_23_12_2019.pdf
- Costa, B. V., de Oliveira, I. V., Miranda, J. S., de Lima, D. C. N., Benevenuto, W. C. A. do N., Martins, M. L., Leite Júnior, B. R. de C., da Silva, R. R., & Martins, E. M. F. (2024). Development and characterization of probiotic pectin candy with jussara and passion fruit. *Ciência e Natura*, 46, e84112. <https://doi.org/10.5902/2179460X84112>

- 515 Delgado Junior, M. A., Vilela, D. M., & Brabes, K. C. S. (2024). Evaluation of the
516 viability of *Bacillus clausii* spores in a simulation of the human gastrointestinal tract.
517 *Revista Brasileira Multidisciplinar*, 27(3), 103–110. [https://doi.org/10.25061/2527-](https://doi.org/10.25061/2527-2675/ReBraM/2024.v27i3.1973)
518 2675/ReBraM/2024.v27i3.1973
- 519 Dragomir, N., Grigore, D.-M., & Pogurschi, E. N. (2025). Beyond sugar: A holistic
520 review of sweeteners and their role in modern nutrition. *Foods*, 14(18), 3182.
521 <https://doi.org/10.3390/foods14183182>
- 522 Food and Agriculture Organization of the United Nations, & World Health
523 Organization. (2001). *Health and nutritional properties of probiotics in food including*
524 *powder milk with live lactic acid bacteria: Report of a Joint FAO/WHO Expert*
525 *Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food*
526 *including Powder Milk with Live Lactic Acid Bacteria*. FAO/WHO.
527 <https://www.fao.org/4/y6398e/y6398e.pdf>
- 528 Gunes, R., Sözeri Atik, D., Palabiyik, I., Konar, N., Karahan, D., & Tayyem, R. (2025).
529 Plant-derived gum as a gelatin alternative in the formulation of probiotic-enriched toffee
530 candy. *Applied Food Research*, 5(2), 101137.
531 <https://doi.org/10.1016/j.afres.2025.101137>
- 532 Instituto Adolfo Lutz. (2008). *Métodos físico-químicos para análise de alimentos:*
533 *Normas analíticas do Instituto Adolfo Lutz* (4th ed., 1st digital ed.). Instituto Adolfo
534 Lutz.
- 535 International Organization for Standardization, & Commission Internationale de
536 l'Éclairage. (2019). *ISO/CIE 11664-4:2019: Colorimetry—Part 4: CIE 1976 Lab colour*
537 *space**. ISO.
- 538 Kahraman, B., Korkmaz, K., Daştan, D., Toker, Ö. S., Dertli, E., & Arici, M. (2023).
539 Production and characterization of probiotic jelly candy containing *Bacillus* species.
540 *Journal of Food Measurement and Characterization*, 17(6), 5864–5873.
541 <https://doi.org/10.1007/s11694-023-02076-3>
- 542 Liu, X., Mao, B., Tang, X., Zhang, Q., Zhao, J., Chen, W., & Cui, S. (2025). Bacterial
543 viability retention in probiotic foods: A review. *Critical Reviews in Food Science and*
544 *Nutrition*, 65(32), 7964–7986. <https://doi.org/10.1080/10408398.2025.2488228>
- 545 Meira, S. M. M., Silva, T. M., Jacques, A. C., Moura, C. M. de, Nalerio, E. S., Giongo,
546 C., & Cogo, S. L. (2024). Desenvolvimento de balas de gelatina probióticas sem açúcar
547 adicionado. *Brazilian Journal of Health Review*, 7(9), e74650.
548 <https://doi.org/10.34119/bjhrv7n9-126>.
- 549 Perotti, S., Mantegazza, G., Pierallini, E., Kirika, N., Duncan, R., Telesca, N., Sarrica,
550 A., & Guglielmetti, S. (2024). Human in vivo assessment of the survival and
551 germination of *Heyndrickxia coagulans* SNZ1969 spores delivered via gummy candies.
552 *Current Research in Food Science*, 9, 100793.
553 <https://doi.org/10.1016/j.crfs.2024.100793>

- 554 Rune, C. J. B., Clausen, M. P., & Giacalone, D. (2026). Sensory evaluation of plant-
555 based cheese: A systematic review with a focus on texture and mouthfeel. *Critical*
556 *Reviews in Food Science and Nutrition*, 66(4), 754–779.
557 <https://doi.org/10.1080/10408398.2025.2531220>
- 558 Sadrimovahed, M., & Ulusoy, B. H. (2024). *Bacillus clausii*: A review into story of its
559 probiotic success and potential food applications. *Fermentation*, 10(10), 522.
560 <https://doi.org/10.3390/fermentation10100522>
- 561 Silva, N. D., et al. (2017). *Manual de métodos de análise microbiológica de alimentos e*
562 *água* (5th ed.). Blucher.
- 563 Tarahi, M., et al. (2023). Physicochemical and sensory properties of vegan gummy
564 candies enriched with high-fiber Jaban watermelon exocarp powder. *Foods*, 12(7),
565 1478. <https://doi.org/10.3390/foods12071478>
- 566 Treven, P., et al. (2024). The effect of food matrix taken with probiotics on the survival
567 of commercial probiotics in simulation of gastrointestinal digestion. *Foods*, 13(19),
568 3135. <https://doi.org/10.3390/foods13193135>.
- 569

Conclusão

Este trabalho demonstrou a viabilidade do chocolate e da bala de goma como matrizes alimentares não lácteas para a incorporação e veiculação de endósporos de *Alkalihalobacillus clausii*. Ambas as matrizes possibilitaram a manutenção da viabilidade do microrganismo durante os 120 dias de armazenamento avaliados, com contagens situadas entre 8,8 e 9,5 log UFC/g. A manutenção de elevadas contagens ao longo do período experimental evidencia a estabilidade dos endósporos nas condições estudadas e reforça seu potencial de aplicação em produtos alimentícios submetidos a diferentes condições de processamento e conservação.

A incorporação dos endósporos, tanto de forma isolada quanto associada às vitaminas D3 e K2 e ao magnésio na forma de malato, não comprometeu de maneira substancial as características físico-químicas e tecnológicas das formulações. Os resultados obtidos para composição centesimal, textura, cor e valor energético demonstraram que foi possível incorporar os componentes funcionais às diferentes matrizes, preservando, de modo geral, suas propriedades características.

Os ensaios de simulação gastrointestinal *in vitro* demonstraram a capacidade dos endósporos de *A. clausii* de permanecerem viáveis após a exposição às condições experimentais avaliadas, incluindo variações de pH e a ação de pepsina, pancreatina e sais biliares. Esse comportamento evidencia uma característica relevante para sua utilização em alimentos destinados à veiculação de microrganismos probióticos. Entretanto, por se tratar de um modelo *in vitro*, os resultados devem ser interpretados como evidência de resistência às condições simuladas, não permitindo, isoladamente, estabelecer a sobrevivência do microrganismo no trato gastrointestinal humano ou confirmar efeitos benéficos à saúde. Para isso, são necessários estudos *in vivo* e, posteriormente, ensaios clínicos adequadamente delineados.

Do ponto de vista higiênico-sanitário, as formulações apresentaram resultados compatíveis com os padrões estabelecidos pela legislação vigente para os parâmetros microbiológicos analisados, indicando conformidade microbiológica nas condições avaliadas. Esse resultado, associado à estabilidade dos endósporos durante o armazenamento, demonstra que as formulações desenvolvidas apresentaram condições

favoráveis à incorporação do microrganismo, sem comprometer os parâmetros de segurança microbiológica avaliados.

A contribuição desta tese reside na integração de diferentes abordagens experimentais para avaliar a aplicação de *A. clausii* em duas matrizes distintas, abrangendo aspectos microbiológicos, físico-químicos, tecnológicos e de resistência às condições gastrointestinais simuladas. Os dados obtidos fornecem subsídios experimentais para o desenvolvimento de estratégias de veiculação de endósporos em alimentos não lácteos e constituem uma base para investigações posteriores relacionadas à estabilidade, funcionalidade e aplicabilidade dessas formulações.

Apesar dos avanços alcançados, algumas etapas ainda são necessárias para avançar da viabilidade experimental para uma eventual aplicação comercial. Estudos adicionais de estabilidade sob diferentes condições de armazenamento e temperatura, avaliações sensoriais e de aceitabilidade, além da investigação da estabilidade dos compostos bioativos e micronutrientes ao longo do tempo, poderão ampliar a compreensão sobre o comportamento das formulações. Essas investigações serão importantes para determinar não apenas a estabilidade do microrganismo, mas também sua efetiva funcionalidade e possíveis efeitos fisiológicos após o consumo.

Por fim, a presente tese está alinhada à perspectiva interdisciplinar do Programa de Pós-Graduação em Ciência e Tecnologia Ambiental da Universidade Federal da Grande Dourados, ao integrar conhecimentos de microbiologia, biotecnologia, ciência e tecnologia de alimentos e segurança alimentar na abordagem de um problema de natureza científica e tecnológica. O desenvolvimento e a caracterização das matrizes propostas exigiram a articulação de diferentes áreas do conhecimento, desde a avaliação da estabilidade e viabilidade microbiológica até a caracterização físico-química, tecnológica e da resistência dos endósporos às condições gastrointestinais simuladas. Essa integração evidencia a possibilidade de abordar desafios relacionados ao desenvolvimento de alimentos funcionais a partir de diferentes perspectivas, em consonância com a proposta do Programa de formar profissionais capazes de transitar entre áreas do conhecimento e pensar interdisciplinarmente problemas e desafios científicos e tecnológicos.