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CONTRIBUTION TO THE STUDY OF KEFIR AND ITS BY-PRODUCTS ON SKIN HEALTH

Tesis Doctoral presentada por

EMILIA CONCEIÇÃO AZEITONA BORBA CORREIA ALVES

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CONTRIBUCIÓN AL ESTUDIO DEL KEFIR Y SUS SUBPRODUCTOS EN LA SALUD DE LA PIEL

CONTRIBUIÇÃO PARA O ESTUDO DO KEFIR E SEUS SUBPRODUTOS NA SAÚDE DA PELE

Tesis Doctoral presentada por

Emilia Conceição Azeitona Borba Correia Alves

Directores:
Dra. Catarina Batista Fialho Rosado
Dra. Patricia Dias Mendonça Rijo
Dra. Maria Victorina Aguilar-Vilas

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Alcalá de Henares

Para as minhas adoradas filhas.

O caminho faz-se caminhando.
Fernando Pessoa

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RESUMEN AND ABSTRACT

RESUMEN

La piel es un órgano funcional con un papel fundamental en el mantenimiento de la homeostasis corporal. La disfunción de la barrera cutánea se correlaciona con la gravedad clínica de la dermatitis atópica (DA) y, junto con la desregulación inmunitaria, están en el inicio de la DA. La ingestión de probióticos ha mostrado efectos beneficiosos sobre la salud de la piel, concretamente en la reducción de la gravedad de la DA.

El kéfir es un alimento fermentado tradicional con numerosos supuestos beneficios para la salud, atribuidos a su composición microbiana única y excelente valor nutricional, por lo que se presenta como un probiótico de gran interés en la relación entre el intestino y la piel. Sin embargo, en la literatura aún no existe evidencia sobre el impacto de una dieta que contiene kéfir en la salud de la piel, ya sea sana o atópica.

Este trabajo exploró el efecto de la ingestión de kéfir en la piel de individuos sanos y atópicos, así como su efecto adicional sobre los síntomas gastrointestinales funcionales, como estreñimiento o diarrea.

En primer lugar, se examinó el perfil fisicoquímico y nutricional del kéfir producido en condiciones domésticas tradicionales mediante la fermentación de leche de vaca pasteurizada semidesnatada utilizando granos de kéfir. Se evaluó adicionalmente la estabilidad y la conformidad nutricional del kéfir fresco y refrigerado. Además, también se exploró la aceptación de la bebida de kéfir en una muestra de consumidores portugueses, una vez que el kéfir se caracteriza por un sabor y aroma únicos y no se consume tradicionalmente en Portugal.

Los resultados de esta investigación mostraron que la ingestión de kéfir producido en condiciones domésticas promovió una mejora de la función de barrera cutánea tanto en pieles sanas como atópicas, con una mejora adicional en el grado de severidad de la DA, todas las cuales fueron verificado solo en los grupos que ingirieron kéfir. Este estudio también mostró una mejora en los síntomas gastrointestinales funcionales, solo en los grupos de ingesta de kéfir, tanto en sujetos de piel sana como atópica.

Hasta donde sabemos, este fue el primer estudio *in vivo*, realizado en humanos, para proporcionar información sobre el impacto de la ingestión de kéfir casero en la salud de la piel y los síntomas gastrointestinales funcionales, en personas con piel sana y atópica.

En general, este trabajo hizo una contribuición crucial a la caracterización de un producto alimenticio que se consume ampliamente en todo el mundo, centrándose en el kéfir que se produce en un ambiente doméstico típico; proporcionó información valiosa sobre la evaluación de la salud de la piel, particularmente la piel atópica; y contribuyó fundamentalmente a reforzar la hipótesis del efecto beneficioso del kéfir sobre la piel a través del eje intestino-piel, por lo tanto estableciendo y abriendo la posibilidad de continuar la investigación sobre el impacto del probiótico kéfir en la salud de la piel y su mecanismo de acción a saber, posible a través del eje intestino-piel.

Palabras clave: kéfir, probiótico, salud de la piel, dermatitis atópica, eje intestino-piel

ABSTRACT

The skin is a functional organ with a fundamental role in the maintenance of body homeostasis. Skin barrier dysfunction correlates with atopic dermatitis (AD) clinical severity and together with immune dysregulation are at the onset of AD. Ingestion of probiotics has shown beneficial effects on skin health, namely in the reduction of severity in AD.

Kefir is a traditional fermented food with numerous putative health benefits, attributed to its unique microbial composition and excellent nutritional value, thereby presenting itself as a probiotic of the utmost interest in the gut-skin relationship. However, in the literature there is still no evidence on the impact of a diet containing kefir on skin health, of either healthy or atopic.

This work explored the effect of kefir ingestion on the skin of healthy and atopic individuals, as well as its additional effect on the functional gastrointestinal symptoms, such as constipation or diarrhea. Firstly, the physicochemical and nutritional profile of kefir produced under traditional domestic conditions by fermentation of pasteurized semi-skimmed cow's milk using kefir grains was examined. The stability and nutritional compliance of the kefir freshly made and refrigerated was further evaluated. Additionaly, the acceptance of the kefir drink in a sample of Portuguese consumers was also explored, once kefir is characterized by a unique flavor and aroma and it is not traditionally consumed in Portugal.

The results of this research showed that the ingestion of kefir produced under household conditions promoted an improvement in the cutaneous barrier function in both healthy and atopic skin, with an additional improvement in the degree of severity of AD, all of which were verified only in the groups that ingested kefir. This study also showed an improvement on functional gastrointestinal symptoms, only in the kefir intake groups, on both healthy and atopic skin subjects.

To our knowledge, this was the first *in vivo* study, performed in humans, to provide information on the impact of homemade kefir ingestion on skin health and functional gastrointestinal symptoms, in individuals with healthy and atopic skin.

Overall, this work made a crucial contribution to the characterization of a food product so widely consumed around the world by focusing on kefir that was produced in a typical domestic environment; provided valuable information on the assessment of skin health, particularly atopic skin; and fundamentally contributes to reinforcing the hypothesis of the beneficial effect of kefir on the skin through the intestine-skin axis, thus establishing and opening up the possibility of continuing the research on the impact of the probiotic kefir on skin health and its mechanism of action, possibly via the gut-skin axis.

Keywords: kefir, probiotic, skin health, atopic dermatitis, gut-skin axis

This work gave rise to the following publications:

1. Alves, E.; Rijo, P.; Rodrigues, L.M.; Rosado, C. Determination of Relevant Endpoints to Evaluate the *in Vivo* Barrier Function in Cutaneous Health. Biomed. Biopharm. Res. 2019, 16, 80–88, doi:10.19277/bbr.16.1.201.

2. Alves, E.; Rijo, P.; Rodrigues, L.M.; Rosado, C. Probiotics in the Gut-Skin Axis – the Case of Kefir. *Biomed. Biopharm. Res.* 2021, *18*, 1–15, doi:10.19277/bbr.18.261.

3. Alves, E.; Ntungwe, E.N.; Gregório, J.; Rodrigues, L.M.; Pereira-Leite, C.; Caleja, C.; Pereira, E.; Barros, L.; Aguilar-Vilas, M.V.; Rosado, C.; et al. Characterization of Kefir Produced in Household Conditions: Physicochemical and Nutritional Profile, and Storage Stability. Foods 2021, 10, 1–16, doi:10.3390/foods10051057.

4. Alves, E.; Rijo, P.; Rodrigues, L. M.; Rosado, C. Acceptability of kefir produced by fermentation of Portuguese milk with CIDCA AGK1 grains in a sample of Portuguese consumers. Biomed. Biopharm. Res. 2021, 18(1), 1–9, doi: 10.19277/bbr.18.1.252.

5. Alves, E.; Gregório, J.; Baby, A.R.; Rijo, P.; Rodrigues, L.M.; Rosado, C. Homemade Kefir Consumption Improves Skin Condition - A Study Conducted in Healthy and Atopic Volunteers. Foods 2021, 10, 2794, doi: 10.3390/foods10112794.

6. Alves, E.; Gregório, J.; Rijo, P.; Rodrigues, L.M.; Rosado, C. Kefir as a modulator of the gut-skin axis: a study conducted in healthy and atopic volunteers. Foods 2021 (In *Submission)*.

This work gave rise to the following presentations at scientific events:

Emília Alves, João Gregório, Patrícia Rijo, Luís Monteiro Rodrigues, Catarina Rosado. Can the consumption of probiotic drinks improve skin health?. 13th International Congress of Pharmaceutical Sciences (CIFARP), Brazil, 2021.

Emília Alves, João Gregório, Patrícia Rijo, Luís Monteiro Rodrigues, Catarina Rosado. Kefir intake can improve skin health?. 33rd Congresso Brasileiro de Cosmetologia. Associação Brasileira de Cosmetologia, Brazil ,2021.

Emília Alves, João Gregório, Patrícia Rijo, Luís Monteiro Rodrigues, Catarina Rosado. Variabilidade na função de barreira da pele de indivíduos saudáveis, induzida pela ingestão de kefir. XXV Congresso Latino-Americano e Ibérico de Quimicos Cosmeticos-COLAMIQC- I Virtual, 2021.

Alves E, Gregório J, Rijo P, Rodrigues LM, Rosado C. Probiotics and skin health – impact of kefir consumption assessed by an SLS-inducing lesion model. IV CBIOS Science Seminar 2020, Lisbon. Portugal.

Alves E, Rijo P, Rodrigues LM, Rosado C. Assessment of the acceptability of kefir fermented drink in a panel of Portuguese consumers. IV CBIOS Science Seminar 2020, Lisbon. Portugal.

Alves E, Gregório J, Rijo P, Rodrigues LM, Rosado C. Impact of kefir consumption on cutaneous health assessed using an SLS-induced lesion model. 55th Annual Congress of SBFIS. Sao Paulo, Brazil, 2020.

Alves E, Rijo P, Rodrigues LM, Rosado C. Assessment of the impact of oral intake of the probiotic kefir in cutaneous health. Physioma 2019 - 1st International Meeting of the Portuguese Society of Physiology. Lisbon, Portugal, 2019.

Alves E, Nicolai M, Fonte P, Costa J, Rodrigues LM, Rijo P, Rosado C. Characterization of Kefir beverage produced from Portuguese cow milk. I Bio.Natural - Bioactive Natural Products Research Meeting. Lisbon, Portugal 2019.

Alves E, Rijo P, Rodrigues LM, Rosado C. Compliance with the *Codex alimentarius* of kefir beverage produced from Portuguese cow milk. I Bio.Natural - Bioactive Natural Products Research Meeting. Lisbon, Portugal, 2019.

Alves E, Costa MC, Rodrigues LM, Rijo P, Rosado C. Contribuição para o estudo dos efeitos do kefir e seus subprodutos sobre a saúde da pele. Conferência Conjunta sobre Investigação em Produtos Naturais e Avícolas-Iniciativa conjunta CBIOS – CYTED. Lisboa, Portugal, 2018.

BACKGROUND

Article 1

Probiotics in the gut-skin axis – the case of kefir

Emilia Alves[1,2], Patricia Rijo[1,3], Luis Monteiro Rodrigues[1*],Catarina Rosado[1*]

[1] CBIOS - Universidade Lusófona's Research Center for Biosciences and Health Technologies, Av. Campo Grande 376, 1749-024 Lisbon, Portugal.
[2] Department of Biomedical Sciences, Faculty of Pharmacy, University of Alcalá, Campus Universitario, 28871 Alcalá de Henares, Spain.
[3] Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisbon, Portugal

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Probiotics in the gut-skin axis – the case of kefir

Probióticos no eixo intestino-pele - o caso do kefir

Emilia Alves [1,2], Patricia Rijo [1,3], Luis Monteiro Rodrigues [1⌘],Catarina Rosado [1*⌘]

[1]CBIOS - Universidade Lusófona's Research Center for Biosciences and Health Technologies, Av. Campo Grande 376, 1749-024 Lisbon, Portugal; [2]Department of Biomedical Sciences, Faculty of Pharmacy, University of Alcalá, Campus Universitario, 28871 Alcalá de Henares, Spain; [3]Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisbon, Portugal

*corresponding author: catarina.rosado@ulusofona.pt

[⌘]shared senior mentorship / supervisão senior partilhada

Abstract

The intestinal microbiota is linked to important functions in the host. Alterations in its composition and/or its by-products, causing loss of homeostasis, contribute to dysfunctions in other organic systems, including the skin, hence suggesting a gut-skin relationship. The oral administration of probiotics, widely associated with improved intestinal health, can act through an immunomodulatory response, both locally and systemically, presenting itself as potentially beneficial in inflammatory skin diseases such as atopic dermatitis. Traditional kefir, consumed for centuries as a health-promoting natural food, has its biological activity attributed both to the presence of a complex microbiota and to the action of the metabolites released during fermentation. The biological activity of kefir has been demonstrated in part by its ability to positively impact the intestinal microbiota, mainly based on animal models and *in vitro*, thus providing limited information. The nutritional and microbiological value of kefir makes its application as a probiotic in the gut-skin relationship a topic of of significant interest.
This review aimed to explore the impact of probiotics as regulators of the gut-skin axis, focusing on the current knowledge of kefir as a health-promoting food.

Keywords: kefir, probiotic, gut-skin axis, skin health, atopic dermatitis

Resumo

A microbiota intestinal está ligada a importantes funções no hospedeiro. Alterações em sua composição e / ou subprodutos, causando perda da homeostase, contribuem para disfunções em outros sistemas orgânicos, incluindo a pele, sugerindo uma relação intestino-pele. A administração oral de probióticos, amplamente associada à melhora da saúde intestinal, pode atuar por meio de uma resposta imunomoduladora, quer local quer sistemicamente, apresentando-se como potencialmente benéfica em doenças inflamatórias da pele como a dermatite atópica. O kefir tradicional, consumido durante séculos como um alimento natural promotor de saúde, tem a sua actividade biológica atribuída à presença de uma microflora complexa, bem como à acção dos metabolitos libertados durante a fermentação. A actividade biológica de kefir parcialmente demonstrada pela sua capacidade de influenciar positivamente a microbiota intestinal, tem sido baseada principalmente em modelos animais e *in vitro*, proporcionando assim informação limitada. O valor nutricional e microbiológico do kefir torna sua aplicação como um probiótico na relação intestino-pele de grande interesse.
Esta revisão teve como objetivo explorar o impacto dos probióticos enquanto reguladores do eixo intestino-pele, focando o conhecimento atual do kefir como um alimento promotor de saúde.

Palavras-chave: kefir, probiótico, eixo intestino-pele, saúde da pele, dermatite atópica

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Introduction

The adult human intestine includes a complex ecosystem of microorganisms referred to as gut microbiota (1). The intestinal microbiota is linked to important functions in the host, including digestion of fermentable carbohydrates into short-chain fatty acids (SCFAs) used as an energy source for intestinal cells; production of key nutrients such as essential vitamins and amino acids; protection against pathogens and regulation of the immune system (2,3,4,5). Under normal conditions, intestinal barrier function is highly efficient due to a complex network of mechanisms including a mucus layer, junction proteins, antimicrobial factors, and adaptive immune cells (5,6). However, changes in both the quantitative and qualitative composition of the microbiota, designated as intestinal dysbiosis, potentiates the disruption of these conditions, resulting in loss of homeostasis and, consequently, contributing to a disease state (4,7,8,9). This connection between the gut microbiome and human health foresees that the factors affecting microbial composition can indirectly modulate disease states. Among these, the inclusion of probiotics in the host's diet plays a prominent role, both for its nutritional value and easy digestion, and for the growing predisposition of individuals to consume foods perceived as healthy (3,6,10,11,12). Traditional kefir, originating in the Caucasus Mountains, has been consumed for centuries. Current knowledge supports the historical consideration of kefir as a health-promoting natural food (13,14,15), and this review aimed to explore the impact of probiotics from kefir as regulators of the gut-skin axis.

Gut-Skin Axis

The intestinal microbiota and its by-products have been shown to affect other organic systems, including the skin, thus demonstrating the existence of a gut-skin relationship (16,17,18). This influence can manifest itself directly via modulation of the immunological response, or indirectly through the secretory activity of the intestinal epithelium and the impact of the host's diet (19,20,21).

Alterations in the balance of the gut-skin relationship are associated with dysfunctions both at the gastrointestinal and skin levels. Changes in the intestinal microbiota associated with increased intestinal permeability can impact the immune system, thus promoting systemic inflammation, and allowing the direct migration of

Introdução

O intestino humano adulto inclui um ecossistema complexo de microrganismos conhecidos como microbiota intestinal (1). A microbiota intestinal está ligada a funções importantes no hospedeiro, como digestão de hidratos de carbono fermentáveis em ácidos gordos de cadeia curta (AGCCs) que são usados como fonte de energia para as células intestinais; produção de nutrientes, como síntese de vitaminas e aminoácidos essenciais; proteção contra patógenos e regulação do sistema imunológico (2,3,4,5). Em condições normais, a função da barreira intestinal é altamente eficiente devido a uma complexa rede de mecanismos como a presença de uma camada de muco, proteínas de junção, fatores antimicrobianos e células imunes adaptativas (5,6). Porém, alterações, quer quantitativas quer qualitativas, na composição da microbiota, denominadas disbiose intestinal, potenciam a perturbação dessas condições, resultando na perda da homeostase, contribuindo consequentemente para o estado de doença (4,7,8,9). Esta conexão entre o microbioma intestinal e a saúde humana prevê que os fatores que afetam a composição microbiana possam indiretamente modular os estados de doença. Dentre estes, a dieta do hospedeiro, incluindo a ingestão de probióticos, desempenha papel de destaque, tanto pelo seu valor nutricional e de fácil digestão, quanto pela crescente predisposição dos indivíduos a consumir alimentos percebidos como saudáveis (3,6,10,11,12). O kefir tradicional, originário da Cordilheira do Cáucaso, é consumido há séculos. O conhecimento atual corrobora a visão histórica do kefir como um alimento natural promotor da saúde (13,14,15), pelo que esta revisão teve como objetivo explorar o impacto dos probióticos do kefir como reguladores do eixo intestino-pele.

Eixo intestino-pele

A microbiota intestinal e seus subprodutos têm a capacidade de afetar outros sistemas orgânicos, incluindo a pele, demonstrando assim a existência de uma relação intestino-pele (16,17,18). Essa influência pode manifestar-se diretamente pela modulação da resposta imunológica, ou indiretamente, pela atividade secretora do epitélio intestinal e pelo impacto da dieta do hospedeiro (19,20,21). Alterações no equilíbrio da relação intestino-pele estão associadas a disfunções tanto no nível gastrointestinal quanto na pele. Alterações na microbiota intestinal associadas ao aumento da permeabilidade intestinal podem impactar o sistema

inflammatory products into the circulation. When these products reach the skin, skin homeostasis can be impaired, thus reinforcing the existence of a link between the intestinal microbiota and dermatological diseases (1,3,4,22,23). Although the mechanisms of action are still unclear, considering the current knowledge, the response to intestinal environmental changes seems to involve a combination of factors that lead to a state of systemic inflammation, thus affecting the skin. Moreover, intestinal dysbiosis has been found to be a common factor in inflammatory skin diseases such as atopic dermatitis, rosacea, acne, and psoriasis, thus supporting the bidirectionality of this axis (22,24,25,26).

Probiotics in the modulation of the gut

By definition, probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts (27). However, growing evidence suggests that non-microbial components, such as microbial metabolites and cell wall compounds, can also positively affect human health (16,28,29). In addition to their nutritional benefits, the use of probiotics has been widely associated with improved intestinal health, whether by improving the intestinal barrier function, modulating the immune system and antimicrobial effect against intestinal pathogens, or by producing metabolites with anti-inflammatory action, such as SCFA acetate, propionate, and butyrate (5,28,30,31,32,33,34,35). The mechanisms of action, however, have yet to be fully identified. Moreover, no product with health claims associated with the administration of probiotics has yet been approved by the European Food Security Authority (EFSA) (36).

Probiotics and skin health

The integrity of the skin barrier is critical for skin defense and immune performance (35,37,38). Immune skin diseases such as rosacea, acne, and atopic dermatitis are associated with the breakdown of the skin barrier, whereas its restoration is associated with an improvement in clinical outcomes (38,39,40,41).

Probiotics can modulate the immune response locally or systemically (1,6,23,24,40). Topical application of probiotics reduces pro-inflammatory molecules, hence controlling the spread of skin inflammation in acne, and produces anti-inflammatory molecules via

imunológico, promovendo inflamação sistémica, além de permitir a migração direta de produtos inflamatórios para a circulação. Quando esses produtos atingem a pele pode ocorrer perturbação na homeostase cutânea, reforçando a existência de um elo entre a microbiota intestinal e as doenças dermatológicas (1,3,4,22,23). Embora os mecanismos de ação ainda sejam obscuros, considerando-se os conhecimentos atuais, a resposta às alterações ambientais intestinais parece envolver uma combinação de fatores que levam a um estado de inflamação sistémica, afetando a pele. Além disso, em doenças inflamatórias da pele como dermatite atópica, rosácea, acne e psoríase, a disbiose intestinal é considerada um fator comum, apoiando desse modo a bidirecionalidade deste eixo (22,24,25,26).

Probióticos na modulação do intestino

Probióticos são, por definição, microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefício à saúde do hospedeiro (27). No entanto, evidências crescentes sugerem que componentes não microbianos, como metabolitos microbianos e compostos da parede celular, também podem afetar positivamente a saúde humana (16,28,29). O uso de probióticos, além dos seus benefícios nutricionais, tem sido amplamente associado à melhoria da saúde intestinal, seja pela melhoria da função de barreira intestinal, pela modulação do sistema imunológico e pelo efeito antimicrobiano contra patógenos intestinais, seja pela produção de metabolitos com ação antiinflamatória, como os AGCC, acetato, propionato e butirato, apesar dos seus mecanismos de ação não estarem ainda totalmente identificados (5,28,30,31,32,33,34,35). Além disso, nenhum produto com alegações de saúde associadas à administração de probióticos foi ainda aprovado pela Autoridade Europeia de Segurança Alimentar (EFSA) (36).

Probióticos e a saúde da pele

A integridade da barreira cutânea é crítica para a defesa da pele e para o desempenho imunológico (35,37,38). Doenças imunológicas da pele, como rosácea, acne e dermatite atópica, estão associadas à quebra da barreira cutânea, enquanto que a sua restauração está associada a uma melhoria nos desfechos clínicos (38,39,40,41). Os probióticos podem modular a resposta imune, local ou sistemicamente (1,6,23,24,40). A aplicação

dendritic cells in atopic dermatitis (AD) (16,22,42). In addition to improving the intestinal barrier function, oral administration of probiotics can modulate the immune response and reduce systemic inflammation, thereby improving skin health through the gut-skin axis (22,23,41). Research on the contribution of probiotics to skin health has focused on skin conditions such as AD, acne, wound healing, and skin barrier improvement (17,18,22,26,41,43,44). Furthermore, increasing evidence suggests that in addition to the microbial effect, non-microbial components such as microbial metabolites and cell wall compounds may also have beneficial effects, including benefits to skin health (16,28,29,45).

Immunological modulation of probiotics and skin health

The immunological impact of probiotics has been demonstrated by their ability to upregulate regulatory T cells (Treg) and Type-1 T-helper (Th1) cells, responsible for the production of anti-inflammatory cytokines such as interleukin-10 (IL-10), in addition to their ability to downregulate Type-2 and Type-17 T-helper cells (Th2 and Th17, respectively) responsible for the production of pro-inflammatory cytokines such as interferon (INF)-γ, interleukin-4 (IL-4) and interleukin-5 (IL-5) (5,46). Furthermore, probiotics also suppress the maturation of dendritic cells leading to inhibition of naive T cell differentiation into Th2 cells, thus fighting skin inflammation (35,47,48,49).

The role of microbial metabolites of probiotics in skin health

Several bacterial metabolites can enhance immunological response, thus leading to beneficial dermal effects (29). Lactic acid is the major product of metabolization of carbohydrates by either homo- or heterofermentative lactic-acid bacteria (LAB), which can be produced in sufficient concentrations to exhibit antibacterial activity against most pathogenic dermal bacteria (29,45,50,51). Lactic acid has been documented as part of the natural moisturizing factor (NMF) that retains moisture in the skin, and it plays important roles in the physical properties of the stratum corneum (29). Acetic acid, also produced by heterofermentative LAB, has been shown to exert antibacterial effects on different bacterial species, likely due to its pH lowering capability, thereby creating an environment unsuitable for pathogen growth (29,45). Diacetyl can also be produced by some strains of *Lactobacilli* and *Bifidobacteria* at concentrations that

tópica de probióticos reduz a produção de moléculas pró-inflamatórias, controlando assim a propagação da inflamação da pele no acne, além de produzir moléculas anti-inflamatórias, via células dendríticas, na dermatite atópica (DA) (16,22,42). A administração oral de probióticos, além de melhorar a função de barreira intestinal, pode modular a resposta imune e reduzir a inflamação sistémica, melhorando a saúde da pele através do eixo intestino-pele (22,23,41). Pesquisas sobre a contribuição dos probióticos para a saúde da pele focaram doenças da pele como DA, acne, cicatrização de feridas e melhoria da barreira cutânea (17,18,22,26,41,43,44). Adicionalmente, evidências crescentes sugerem que, além do efeito microbiano, componentes não microbianos, como metabolitos microbianos e compostos da parede celular, também podem exercer efeitos benéficos para a saúde, inclusive na saúde da pele (16,28,29,45).

Modulação imunológica de probióticos e saúde da pele

O impacto imunológico dos probióticos tem sido demonstrado pela sua capacidade de regular positivamente as células T reguladoras (Treg) e as células T auxiliares do tipo 1 (Th1), responsáveis pela produção de citocinas antiinflamatórias, como a interleucina-10 (IL-10), além de regular negativamente as células T auxiliaries do tipo 2 (Th2) e do tipo 17 (Th17), responsáveis pela produção de citocinas pró-inflamatórias, como interferon (INF)-γ, interleucina-4 (IL-4) e interleucina-5 (IL-5) (5,46). Adicionalmente, os probióticos também suprimem a maturação das células dendríticas, levando à inibição da diferenciação das células T naïve em células Th2, combatendo assim a inflamação da pele (35,47,48,49).

O papel dos metabólitos microbianos dos probióticos na saúde da pele

Vários metabolitos bacterianos podem aumentar a resposta imunológica, conduzindo a efeitos dérmicos benéficos (29). O ácido láctico é o principal produto da metabolização de hidratos de carbono por bactérias ácido-lácticas homo e heterofermentativas (BAL), que podem produzi-lo em concentrações suficientes para exibir atividade antibacteriana contra a maioria das bactérias dérmicas patogénicas (29,45,50,51). O ácido lático foi documentado como fazendo parte do fator de hidratação natural (NMF), que retém a humidade da pele e desempenha papéis importantes nas propriedades físicas do estrato córneo (29). O ácido acético, também produzido por BAL heterofermentativas, demonstrou exercer efeitos antibacterianos em diferentes espécies

suggest its potential dermal antimicrobial activities (45). Lipoteichoic acid (ALT) and peptidoglycan (PG) are structural components of cell walls and play a vital role in their growth and physiology, with evidence that their production by LAB can be sufficient to increase the dermal cell defense against bacterial infection (29,45). Moreover, PG from *Lactobacilli* demonstrated the ability to stimulate an immune response, thus contributing to skin protection (2,9,23), and can be effective even at low concentrations by synergism with LTA (25). Hyaluronic acid (HA), widely utilized in dermatology as a biomaterial and in the promotion of wound healing due to its highly osmotic nature, is relevant in controlling tissue hydration during inflammatory processes (52). To date, only certain strains of *Lactobacilli* are known to produce HA (45). Finally, sphingomyelinase (SMase), an enzyme that generates ceramides and sphingomyelin precursors necessary for the development of extracellular lipid bilayers in the stratum corneum, has demonstrated important activity for skin barrier function (29,53). SMase can be produced by strains of *Lactobacilli* and *Bifidobacteria* at sufficient concentrations to promote ceramide production in skin cells with the possibility to improve barrier properties (45).

Probiotics in Atopic Dermatitis

AD is a chronic inflammatory skin disease associated with an exacerbated skin response to environmental agents that, together with the disruption of the skin barrier integrity, promote a decrease in the antimicrobial response, thus enabling abnormal skin inflammation (38,54). Although the etiology remains unclear, AD onset points towards a complex interaction between skin barrier dysfunction, immune dysregulation, environmental risk factors, and dysbiosis of the intestinal and skin microbiota, which correlates with its clinical severity (54,55,56,57).

Immunological imbalance has been reported in AD patients, namely a decrease in Treg cells and an increase in Th2 cells and Th17 cells in the acute phase of the disease, whereas Th1 cells were associated with the chronic phase (23,26). Th17 cells were also positively correlated with AD severity (58). Current research has focused on the immunomodulatory effect of probiotics, as they are able to stimulate Treg cells and suppress Th2 cells mediated responses, which are the predominant immune responses in AD (23,26,41,46,59,60,61,62,63). However, evidence supporting their use for the treatment and prevention of AD is limited (40,41,61,64,65,66,67,68).

bacterianas, provavelmente devido à sua capacidade de diminuir o pH, criando assim um ambiente inadequado para o crescimento de patógenos (29,45). Também o diacetil pode ser produzido por algumas estirpes de *Lactobacilli* e *Bifidobacteria* em concentrações que sugerem sua potencial atividade antimicrobiana dérmica (45). O ácido lipoteicóico (ALT) e o peptidoglicano (PG) são componentes estruturais das paredes celulares bacterianas e desempenham um papel vital no seu crescimento e fisiologia, existindo evidência de que a sua produção por BAL consegue atingir quantidades suficientes para aumentar a defesa celular dérmica contra infecção bacteriana (29,45). Adicionalmente, o PG de *Lactobacilli* demonstrou capacidade para estimular a resposta imune, contribuindo assim para a proteção da pele (2,9,23), podendo ainda ser eficaz mesmo em baixas concentrações por sinergismo com o LTA (25). O ácido hialurónico (AH), amplamente utilizado na dermatologia como biomaterial e também na promoção da cicatrização de feridas devido à sua natureza altamente osmótica, é relevante no controle da hidratação dos tecidos durante os processos inflamatórios (52). Até o momento, apenas certas estirpes de *Lactobacilli* são conhecidas por produzir AH (45). Por fim, a esfingomielinase (SMase), uma enzima que gera ceramidas e precursores de esfingomielina para o desenvolvimento de bicamadas lipídicas extracelulares no estrato córneo, demonstrou atividade importante para a função de barreira da pele (29,53). SMase pode ser produzida por estirpes de *Lactobacilli* e *Bifidobacteria* em concentrações suficientes para promover a produção de ceramidas nas células da pele com a possibilidade de melhorar as propriedades de barreira (45).

Probióticos na Dermatite Atópica

A DA é uma doença inflamatória crónica da pele associada a uma resposta exacerbada da pele a agentes ambientais que, juntamente com a quebra da integridade da barreira cutânea, promovem uma diminuição na resposta antimicrobiana, possibilitando, assim, uma inflamação cutânea anormal (38,54). Embora a etiologia permaneça obscura, o início da DA aponta para uma complexa interação entre a disfunção da barreira cutânea, a desregulação imunológica, fatores de risco ambientais e disbiose da microbiota intestinal e cutânea, que se correlacionam com a sua gravidade clínica (54,55,56,57).

Desequilíbrios imunológicos têm sido relatados em pacientes com DA, nomeadamente a diminuição das células Treg e o aumento das células Th2 e Th17, na

The composition of the gut microbiota has been shown to be different in AD patients, which helps support the hypothesis that aberrant gut microbiota may underlie the onset or worsening of AD (23,59,69). Through the gut-skin axis, intestinal dysbiosis has the ability to negatively impact skin function, either by increasing epithelial permeability via pro-inflammatory cytokines, thus promoting immune dysregulation and contributing to the chronic systemic inflammation in AD, or by perpetuating pruritus via secretion of neuroendocrine itch mediators, leading to a chronic itch-scratch cycle, thus further disrupting the skin barrier (47,54,70,71). Consequently, the gut-skin axis may be receptive to modulation via dietary modification, which represents a potential complementary alternative in AD therapy (6,13). Despite growing evidence that probiotics can improve the intestinal disorders associated with AD, their use has not always proven to be effective, as the observed decrease in gut permeability may be insufficient to cause a discernible disease improvement (59,63,65).

To date, research conducted *in vivo* in human adults on the impact of probiotics on AD is scarce (40,41,48,59,61). Typically studied probiotics are *Lactobacillus*, *Bifidobacterium* and *Saccharomyces boulardii*, both isolated or in combination. In this sense, and due to the lack of consistency of the results obtained, it is plausible that some of the observed effects may be dependent on strains or species used, as well as on the microbial diversity and potential synergisms between microbes (26,44,63,72,73,74).

fase aguda da doença, enquanto as células Th1 foram associadas à sua fase crónica (23,26). As células Th17 também foram positivamente correlacionadas com a gravidade da DA (58). Investigação recente tem-se concentrado no efeito imunomodulador dos probióticos, face à sua capacidade de estimular as células Treg e suprimir as respostas mediadas pelas células Th2, que são as respostas imunes predominantes na DA (23,26,41,46,59,60,61,62,63). No entanto, as evidências atuais que apoiam o seu uso para o tratamento e prevenção da DA são limitadas (40,41,61,64,65,66,67,68).

A composição da microbiota intestinal mostrou ser diferente em pacientes com DA, o que ajuda a apoiar a hipótese de que uma microbiota intestinal aberrante pode estar subjacente ao início ou agravamento da DA (23,59,69). Através do eixo intestino-pele, a disbiose intestinal tem a capacidade de impactar negativamente a função da pele, seja pelo aumento da permeabilidade epitelial via citocinas pró-inflamatórias, promovendo assim a desregulação imunológica e contribuindo para a inflamação sistémica crónica na DA, ou perpetuando o prurido via secreção de mediadores neuroendócrinos, levando a um ciclo crónico de prurido-coçar, danificando ainda mais a barreira da pele (47,54,70,71). Consequentemente, o eixo intestino-pele pode ser receptivo à modulação por meio de alterações dietéticas, representando, portanto, uma potencial alternativa complementar na terapia da DA (6,13). Apesar das evidências crescentes de que os probióticos podem melhorar os distúrbios intestinais associados à DA, a sua utilização nem sempre se mostrou eficaz, pois a diminuição observada na permeabilidade intestinal pode ser insuficiente para causar uma melhora perceptível da doença (59,63,65).

Até o momento, pesquisas conduzidas *in vivo,* em humanos adultos, são escassas sobre o impacto dos probióticos na DA (40,41,48,59,61). Os probióticos normalmente estudados são *Lactobacillus, Bifidobacterium* e *Saccharomyces boulardii*, geralmente isolados ou em combinação. Nesse sentido, e devido à falta de consistência dos resultados obtidos, é plausível que alguns dos efeitos observados possam ser dependentes de estirpes ou espécies usadas, bem como da diversidade microbiana, devido a possíveis sinergismos entre micróbios (26,44, 63,72,73,74).

Kefir, a traditional and trendy probiotic beverage

Traditional kefir production uses kefir grains as a starter culture for the fermentation of milk, differentiating it from other fermented foods (75). The microorganisms present in the grains are responsible for the lactic, acetic, and alcoholic fermentation of the milk, originating a product with a viscous texture, a sour and slightly acidic taste, and a low alcohol and carbonation content (76,77). Although these microbiological mixtures may not be fully defined, this product is considered acceptable for human consumption by EFSA due to its long tradition of food production using the traditional fermentation substrate (e.g., cow milk) (36,78).

Currently, the growing demand for healthy foods has encouraged the consumption of kefir, drawing the attention of the food industry into its industrial production. However, due to the microbiological complexity of the kefir grains, maintaining the product quality in industrial production is problematic (79). Additionally, secondary yeast fermentation during storage compromised attempts to package traditionally produced kefir, further contributing to limit its large-scale production (80). Thus, pure cultures, composed of a mixture of bacteria with or without yeast, are used in current kefir-like industrial products. Despite their similar flavor, some of the health benefits typically ascribed to traditional kefir may not occur, in part due to this change in microbial diversity (77,81,82).

The microbiological composition of the fermented beverage is different from that of the grains and varies depending on its origin and cultivation method (75,83). The nutritional composition is influenced by the type of milk, the time and temperature of fermentation, and the storage conditions (51,84,85). Even so, traditionally produced kefir fulfills both microbiological and nutritional requirements (86,87).

The lactic acid produced by LAB and the presence of acetic acid produced by acetic-acid bacteria act as natural preservatives, resulting in a low contamination risk for the traditional homemade product (31,88). LAB also contribute to the organoleptic properties of the beverage by producing volatile compounds (e.g., acetaldehyde and acetyl), exopolysaccharides, and free amino acids (89,90). Yeasts produce alcohol and carbon dioxide that contribute to the characteristic mouth feel and taste of kefir (51). The biochemical composition of kefir is reflected in its nutritional value, which is typically around 3% protein, less than 10% fat, and at

Kefir, uma bebida probiótica tradicional e moderna

A produção tradicional de kefir usa grãos de kefir como cultura inicial para a fermentação do leite, diferenciando-o de outros alimentos fermentados (75). Os microrganismos presentes nos grãos são responsáveis pela fermentação láctica, acética e alcoólica do leite, originando um produto de textura viscosa, sabor azedo e ligeiramente ácido, baixo teor alcoólico e carbonatado (76,77). Uma longa tradição de produção de alimentos usando essas misturas microbiológicas não totalmente definidas e sendo o substrato de fermentação consistente com essa tradição (por exemplo, leite de vaca), torna este produto como aceitável para consumo humano pela EFSA (36,78).

Atualmente, a crescente demanda por alimentos saudáveis tem estimulado o consumo do kefir, atraindo a atenção da indústria alimentar para sua produção industrial. No entanto, devido à complexidade microbiológica dos grãos de kefir, manter a qualidade do produto na produção industrial é problemático (79). Além disso, as tentativas de embalar o kefir tradicionalmente produzido foram comprometidas pela fermentação secundária de leveduras durante o armazenamento, o que contribuiu ainda mais para limitar sua produção em larga escala (80). Assim, o uso de culturas puras, compostas por uma mistura de bactérias com ou sem leveduras, está na base dos atuais produtos industriais do tipo kefir. Apesar de seu sabor semelhante, alguns dos benefícios para a saúde, tipicamente atribuídos ao kefir tradicional, podem não ocorrer, em parte devido a esta diferença na diversidade microbiana (77,81,82).

A composição microbiológica da bebida fermentada é diferente da dos grãos e varia em função da origem dos grãos e do seu método de cultura (75,83). Já a composição nutricional é influenciada pelo tipo de leite, pelo tempo e temperatura de fermentação e pelas condições de armazenamento (51,84,85). No entanto, o kefir tradicionalmente produzido cumpre os requisitos microbiológicos e nutricionais (86,87).

O ácido lático produzido pelas BAL, potencializado pela presença do ácido acético produzido por bactérias ácido-acéticas, atua como conservante natural, permitindo que o produto caseiro tradicional tenha baixo risco de contaminação (31,88). As BAL também contribuiem para as propriedades organolépticas da bebida, produzindo compostos voláteis (por exemplo, acetaldeído e acetil), exopolissacarídeos e aminoácidos

least 0.6% lactic acid, according to the Codex criteria (86). Kefir can be refrigerated to maintain a shelf life of 3–12 days (77,81,91).

Health effects of kefir

Several health-promoting properties have been ascribed to kefir consumption (14,50,51,84,92,93,94,95). However, to date, most studies have been conducted *in vitro* with undigested kefir, or in animal models, thus limiting the prediction of the biological activity of kefir in humans (96,97,98). Among the reported health activities of kefir are the improved lactose digestion, hypocholesterolemic effect, reduction of insulin resistance and antihypertensive effect, anti-inflammatory effect, antimicrobial activity, antioxidant activity, antitumor activity, endothelial dysfunction, wound healing, modulation of the immune system and inhibition of pathogenic microorganisms (28,31,52,88,99,100,101,102,103,104). *In vivo* human studies using kefir, however scarce, have been able to support some of these health benefits such as anti-inflammatory activity, hypocholesterolemic effect, and intestinal integrity conditions (107,109,126,127).

These putative beneficial health properties can be attributed both to the complex microbial fraction of kefir that has shown *in vitro* an ability to colonize the human gut and modulate intestinal microbiota composition (98,112,124), and to the non-microbial fraction containing bioactive metabolites resulting from fermentation (32,76,100,102,105,106), including lactic acid, acetic acid, ethanol and CO_2, vitamins, peptides, polysaccharides (such as kefiran), bacteriocins, acetaldehyde and diacetyl (74,98,108,110,111). The role of the lactic acid has been highlighted. In addition to down-regulating pro-inflammatory responses at intestinal level (33,34,108), lactic acid can be used by the gut microbiota to produce acetate, propionate, and butyrate. These SCFAs are highly associated with intestinal health and the modulation of the immune response (5,62,98,112). Furthermore, the antimicrobial capacity of kefir, mainly attributed to the presence of organic acids and other inhibitor compounds such as bacteriocins, has also been demonstrated *in vitro* (28,31,106). Its peptides have been linked to anti-hypertensive, antimicrobial, immunomodulatory, and anti-oxidative properties (14,108,113,114). Moreover, the water-soluble polysaccharide kefiran has demonstrated *in vitro* resistance to enzymatic intestinal hydrolysis (77,89,108,115,116,117), therefore becoming available to act as a substrate to the beneficial gut microbiota (118). Additionally, anti-tumor, anti-

livres (89,90). As leveduras produzem álcool e dióxido de carbono que contribuem para a sensação na boca e sabor caracteristicos do kefir (51). A composição química do kefir reflete-se no seu valor nutricional, que é normalmente cerca de 3% de proteína, menos de 10% de gordura e pelo menos 0,6% de ácido lático, de acordo com os critérios do Codex (86). O kefir pode ser refrigerado mantendo uma vida útil de 3–12 dias (77,81,91).

Efeitos do kefir na saúde

Várias propriedades de promoção da saúde foram atribuídas ao consumo de kefir (14,50,51,84,92,93,94,95). No entanto, até o momento, a maioria dos estudos foi realizada *in vitro* usando kefir não digerido ou em modelos animais, limitando assim a previsão da atividade biológica do kefir em humanos (96,97,98). Entre as atividades de saúde relatadas sobre o kefir estão a melhoria da digestão da lactose, efeito hipocolesterolémico, redução da resistência à insulina e efeito anti-hipertensivo, efeito antiinflamatório, atividade antimicrobiana, atividade antioxidante, atividade antitumoral, disfunção endotelial, cicatrização de feridas, modulação do sistema imunológico e inibição de microorganismos patogénicos (28,31,52,88,99,100,101,102,103,104). Estudos *in vivo,* em humanos, usando kefir, embora escassos, têm sido capazes de apoiar alguns desses benefícios para a saúde, como a atividade antiinflamatória, efeito hipocolesterolémico e integridade das condições intestinais (107,109,126,127).

Essas supostas propriedades benéficas para a saúde podem ser atribuídas tanto à complexa fração microbiana do kefir, que demonstrou, *in vitro,* uma capacidade de colonizar o intestino humano e modular a composição da microbiota intestinal (98,112,124), quanto à fração não microbiana que contém todos os metabolitos bioativos resultantes da fermentação (32,76,100,102,105,106), como ácido lático, ácido acético, etanol e CO_2, vitaminas, peptídeos, polissacarídeos (como o kefirano), bacteriocinas, acetaldeído e diacetil (74,98,108,110,111). O papel do ácido láctico tem sido destacado, pois além de regular as respostas pró-inflamatórias ao nível intestinal (33,34,108), também pode ser usado pela microbiota intestinal para produzir acetato, propionato e butirato, que são AGCC altamente associados à saúde intestinal e à modulação da resposta imune (5,62,98,112). Adicionalmente, a capacidade antimicrobiana do kefir, atribuída principalmente à presença de ácidos orgânicos e outros compostos inibidores, como bacteriocinas, também foi demonstrada *in vitro* (28,31,106). Os seus peptídeos têm sido relacionados com propriedades anti-hipertensivas,

fungal, anti-bacterial, anti-hypertensive, anti-glycemic, laxative, immunomodulatory, anti-inflammatory, healing, and antioxidant properties of kefiran have been reported (100,102,116,118,119,120).

Modulation of the gut by kefir

The ability of kefir to positively impact both the intestinal microbiota and the general condition of the digestive system has been demonstrated *in vitro*, in animal models, and in a limited number of human trials, where its potent anti-inflammatory effect has been frequently noted (93,96,98,108,112,121,122,123,124,125). Recent research in humans on the putative modulation of intestinal microbiota showed that after the consumption of kefir, individuals with metabolic syndrome presented positive correlations between the composition of the intestinal microbiota and improvement of the insulin profile, decreased levels of pro-inflammatory cytokines (such as Tumoral Necrosis Factor (TNF)-α and IFN-γ) and lower blood pressure (126). In addition, another study in humans demonstrated the ability of kefir to modulate the composition of the intestinal microbiota by increasing the concentration of serum zonulin, thus avoiding disruption of the intestinal permeability (109). Thereby, the positive impact of kefir in the host's gut microbiota suggests that regular kefir consumption may reduce the risk of intestinal dysbiosis and, consequently, could improve the outcome of diseases, such as those with an inflammatory component (127,128).

Effect of kefir on the skin

Research regarding the impact of kefir on the skin thus far has been limited to *in vitro* and animal studies and to the beneficial effect of its topical application on wound healing (52,102) and anti-inflammatory and antimicrobial activity (52,102,119). A recent study exploring the impact of oral administration of a kefir yeast (*Kazachstania turicensis*) in AD using an animal model verified a beneficial effect on the modulation of the gut microbiota as well as in the immune response, thus increasing the potential of kefir as a possible application in AD (121).

Noteworthy, none of the *in vivo* human studies found in the literature assessed the impact of a diet containing traditionally homemade kefir as the probiotic, neither in healthy nor atopic skin (5,112).

antimicrobianas, imunomoduladoras e antioxidantes (14,108,113,114). O polissacarídeo hidrossolúvel, kefirano, demonstrou *in vitro* resistência à hidrólise enzimática intestinal (77,89,108,115,116,117), tornando-se, desse modo, disponível para atuar como um substrato para a microbiota intestinal benéfica (118). Por fim, o kefirano tem sido também evidenciado pelas suas propriedades antitumorais, antifúngicas, antibacterianas, anti-hipertensivas, anti-glicémicas, laxantes, imunomoduladoras, antiinflamatórias, cicatrizantes e antioxidantes (100,102,116,118,119,120).

Modulação do intestino pelo kefir

A capacidade do kefir de impactar positivamente a microbiota intestinal e a condição geral do sistema digestivo foi demonstrada *in vitro*, em modelos animais e num número limitado de testes em humanos, onde o seu forte efeito antiinflamatório se destacou (93,96,98, 108,112,112,121,122,123,124,125). Pesquisas recentes em humanos sobre a potencial modulação da microbiota intestinal mostraram que, após o consumo do kefir, indivíduos com síndrome metabólica apresentaram correlações positivas entre a composição da microbiota intestinal e melhoria do perfil insulínico, diminuição de citocinas pró-inflamatórias (como a Tumoral Fator de necrose (TNF)-α e IFN-γ) e na pressão arterial (126). Adicionalmente, outro estudo em humanos foi capaz de demonstrar a capacidade do kefir em modular a composição da microbiota intestinal, aumentando a concentração de zonulina sérica, evitando assim a ruptura da permeabilidade intestinal (109). Deste modo, o impacto positivo do kefir na microbiota intestinal sugere a possibilidade de que o consumo regular de kefir pode reduzir o risco de disbiose intestinal e, consequentemente, melhorar o resultado de doenças, como aquelas com um carácter inflamatório (127,128).

Efeito do kefir na pele

A investigação sobre o impacto do kefir na pele está até agora limitada a estudos *in vitro* e em animais, e ao efeito benéfico de sua aplicação tópica na cicatrização de feridas (52,102) e atividade antiinflamatória e antimicrobiana (52,102,119). Um estudo recente explorando o impacto da administração oral de uma levedura de kefir (*Kazachstania turicensis*) na DA, usando um modelo animal, verificou um efeito benéfico na modulação da microbiota intestinal, bem como na resposta imune, aumentando assim o potencial do kefir como uma possível aplicação em AD (121).

Conclusion

This work provided an overview of the impact of probiotics on the gut, and their potential effects on the skin, as a gut-skin axis appears to exist. In addition, current knowledge regarding the role of probiotics in skin health was presented.

Intestinal dysbiosis promotes the occurrence of low-grade systemic chronic inflammation; hence modulation of the intestinal microbiota may represent a promising strategy for the prevention and treatment of cutaneous and non-cutaneous disease states. The use of fermented foods with probiotic activity, such as kefir, may represent an excellent nutritionally based alternative therapeutic strategy via intestinal modulation. Kefir stands out as a probiotic with potential to regulate the gut-skin axis, both for its nutritional and microbiological value, and supported by the historical safety of its consumption and its wide availability and growing popularity. However, scientific literature regarding the impact of a diet containing kefir on skin health is limited, making it essential to identify the effects of kefir in greater depth, as well as its mechanisms of action, in well-controlled human intervention studies. Thus, this review demonstrates the need for further *in vivo* studies in humans to assess the impact of traditional kefir on skin conditions both in healthy and diseased skin, particularly in individuals presenting AD.

Conflict of Interests

The editors involved in this manuscripts' authorship had no participation in the review or decision process. All authors have stated that there are no financial and/or personal relationships that could represent a potential conflict of interest.

Author Contributions Statement

CR, LMR, PR and EA: conceptualization and study design; EA: drafting; EA, PR and CR: drafting editing and reviewing.

Digno de nota, nenhum dos estudos em humanos, *in vivo,* encontrados na literatura avaliou o impacto de uma dieta contendo kefir tradicionalmente produzido, como probiótico, quer em pele saudável, quer atópica (5,112).

Conclusão

Este trabalho forneceu uma visão geral do impacto dos probióticos no intestino e dos seus potenciais efeitos na pele, dada a aparente existência de um eixo intestino-pele. Além disso, foi fornecido o estado da arte sobre o papel dos probióticos na saúde da pele.

A disbiose intestinal promove a ocorrência de inflamação crónica sistémica de baixo grau, portanto a modulação da microbiota intestinal pode representar uma estratégia interessante para a prevenção e tratamento de estados de doença, incluindo as cutâneas. O uso de alimentos fermentados com atividade probiótica, como o kefir, pode representar uma excelente alternativa de base nutricional, como modulador intestinal. O kefir destaca-se como um probiótico com potencial para regular o eixo intestino-pele, seja pelo seu valor nutricional e microbiológico, aliado à sua segurança evidenciada pelo seu histórico de consumo humano, seja pela sua ampla disponibilidade e crescente popularidade. No entanto, a literatura ainda é escassa sobre o impacto de uma dieta contendo kefir na saúde da pele, sendo imprescindível identificar todos os envolvidos nos efeitos do kefir, bem como os seus mecanismos de ação, em estudos de intervenção humana bem controlados. Assim, esta revisão demonstra a necessidade de mais estudos *in vivo*, em humanos, sobre o impacto do kefir tradicional nas condições da pele, tanto na pele saudável quanto na doente, particularmente naquela que apresenta DA.

Conflito de interesses

Os editores envolvidos na autoria deste manuscrito não participaram do processo de revisão ou decisão. Todos os autores afirmaram que não existem relações financeiras e / ou pessoais que possam representar um potencial conflito de interesses.

Declaração sobre as contribuições do autor

CR, LMR, PR e EA: conceptualização e desenho de estudo; EA: redação; EA, PR e CR: edição e revisão da redação.

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HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

The use of probiotics is widely associated with improved intestinal health. Changes in the composition of the intestinal microbiota, known as intestinal dysbiosis, are associated with decreased bacterial function and diversity, weakened intestinal barrier function, increased inflammation and changes in the immune system, thus having a negative impact on health. The ability of the intestinal microbiota and its by-products to affect other organ systems, including the skin, evidences the existence of an intestine-skin relationship. Changes in the balance of the gut-skin relationship are associated with dysfunctions both at the gastrointestinal and skin levels. The oral administration of probiotics has been shown to modulate the immune response, both locally and systemically, thus improving the intestinal barrier function and contributing to the prevention and treatment of inflammatory diseases such as atopic dermatitis.
Kefir is a fermented milk obtained from grains made up of a matrix of polysaccharides and proteins densely populated by lactic-acid, acetic-acid and yeast bacteria, with probiotic characteristics and that live in symbiotic association. Kefir consumption has been associated with a variety of health benefits and its biological activity can be attributed both to the presence of a complex microbiota, as well as the action of some organic acids released during fermentation. Therefore, and also taking into account its easy availability, the application of kefir as a modulator of the gut-skin axis is of the utmost interest.

Aiming to contribute to the increase of knowledge of the effects of kefir consumption on skin health, this work proposes to carry out a study on the effects of kefir on skin health, in individuals with and without a history of atopy and its possible relationship with gastrointestinal effects.

In this way, the following hypotheses are fundamented:

Does the regular intake of kefir, produced in homemade conditions, produce an impact:

- on the cutaneous health of healthy and atopic skin individuals?
- on the functional gastrointestinal symptoms of healthy and atopic skin individuals?

And if so, can these effects be related to each other, and be modulated by kefir?

OBJECTIVES

In order to respond to the hypothesis previously established, the following objectives have been set up for the progress of this work:

Objective 1

Perform a literature review regarding the impact of probiotics in the gut-skin axis, including the current evidence about kefir and its health effects.

At this stage, we performed an extensive review of the literature concerning the current knowledge on the gut-skin axis, on the effect of probiotics in the modulation of the gut, and on skin health, including in AD. Furthermore, the health effects of kefir, its role as a gut modulator and its skin effect were also reviewed. The review can be consulted at:

Background / Article 1: Alves, E.; Rijo, P.; Rodrigues, L.M.; Rosado, C. Probiotics in the Gut-Skin Axis – the Case of Kefir. *Biomed. Biopharm. Res.* 2021, *18*, 1–15, doi:10.19277/bbr.18.261.

Objective 2

Produce and characterize the physicochemical and nutritional profile of the kefir beverage and its stability during storage.

In this chapter, the production of the kefir drink that would be used in the intervention phase began, therefore, the characterization of the physicochemical and nutritional profile of the kefir produced under typical domestic conditions and its storage stability was carried out. Furthermore, the acceptability of kefir produced under conditions of domestic use was also evaluated in a sample of Portuguese consumers. The respective results can be consulted at:

Article 2: Alves, E.; Ntungwe, E.N.; Gregório, J.; Rodrigues, L.M.; Pereira-Leite, C.; Caleja, C.; Pereira, E.; Barros, L.; Aguilar-Vilas, M.V.; Rosado, C.; et al. Characterization of Kefir Produced in Household Conditions: Physicochemical and Nutritional Profile, and Storage Stability. Foods 2021, 10, 1–16, doi:10.3390/foods10051057.

Article 3: Alves, E.; Rijo, P.; Rodrigues, L. M.; Rosado, C. Acceptability of kefir produced by fermentation of Portuguese milk with CIDCA AGK1 grains in a sample of Portuguese consumers. Biomed. Biopharm. Res. 2021, 18(1), 1–9, doi: 10.19277/bbr.18.1.252.

Objective 3

Evaluate the impact of the regular ingestion of kefir on the skin barrier function of healthy and atopic individuals.

In order to achieve this goal, changes on the skin barrier function after 8 weeks of daily kefir intake were evaluated in healthy and atopic volunteers, and compared with the respective control group.

In addition, the impact of drinking kefir on AD severity was also assessed. All results were reported in Article 4, which can be consulted at:

Article 4: Alves, E.; Gregório, J.; Baby, A.R.; Rijo, P.; Rodrigues, L.M.; Rosado, C. Homemade Kefir Consumption Improves Skin Condition - A Study Conducted in Healthy and Atopic Volunteers. Foods 2021, 10, 2794, doi: 10.3390/foods10112794.

Additionally, an exploratory study was conducted in order to determine relevant endpoints to evaluate the *in vivo* barrier function and an induced lesion model was used in healthy and atopic volunteers, which can be consulted at:

Supplementary material, Article 5: Alves, E.; Rijo, P.; Rodrigues, L.M.; Rosado, C. Determination of Relevant Endpoints to Evaluate the *in Vivo* Barrier Function in Cutaneous Health. Biomed. Biopharm. Res. 2019, 16, 80–88, doi:10.19277/bbr.16.1.201.

Objective 4

Evaluate the impact of kefir ingestion as a modulator of the gut-skin axis in healthy and atopic individuals.

In order to accomplish this goal, changes on gastrointestinal symptons after the 8 weeks of daily kefir intake were assessed in healthy and atopic volunteers, and compared with the respective control group. In addition, the potential relationship between changes in the skin barrier and gastrointestinal changes resulting from the ingestion of kefir was evaluated. All results were reported in the article under submission presented in Chapter III:

Article 6: Alves, E.; Gregório, J.; Rijo, P.; Rodrigues, L.M.; Rosado, C. Kefir as a modulator of the gut-skin axis: a study conducted in healthy and atopic volunteers. Foods 2021 (*In Submission)*.

CHAPTER I

Article 2

Characterization of Kefir Produced in Household Conditions: Physicochemical and Nutritional Profile, and Storage Stability

Emília Alves [1,2] , Epole N. Ntungwe [1,2] , João Gregório [1] , Luis M. Rodrigues [1] , Catarina Pereira-Leite [1,3] , Cristina Caleja [4] , Eliana Pereira [4], Lillian Barros [4] , M. Victorina Aguilar-Vilas [2], Catarina Rosado [1] and Patrícia Rijo [1,5]

[1] CBIOS – Universidade Lusófona's Research Center for Biosciences & Health Technologies, Campo Grande 376, 1749-024 Lisboa, Portugal.
[2] Department of Biomedical Sciences, Faculty of Pharmacy, University of Alcalá, Carretera Madrid-Barcelona, Km 33.100, 28805 Alcalá de Henares, Madrid, Spain
[3] LAQV, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal
[4] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal.
[5]Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisboa, Portugal.

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Article

Characterization of Kefir Produced in Household Conditions: Physicochemical and Nutritional Profile, and Storage Stability

Emília Alves [1,2], Epole N. Ntungwe [1,2], João Gregório [1], Luis M. Rodrigues [1], Catarina Pereira-Leite [1,3], Cristina Caleja [4], Eliana Pereira [4], Lillian Barros [4], M. Victorina Aguilar-Vilas [2], Catarina Rosado [1,*] and Patrícia Rijo [1,5,*]

1 CBIOS—Research Center for Biosciences & Health Technologies, Universidade Lusófona's, Campo Grande 376, 1749-024 Lisboa, Portugal; emilia.alves@ulusofona.pt (E.A.); p5999@ulusofona.pt (E.N.N.); joao.gregorio@ulusofona.pt (J.G.); monteiro.rodrigues@ulusofona.pt (L.M.R.); catarina.leite@ulusofona.pt (C.P.-L.)
2 Department of Biomedical Sciences, Faculty of Pharmacy, University of Alcalá, Carretera Madrid-Barcelona, Km 33.100, 28805 Alcalá de Henares, Madrid, Spain; mvictorina.aguilar@uah.es
3 LAQV, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal
4 Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal; ccaleja@ipb.pt (C.C.); eliana@ipb.pt (E.P.); lillian@ipb.pt (L.B.)
5 Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisboa, Portugal
* Correspondence: catarina.rosado@ulusofona.pt (C.R.); prijo@ff.ulisboa.pt (P.R.)

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Abstract: Kefir, a traditional fermented food, has numerous health benefits due to its unique chemical composition, which is reflected in its excellent nutritional value. Physicochemical and microbial composition of kefir obtained from fermented milk are influenced by the type of the milk, grain to milk ratio, time and temperature of fermentation, and storage conditions. It is crucial that kefir characteristics are maintained during storage since continuous metabolic activities of residual kefir microbiota may occur. This study aimed to examine the nutritional profile of kefir produced in traditional in use conditions by fermentation of ultra-high temperature pasteurized (UHT) semi-skimmed cow milk using argentinean kefir grains and compare the stability and nutritional compliance of freshly made and refrigerated kefir. Results indicate that kefir produced under home use conditions maintains the expected characteristics with respect to the physicochemical parameters and composition, both after fermentation and after refrigerated storage. This work further contributes to the characterization of this food product that is so widely consumed around the world by focusing on kefir that was produced in a typical household setting.

Keywords: kefir; household conditions; storage time influence; nutritional composition; fatty acid profile; particle size; polydispersity index; zeta potential

1. Introduction

Traditional kefir has been consumed for centuries [1,2] due to its high nutritional value and is therefore considered a health promoting food [3]. Several health benefits have been attributed to kefir, mainly justified by the bioactivity of metabolites produced during fermentation [4,5], such as improved lactose digestion and tolerance [6], anti-inflammatory effect [7,8], antimicrobial activity [9], antioxidant activity [10], antitumor activity [11], wound healing [9,12], modulation of the immune system [13], and growth inhibition of pathogenic microorganisms [14,15]. Traditional kefir production uses kefir grains as starter culture, differentiating it from other fermented foods [16]. Kefir grains can maintain their activity as long as they are preserved and incubated under appropriate conditions, due to their extremely stable microbial composition [17–19]. The microorganisms usually found in the grains are homo and heterofermentative lactic acid bacteria (LAB), Lactobacillaceae

family (genera *Lactobacillus* and *Leuconostoc)* and Streptococcaceae family (genera *Lactococcus* and *Streptococcus*), acetic acid bacteria Acetobacteraceae family (genera *Acetobacter*) and yeasts Saccharomycetaceae family (genera *Kluyveromyces* and *Saccaromyces*) [20–24]. The viability of kefir grains is guaranteed through maintenance of the bacterial/yeast ratio achieved by continuous fermentation cycles that lead to their biomass increase [2,20,25]. This increment is dependent on temperature, pH, washing of grains, renewal of milk, and presence of nutrients [16,25–27]. Grain preservation for household kefir production can be achieved either by continuous fermentation cycles, and assured for ten weeks of propagation [17], or by freezing at −20 °C [19]. The microbiological composition of kefir grains depends on their origin [20,28–30].

Kefir's microbiota is different from that of the grains [16]. The physicochemical and microbial composition of kefir fermented milk is influenced by the type of the milk, grain to milk ratio, time and temperature of fermentation, and storage conditions [31–35]. Traditional kefir typically uses cow's milk as substrate [30,33]. Although whole, semi-skimmed, or skimmed milk can be used [32,36], the latter creates a kefir with significantly lower nutritional quality [29]. Grain to milk ratio, usually varying between 2% and 10% (*w*/*v*), influences the kefir microbial profile, and higher rates of grain inoculum increase lactic acid levels, providing sharper pH lowering [14,31,36]. The viscosity is also affected, since higher percentages of kefir grain inoculate produce a more acidic, but less viscous, kefir [31,36]. Lactose content is the main nutritional compound influenced by the amount of grain inoculum and smaller ratios of inoculate results in kefir with higher lactose levels [21,36].

Typical kefir fermentation occurs at temperatures between 20 and 25 °C for approximately 24 h, with pH varying between 4.2 and 4.6 [14,31,37]. During fermentation, the chemical composition of kefir changes mainly due to lactose conversion by homofermentative LAB, first into lactic acid, causing the pH to drop and acidity to increase [21,38], followed by the remaining hydrolyzation into glucose and galactose by the enzymatic activity of β-galactosidase present in the grains [39]. Further into the fermentation cycle heterofermentative LAB convert glucose into CO_2, ethanol and lactic acid, the latter being the most predominant organic acid after fermentation, and in this environment proteins are converted into peptides [34,36,40,41]. The production of lactic acid contributes to the antimicrobial effect of kefir, and since it acts as a natural preservative, allows the homemade product to have a low contamination risk [14,24,42].

The chemical composition of kefir reflects its nutritional value and the recommended quality standards for kefir are at least 2.8% protein, less than 10% fat, and at least 0.6% lactic acid [43]. Kefir can be consumed immediately after grain separation or may be refrigerated for later consumption [4,25,41]. Fermented milk characteristics must be maintained during storage; however, since continuous metabolic activities of residual kefir microbiota may occur, the composition of refrigerated kefir may be affected during storage [19,34,36,44]. Kefir can maintain a shelf life of 3–12 days [25]. During refrigerated storage at 4 °C, viscosity is reported to decrease abruptly with time [34,36], while total fat, lactose, dry matter, and pH remain constant until 14 days of storage [32,36,40] and lactic acid slightly increases after 7 days storage [40]. Although the lipolytic activity in milk fat by LAB is limited, it can still contribute to the production of free fatty acids [45].

This work aimed to study kefir produced in representative household conditions, characterizing the properties, nutritional composition, and stability of a freshly made and 48 h refrigerated beverage. To date, information on homogeneity and stability of traditional kefir is scarce, and to the best of our knowledge, this is the first study to provide information on kefir produced by simulating representative home use conditions. The innovative character of this study also resides in the fact that, in addition to the usual parameters for physicochemical analysis and composition, the fatty acid profile, particle size, polydispersity index (PdI), zeta potential, and Fourier Transform Infrared Spectroscopy (FTIR) spectra analysis were also included in the global evaluation of kefir.

2. Materials and Methods

2.1. Kefir Grains Storage and Kefir Production

Kefir grains CIDCA AGK1 were obtained from the Centro de Investigacíon y Desarrollo en Criotecnologia de Alimentos (CIDCA), La Plata, Argentina. Microbiological characterization of these grains has been described elsewhere [20,46,47]. Kefir grains were maintained in milk and preserved by storage in a freezer at -20 ± 2 °C, which proved to be the best method for grain preservation and can also be used to maintain the grains for household kefir production [19]. After this type of storage, the grains were activated before use in fermentation [27]. For activation, grains were left to defrost at room temperature for 12 h, after which they were placed in semi-skimmed milk at 20 ± 1 °C for 24 h. The activation step was repeated three times.

Kefir beverage samples were produced by fermentation for 24 h of a commercial ultra-high temperature pasteurized (UHT) semi-skimmed cow milk of Portuguese provenance (Nova Açores®, S. Miguel, Portugal), with CIDCA AGK1 kefir grains using a grain inoculum of 10% (*w*/*v*), at a temperature of 20 ± 1 °C. After fermentation, grains were separated from the fermented milk by filtration through a plastic sieve and used as starter culture for the next kefir batch, under the same conditions. Samples of fermented milk kefir were collected after filtration.

2.2. Activity of Kefir Grains

2.2.1. Biomass Growth

Kefir grain biomass increase was measured over 8 days, with daily inoculations in milk. Kefir grains were sub-cultured by successive passage of the total amount of grains in increasing volumes of milk to maintain a concentration of 10% (*w*/*v*) [19]. Rising of the grains was made with milk, because growth of the grains is retarded when they are rinsed with water after each sieving [27]. Kefir grains were separated from fermented milk by filtration using a plastic sieve. Grains were rinsed with milk at room temperature and left to dry on a filter paper at room temperature (20 ± 1 °C), after which kefir grains were weighed using an analytical scale (KERN ALJ220-4NM (KERN & Sohn GmbH, Balingen, Germany)) for gravimetric determination. After weighing, kefir grains were used as a new inoculum, maintaining the grain to milk ratio. Samples were made in duplicate. Biomass growth rate was determined gravimetrically, and increment percentage was calculated. All measurements were made in triplicate.

2.2.2. Acidification Kinetic

Kefir fermentation of milk was carried out at 20 ± 1 °C and samples of fermented milk were collected every 2 h until a stabilized pH value was reached. Measurements were made using a Metrohom 827 pH lab® digital meter (Metrohom AG, Herisau, Switzerland). Samples were made in duplicate. All measurements were made in triplicate.

2.3. Viable Microorganisms and Inhibitory Activity Test

2.3.1. Bacterial and Yeasts Counts

Determination of LAB and yeast counts were made by conventional culture techniques [48]. Tryptone water (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1 g/L was used to prepare the dilutions for the microbiological analyses. Ten-fold dilutions in 0.1% sterile tryptone water were plated in each medium. LAB counts were quantified on De Man, Rogosa and Sharpe (MRS) agar plates (Oxoid, Hampshire, UK), which were incubated at 30 ± 1 °C for 24 h, and then for another 24 h, under the same conditions. Yeast counts were quantified on Yeast-Extract Glucose Chloramphenicol (YGC) agar plates (Oxoid, Hampshire, UK), which were incubated at 30 ± 1 °C for 48 h. Counts were expressed in total colony-forming units per milliliter. Measures were made in duplicate.

2.3.2. Inhibitory Activity Test

Inhibitory activity of kefir fermented milk was evaluated on the growth of *Escherichia coli* using conventional culture techniques [48]. *E. coli* counts were quantified on Eosin Methylene Blue Agar (EMB) agar plates (Oxoid, Hampshire, UK), which were incubated at 37 ± 1 °C for 24 h. A known *E. coli* strain (ATCC 25922) was used as control. Measures were made in duplicate.

2.4. Physicochemical Characteristics of Kefir Beverage

2.4.1. Particle Size, Polydispersity Index, and Zeta Potential

The particle size and PdI were analyzed, by dynamic light scattering, for milk (control), kefir immediately after the 24 h fermentation period (t0), and kefir after storage at 5 ± 1 °C for 24 and 48 h (t24 and t48, respectively). Zeta potential was also evaluated at the same time points by an electrophoretic mobility technique using a Delsa™ Nano C from Beckman Coulter, Inc. (Brea, CA, USA). All analyses were run in triplicate at room temperature (20 ± 1 °C) after diluting the samples with distilled water. Dilutions of 1:50 or 1:125 were used in the case of unfermented or kefir fermented milk, respectively.

2.4.2. Fourier Transform Infrared Spectroscopy (FTIR)

Kefir samples (t0, t24, and t48) obtained after freeze-drying were evaluated by FTIR in a PerkinElmer® Spectrum 400 (PerkinElmer Inc, Waltham, MA, USA) equipped with an attenuated total reflectance (ATR) device. The ATR system was cleaned before each analysis by using dry paper and scrubbing it with methanol and water (50:50). The room air FTIR-ATR spectrum was used as background to verify the cleanliness and to evaluate the instrumental conditions and room interferences due to H_2O and CO_2. The spectra were obtained collecting 100 scans of each sample, between 4000 and 600 cm^{-1}, with a resolution of 4 cm^{-1}. The FTIR analysis was also performed for unfermented milk as a control sample.

2.4.3. Viscosity and pH

The viscosity and pH were evaluated for both control and kefir samples (t0, t24, and t48). Viscosity was measured using a Brookfield Ametek DV3T® rheometer (AMETEK Brookfield, Middleboro, MA, USA) with a SV18 spindle, at 30 rpm. Measurements were performed at 25.2 ± 0.2 °C and readings were recorded for 1 min. pH was measured using a Metrohom 827 pH lab® digital meter (Metrohom AG, Herisau, Switzerland). Samples were made in duplicate, and all measurements were made in triplicate.

2.5. Nutritional Analysis of Kefir Beverage

For chemical analysis, both control and kefir samples (t0, t24, and t48) were frozen at –80 °C for 24 h, after which all samples were freeze-dried in a Labconco FreeZone 25® (Labconco, Kansas City, MO, USA) using a surface condenser temperature of −50 °C and 400 mTorr for 24 h. Samples were weighed before freezing and after freeze-drying for mass determination. The contents of protein, fat, carbohydrates, and ash were determined according to the official analysis methodologies AOAC [49] and following a procedure previously reported by Barros et al. [50]. Protein was determined considering the total nitrogen content and using the specific conversion factor for milk (6.38). Total fat content was analyzed as fatty acids and expressed as triglyceride equivalents. Ash was determined by gravimetry. Total carbohydrate content was determined by difference, as follows: 100-(weight in grams (protein + fat + water + ash + alcohol) in 100 g of food) [51]. Dry matter was calculated as the sum of total fat, protein, ash, and carbohydrates content. Total energy was calculated following the Equation:

$$\text{Energy (kcal)} = 4 \times (\text{g protein} + \text{g carbohydrates}) + 9 \times (\text{g fat}).$$

All samples were also evaluated regarding the sugar content, following an extraction procedure previously described [50]. Samples were then filtered through 0.2 µm Whatman nylon filters into a 1.5 mL vial for liquid chromatography analysis. The HPLC system was coupled to a refraction index (RI) detector and the free sugars were identified by comparison with standards and further quantified considering the internal standard and results were expressed in g per 100 g [50]. In addition, all samples were also evaluated for fatty acids content, which were extracted from all the samples and determined by gas chromatography coupled with a flame ionization detector (GC-FID, DANI model GC 1000, Contone, Switzerland) using a procedure previously described by Barros et al. [50]. The results were expressed as relative percentage of each fatty acid (%). Two batches of all samples were made, and all analyses were performed in duplicate.

2.6. Statistical Analysis

Results were expressed as mean ± standard deviation (SD). Linear regression was used to assess grains biomass growth. Differences over the groups were identified using one-way ANOVA analysis of variance. Different letters show significant differences by Tukey Post hoc multiple comparison tests. When homogeneity was not guaranteed, Games-Howell post-hoc tests were used. All analyses were performed using the SPSS statistical package version 25 (SPSS Inc., Chicago, IL, USA) with a level of significance of 0.05.

3. Results

3.1. Activity of Kefir Grains

3.1.1. Biomass Growth

Biomass growth of kefir grains, incubated at 20 ± 1 °C, for successive 24 h periods over 8 days, expressed in weight (g), is showed in Figure 1.

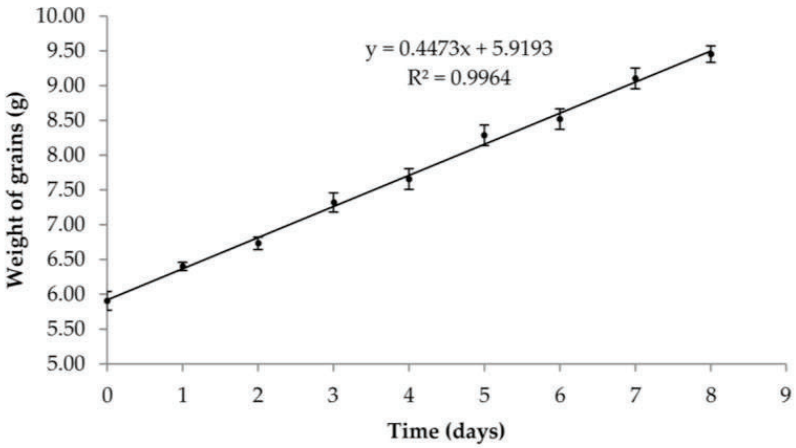

Figure 1. Increment of kefir grains biomass (g), incubated at 20 °C for 24 h periods, over 8 days (mean ± SD, $n = 3$).

Our results showed that after 8 days of successive fermentations the biomass grains had an increment of 60% when compared to the initial weight of the grains. We found that our CIDCA AGK1 grains had a mean 24 h biomass growth of 6 ± 2%, after fermentation at 20 °C.

3.1.2. Acidification Kinetic

The acidification rate of milk measured during kefir fermentation are showed in Figure 2.

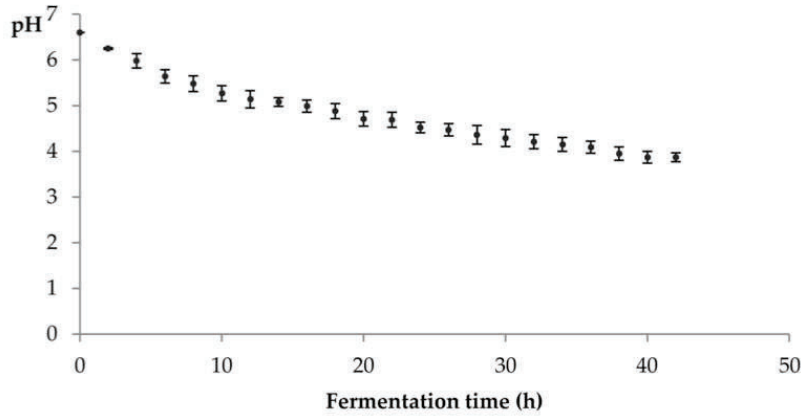

Figure 2. Acidification rate during fermentation at 20 °C, until pH stabilization (mean ± SD, $n = 3$).

As expected, during fermentation the pH value of kefir dropped from the value of 6.6 of unfermented milk, reaching a mean value of 4.5 ± 0.1 at the end of 24 h. After 42 h, the mean pH value of the kefir beverage stabilized at 3.9 ± 0.1.

3.2. Viable Microorganisms and Inhibitory Activity Test

The viable LAB and yeast counts, as well as coliforms found in the kefir analysis are presented in Table 1.

Table 1. Viable LAB and yeast counts (CFU/mL) and coliforms (CFU/mL) of kefir made from CIDCA AGK1 grains.

	Kefir Beverage
LAB (CFU/mL)	7×10^7
Yeasts (CFU/mL)	2×10^6
Coliforms (CFU/mL)	Absent

The microbiological analysis of our kefir revealed 7×10^7 CFU/mL of LAB and 2×10^6 CFU/mL of yeast. Furthermore, the absence of coliforms (*E. coli*) was also confirmed (Table 1).

3.3. Physicochemical Characteristics of Kefir Beverage

3.3.1. Particle Size, PdI, and Zeta Potential

The hydrodynamic diameter, PdI, and zeta potential of unfermented milk and kefir beverages, according to storage conditions, are presented in Table 2. In all cases, nanometric diameters (250–439 nm) were found for all beverages, with PdI values lower than 0.3, and zeta potential values smaller than −30 mV. Kefir at t0 showed a particle size and a PdI significantly higher ($p < 0.0001$ and $p < 0.0001$, respectively) than control, but no statistical difference was observed for zeta potential ($p = 0.483$). 24 h refrigerated kefir presented a smaller particle size ($p < 0.0001$), a smaller PdI ($p = 0.001$) and also a smaller zeta potential ($p = 0.013$) compared to kefir at t0, but no differences were observed throughout storage for these parameters ($p = 0.975$, $p = 0.575$, and $p = 0.996$, respectively).

Table 2. Hydrodynamic diameter, PdI, and zeta potential of control and kefir samples (t0, t24, and t48) (mean ± SD, $n = 3$).

	Control	Kefir		
		t0	t24	t48
Diameter (nm)	280 ± 5^{b}	439 ± 42^{a}	256 ± 6^{b}	249 ± 1^{b}
PdI	0.18 ± 0.01^{b}	0.295 ± 0.006^{a}	0.231 ± 0.008^{c}	0.22 ± 0.02^{c}
Zeta potential (mV)	-35 ± 2^{a}	-38 ± 1^{a}	-31 ± 2^{b}	-30 ± 3^{b}

[a–c] Means within the same row with different superscripts are significantly different $p < 0.05$.

3.3.2. FTIR

FTIR spectra were collected for unfermented milk and for kefir samples (t0, t24, and t48) (Figure 3). The control spectrum showed the presence of a broad band at 3335.99 cm^{-1}: it was attributed to -OH stretching in hydroxyl groups associated with carbohydrate structures. The peaks at 2915.49 cm^{-1} are associated with C-H bending in fatty acids, 1639.26 cm^{-1} correlates to the carbonyl (C=O) stretching or N-H and C-H bending vibration of the milk proteins. The band2 2915 and 2856.7 cm^{-1} may be due to the anti-symmetric and symmetric stretching of CH_2 groups from the fatty milk components.

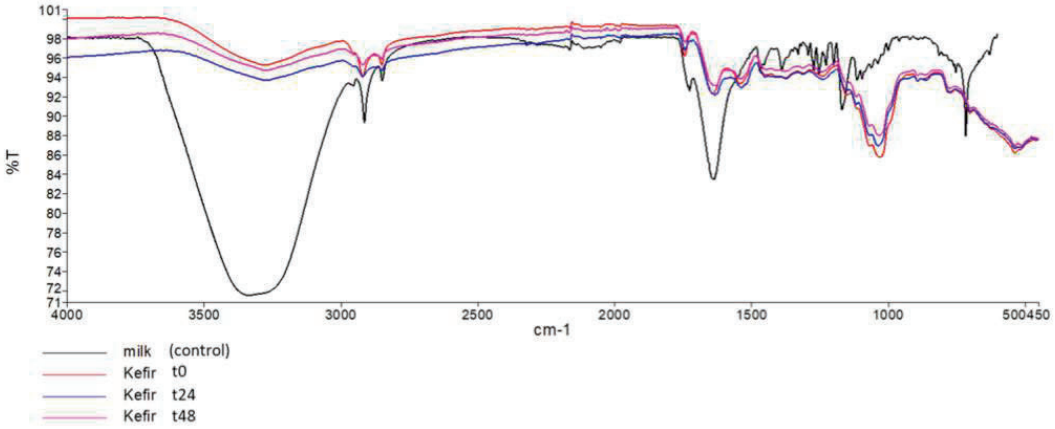

Figure 3. FTIR spectra of control and kefir samples (t0, t24, and t48).

From Figure 3 we can observe a very strong overlap between the spectral signals of milk (control) and kefir samples: t0, t24, and t48. This is evident throughout the full-recorded spectral region, suggesting a high similarity in the composition between the samples.

3.3.3. Viscosity and pH

After a 24 h fermentation, kefir showed a mean pH value of 4.60 ± 0.05, which was significantly lower than that of the control ($p < 0.0001$). No statistical difference in pH values was observed between samples t0 and t24 ($p = 0.116$) and between both refrigerated samples ($p = 0.168$). However, the pH value decreased between t0 and t48 ($p = 0.014$). Kefir at t0 showed a mean viscosity of 32 ± 4 mPa.s, which was significantly higher than that of the control ($p < 0.0001$). The viscosity of kefir decreased after a refrigerated storage period of 24 h ($p = 0.043$). However, no significant difference in viscosity was observed between both refrigerated samples ($p = 0.732$) (Table 3).

Table 3. Physical parameters of control and kefir samples (t0, t24, and t48) (mean ± SD, $n = 6$).

	Control	Kefir		
		t0	t24	t48
pH	6.60 ± 0.00 [a]	4.60 ± 0.05 [b]	4.54 ± 0.02 [b,c]	4.50 ± 0.04 [c]
Viscosity (mPa.s)	2.11 ± 0.01 [b]	32 ± 4 [a]	26 ± 2 [c]	24 ± 4 [c]

[a–c] Means within the same row with the different superscripts are significantly different $p < 0.05$.

3.4. Nutritional Analysis of Kefir Beverage

The nutritional content, evaluated by fat, protein, carbohydrates, ash, lactose, and lactic acid content, as well as the energy value of unfermented milk and the kefir samples immediately after 24 h fermentation at 20 °C, and after 24 h and 48 h of cold storage at 5 ± 1 °C, is shown in Table 4.

Table 4. Nutritional composition of control and kefir samples (t0, t24, and t48) (mean ± SD, $n = 4$).

	Control	Kefir		
		t0	t24	t48
Energy (kcal/100 mL)	48.2 ± 0.4 [a]	43.8 ± 0.6 [b]	44.5 ± 0.8 [b]	44 ± 2 [b]
Carbohydrates (% *w*/*v*)	5.14 ± 0.08	4.9 ± 0.2	5.0 ± 0.1	5.0 ± 0.2
Lactose (% *w*/*v*)	4.74 ± 0.05 [a]	4.1 ± 0.2 [b]	3.75 ± 0.08 [b]	3.8 ± 0.2 [b]
Proteins (% *w*/*v*)	2.8 ± 0.1	3.2 ± 0.2	3.1 ± 0.1	3.15 ± 0.05
Total Fat (% *w*/*v*)	1.81 ± 0.03	1.28 ± 0.04	1.32 ± 0.09	1.3 ± 0.3
Lactic acid (% *w*/*v*)	0.02 ± 0.00 [b]	0.59 ± 0.07 [a]	0.63 ± 0.01 [a]	0.61 ± 0.05 [a]
Ash (% *w*/*v*)	0.50 ± 0.01 [a]	0.58 ± 0.02 [b]	0.59 ± 0.01 [a,b]	0.59 ± 0.02 [b]
Dry matter (% *w*/*w*)	10.28 ± 0.04 [a]	9.9 ± 0.1 [b]	10.05 ± 0.09 [b]	10.0 ± 0.2 [b]

[a,b] Means within the same row with different superscript letters show significant statistical differences ($p < 0.05$).

Kefir at t0 showed a mean nutritional composition of 1.28 ± 0.04 g/100 mL of fat, 3.15 ± 0.19 g/100 mL of protein and 4.91 ± 0.19 g/100 mL of carbohydrates. As macronutrients are concerned no difference was observed in fat, protein, and carbohydrates content due to fermentation or storage ($p = 0.071$, $p = 0.071$ and $p = 0.449$, respectively). Energy, ash, and dry matter (DM) were different between t0 and control ($p = 0.002$, $p = 0.011$ and $p = 0.028$, respectively). Finally, the lactose content in control was significantly higher than in kefir ($p = 0.011$) with a 13.6% decrease during fermentation. Consequently, the lactic acid content in kefir was significantly higher than that of the control ($p = 0.001$). No differences were found between kefir samples for lactose and lactic acid ($p = 0.100$ and $p = 0.580$, respectively).

The content of fatty acids of unfermented milk and kefir beverages was also determined, and the results are presented in Table 5. All samples evidenced the presence of 18 fatty acids, comprising saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids.

Table 5. Fatty acids profile of control and kefir samples (t0, t24, and t48) (relative frequency, mean ± SD, $n = 4$).

	Control	Kefir		
Fatty Acids (%)		**t0**	**t24**	**t48**
C6:0	3.86 ± 0.05	3.6 ± 0.2	3.3 ± 0.1	3.7 ± 0.1
C8:0	2.12 ± 0.03 ^{a}	2.07 ± 0.07 ^{a}	1.95 ± 0.05 ^{b}	2.18 ± 0.05 ^{a}
C10:0	4.41 ± 0.02	4.5 ± 0.3	4.1 ± 0.2	4.6 ± 0.1
C11:0	0.10 ± 0.00	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
C12:0	5.5 ± 0.1 ^{b}	5.3 ± 0.1 ^{b}	5.2 ± 0.2 ^{b}	5.6 ± 0.1 ^{a}
C13:0	0.12 ± 0.01	0.10 ± 0.00	0.10 ± 0.01	0.11 ± 0.02
C14:0	14.62 ± 0.02	14.3 ± 0.2	14.2 ± 0.3	14.1 ± 0.9
C14:1	1.15 ± 0.02	1.18 ± 0.06	1.20 ± 0.07	1.11 ± 0.04
C15:0	1.18 ± 0.00 ^{b}	1.17 ± 0.02 ^{b}	1.14 ± 0.03 ^{b}	1.23 ± 0.01 ^{a}
C15:1	0.25 ± 0.01 ^{b}	0.27 ± 0.01 ^{a,b}	0.26 ± 0.01 ^{b}	0.28 ± 0.01 ^{a}
C16:0	39.34 ± 0.02 ^{b}	38.4 ± 0.3 ^{c}	38.9 ± 0.5 ^{c}	39.9 ± 0.2 ^{a}
C16:1	1.57 ± 0.01 ^{a}	1.61 ± 0.06 ^{a}	1.57 ± 0.08 ^{a}	1.38 ± 0.07 ^{b}
C17:0	0.61 ± 0.02 ^{b}	0.61 ± 0.02 ^{b}	0.61 ± 0.05 ^{b}	0.70 ± 0.05 ^{a}
C18:0	5.71 ± 0.02	5.64 ± 0.06	5.7 ± 0.1	5.74 ± 0.09
C18:1n-9	18.4 ± 0.1 ^{b}	19.4 ± 0.3 ^{a}	19.8 ± 0.3 ^{a}	18.0 ± 0.7 ^{a}
C18:2n-6	0.79 ± 0.03	1.4 ± 0.3	1.52 ± 0.07	0.7 ± 0.7
C18:3n-3	0.023 ± 0.001 ^{b}	0.123 ± 0.003 ^{a}	0.12 ± 0.00 ^{a}	0.15 ± 0.01 ^{a}
C20:0	0.21 ± 0.01	0.32 ± 0.03	0.31 ± 0.06	0.32 ± 0.08
SFA	77.81 ± 0.07 ^{b}	76.1 ± 0.5 ^{a}	75.6 ± 0.3 ^{a}	78.5 ± 1.2 ^{a}
MUFA	21.37 ± 0.09 ^{b}	22.4 ± 0.3 ^{a}	22.8 ± 0.3 ^{a}	20.7 ± 0.7 ^{b}
PUFA	0.82 ± 0.03	1.4 ± 0.4	1.6 ± 0.1	0.8 ± 0.6

SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids. $^{a–c}$ Means within the same row with different superscript letters are significantly different ($p < 0.05$).

SFA content of kefir at t0 was significantly lower than control ($p = 0.012$), which is supported by the differences observed between the samples regarding palmitic acid content ($p = 0.029$). No differences were observed for the remaining SFA, between these samples. Within SFA, palmitic acid (C16:0) stands out as the major fatty acid followed by and myristic acid (C14:0). MUFA content of kefir at t0 was significantly higher than that of control ($p = 0.008$), which may be mainly supported by the difference observed between the samples regarding oleic acid content ($p = 0.014$). No differences were observed for the remaining MUFA, between these samples. Within MUFA, oleic acid (C18:1n-9) represents the major component. PUFA content showed no difference between all samples ($p = 0.050$), which may be supported by the fact that linoleic acid (C18:2n-6), the major PUFA component, also remained constant ($p = 0.083$), despite the content of α-linolenic acid (C18: 3n-3) increased slightly after fermentation ($p = 0.023$).

Between t24 and t0, no difference was observed in SFA and MUFA content ($p = 0.389$ and $p = 0.460$, respectively). Within SFA, only C8:0 evidenced a very small decrease ($p = 0.041$), while within MUFA no change was observed. Between t48 and t0, no difference was observed in the SFA content ($p = 0.083$). Within SFA, lauric acid (C12:0), pentadecylic acid (C15:0), palmitic acid and margaric acid (C17:0) presented a very small increase ($p = 0.022$, $p = 0.009$, $p = 0.002$ and $p = 0.048$, respectively). MUFA content decreased ($p = 0.031$), with only palmitoleic acid (C16:1) reflecting that decrease ($p = 0.002$).

4. Discussion

Kefir grains are traditionally cultured in milk at room temperature, which is considered to be between 20 and 25 °C [36,41]. The traditional use, combined with the fact that 20 °C is in the range of typical indoor conditions of a Portuguese house [52], thus reflecting the domestic scenario of preparation of kefir, justifies the choice of the fermentation temperature in our study. It is widely known that biomass increase and lactose consumption rise at higher incubation temperatures [37]. Nevertheless, Londero et al. [53] found that

biomass growth, acidification capacity, and maintenance of the chemical composition are optimized at a fermentation temperature of 20 °C.

Increment of the grains biomass during fermentation highlights the microbial growth resulting of the balance within the microbiota of the grains [25,53,54]. This biomass increase is mainly due to the production of protein and polysaccharides by its microbiota within the grains matrix, which can be transferred to the fermented milk [54]. Our grains presented a mean biomass increment of 6 ± 2% after a 24 h fermentation of semi-skimmed milk at 20 °C, which is consistent with the results of DeSainz et al. [55], that found a biomass increase of 7.2 ± 0.1% after 24 h fermentation at 35 °C. Interestingly, using a mathematical model Zajšek and Goršek [37] observed a linear trend between fermentation temperature and increase of biomass grains, that predicted an increase of 7.034 g/L in biomass grain growth for a temperature of 20 °C. Our results showed a ten-fold higher growth, which shows a considerable disparity between a mathematical model and a real fermentation scenario. The growth behavior of our grains (Figure 1) was contrary to the results found by Pop et al. [56] using a grain inoculum of 4.5% (*w*/*v*) to ferment skimmed milk at 25 °C and showing a significant biomass decrease after 24 h. This may be justified by the fact that our study used semi-skimmed milk, thus making Pop's justifications, nutrient depletion or increase acidity, less robust arguments to justify growth behavior of our grains. Moreover, the fat content of the milk may be of significant importance, as demonstrated by Schoevers and Britz [27], who reported that higher milk fat content impairs grain growth by inhibition of nutrient exchange. The authors also found that the lowest increase in biomass happened when low fat milk was used, and their results using this milk type and a grain to milk ratio of 1% (*w*/*v*) showed a biomass increase around 50% after 8 days [27]. The increase of 60.07% in biomass that we found may also be justified by the use of a grain inoculum of 10% (*w*/*v*).

The mean pH value of 4.5 ± 0.1 that was verified after 24 h of fermentation is in agreement with that found by Garrote et al. [31], using the same type of grain inoculum. The acidification rate observed during fermentation in our work (Figure 2) is consistent with the literature [21,31,36,37,41] and may reflect the LAB capability to acidify the milk [37,41]. Both pH and lactic acid variation during fermentation of kefir represent an indirect measure of the biological activity of the grains [57]. LAB population present high sensitivity to low pH values, which contributes to their decline, being that the main reason why kefir does not become more acidic through time [31,36].

Interestingly, despite the home use production conditions, the resulting kefir (Table 1) is in conformity with the recommendations of *Codex Alimentarius* for fermented milks (Codex Stan 243-2003), thus complying with a number of total micro-organisms of at least 10^7 colony-forming units (CFU)/mL and a yeast number not less than 10^4 CFU/mL [43].

After fermentation, we found that the mean particle size of kefir (439 ± 42 nm) predictably increased significantly compared to the unfermented milk (280 ± 54 nm) and decreased again after 24 h-refrigerated storage (256 ± 6 nm), remaining stable for another 24 h of cold storage (249 ± 1 nm). According to the literature [58], casein micelles aggregation is promoted by increase of acidification, protein content, fat content and temperature, thus these factors may directly affect particle growth in kefir beverage. The pH decrease observed in freshly made kefir (Table 3) may be at the root of the initial aggregation of casein micelles into larger clusters. After refrigerating the kefir beverage for 24 h, the size of casein micelles probably decreased due to the effect of low temperatures on protein aggregation. In fact, it was already reported that the higher the temperature, the higher the particle size of fermented milk [58]. Moreover, it is noteworthy that, after 24 h of refrigerated storage, the particle size of kefir is in reasonable agreement with the results recently presented by Beirami-Serizkani et al. [59]. Another 24 h of refrigerated storage did not alter the particle size of kefir, probably due to the fact that, during this period, the temperature remained constant, as well as no pronounced alterations were found in pH values (Table 3) and protein or fat content (Table 4) of the kefir beverage.

The degree of non-uniformity of a population's size distribution within a given sample, represented by PdI, suggests the degree of heterogeneity of the sample. A homogeneous sample, perfectly uniform regarding the particle size, shows a PdI value of zero, while a heterogeneous sample, highly polydisperse with multiple particle size populations has a PdI of 1 [60]. The stability of a sample, given by the zeta potential, is a measure of the magnitude of electrostatic repulsion/attraction or charges between particles [58] and increases with the homogeneity of the size distribution [60]. Zeta potential depends on factors like temperature, acidity, and viscosity, and a highly negative/positive zeta potential foresees a more stable dispersion, while values lower than |30| mV can indicate colloidal instability, which can lead to aggregation [61]. Concerning the particle size distribution of the analyzed samples, given by PdI (Table 2), it is remarkable that all beverages display uniform particle size distributions (PdI < 0.3). Despite that, the increase in particle size of kefir in comparison with unfermented milk also resulted in an increase of PdI, which was almost recovered by the decrease of particle size upon refrigerated storage for 24 h and 48 h (Table 2). In addition, the zeta potential values recorded for all samples (<−30 mV, Table 2) indicate that all beverages display good colloidal stability. It is noteworthy that the zeta potential of unfermented milk was in line with a previous report of its variation with milk pH [62]. According to our results, the zeta potential of kefir is similar to that of unfermented milk, slightly increasing with refrigerated storage (Table 2). This is not in agreement with the data reported by Beirami-Serizkani et al. [59], showing that the different preparation procedures of kefir drinks may influence the colloidal stability of the resulting beverage.

FTIR spectrum analysis of unfermented semi-skimmed milk (Figure 3) was consistent with the literature [63]. Using FTIR spectra, we confirm that the physicochemical properties of the milk change during the fermentation process. However, from the strong overlap between the kefir spectral signals (Figure 3) we corroborate that its physicochemical properties are maintained during refrigerated storage, which is consistent with the results obtained from the other analysis performed in this study.

The variations in pH and viscosity found in our kefir samples (Table 3) are similar to those reported the literature [21,36,44]. The pH value of kefir was significantly lower than that of milk, remaining constant in the first 24 h of refrigeration and showing a slight decrease of 2% at the end of 48 h (Table 3). Similar results after 2 days of storage were reported by Leite et al. [21]. Irigoyen et al. [36] also reported no variations in pH during kefir storage, and attributed it to the presence of yeast in the grains, since the production of lactic acid by LAB is slower in the presence of yeasts than in pure culture [38,44].

After a 24 h fermentation, kefir revealed a significantly higher viscosity compared to the unfermented milk (Table 3). This can be in part attributed to the production of kefir's exclusive polysaccharide, kefiran, which, in addition to constituting the grain structure, can also be found dissolved in the liquid, thus contributing to the rheology of the fermented beverage [64]. The decrease observed in kefir's viscosity after the first 24 h refrigerated storage period (Table 3) can be attributed to the hydrolysis of the polysaccharide kefiran together with the reduction observed in the LAB responsible for the polysaccharide's production [34]. Throughout storage, a decrease in viscosity and phase separation (syneresis), due to the aggregation of casein micelles and subsequent precipitation are the most typical events that may impair the quality of kefir [65]; however, these changes only become evident in periods of storage longer than seven days [34,36,44]. Nevertheless, our data showed no difference in viscosity during storage, which may be attributed to a limited storage time (only 48 h).

The nutritional composition of kefir is influenced by milk composition, origin of the grains, temperature, and duration of fermentation and storage conditions [31,36]. As explained previously, our kefir prepared in a typical home use setting fulfills the requirements the *Codex Alimentarius* (Table 4) and is in accordance with data reported by other authors [21,36,66]. Whilst typical cow milk presents a carbohydrate content between 4.7 and 4.9 g/100 mL, reflecting essentially lactose content [67], kefir has a carbohydrate

content around 11.9 g/100 g, also reflecting the presence of polysaccharide kefiran [54]. Our data for unfermented milk were consistent with the literature [67], but no difference was observed in carbohydrate content, neither during fermentation or storage. It is noteworthy that in spite the small lactose decrease observed after fermentation, the carbohydrate profile of kefir is expected to be different from that of the source milk, due to the presence of polysaccharide kefiran in kefir (not quantified in this study).

After 24 h fermentation we observed a decrease of 13.6% in lactose level and an increase in lactic acid content which is consistent with the literature [21,33,36,38,44], and may be explained by the hydrolysis of lactose and production of lactic acid in the initial LAB lactose metabolism [21,44]. These results are in line with those reported by Irigoyen et al. [36], who observed a 20–25% decrease in lactose during 24 h fermentation. Assadi et al. [68] reported much lower levels of lactose after 24 h of fermentation even though producing identical content of lactic acid. Throughout the storage period no changes were observed in lactose and lactic acid content of kefir, which is consistent with results reported for similar time storage [36,40,44]. Guzel-Seydim et al. [40] reported that during cold storage of kefir, lactic acid production may be impaired possible due to the decrease of LAB concentration attributed to pH drop [31,36]. Diversity in results involving lactose degradation and lactic acid production, in kefir fermentation, may be attributed to differences in grain to milk ratio and in different origins of kefir grains [21].

Even though, our data did not reveal any changes in fat, protein; and carbohydrates content, a small decrease in energy content was observed between milk and kefir, possibly due to variation of carbohydrates and fat, despite no statistical significance was found.

DM in freshly made kefir may range between 9.4% and 11.1%, and it is expected to change accordingly with the variation of fat and lactose comparatively with the source milk [36,38]. Our data are consistent with these, once we observed a slightly decrease of DM content after fermentation, which is consistent with the lactose variation also observed. Assadi et al. [68] observed only 5.56% of DM in kefir, however their value was also consistent with the much lower lactose level they found compared with the source milk. After 48 h storage, no differences were observed for both lactose and total fat and consequently also for DM. However, Irigoyen et al. [36] reported a DM content decrease after 48 h storage which is consistent with the fat content decrease verified in their study.

Milk proteins are affected by proteolytic activity of the kefir grains, producing different peptides and nonprotein nitrogen compounds, thus contributing to the protein profile of kefir [69]. However, during fermentation and storage, casein content does not change significantly, suggesting a low degree of casein proteolysis, contrary to the nonprotein nitrogen compounds derived from whey protein, that increase both in fermentation and in storage [70]. Even though the protein profile has not been determined in our work, its results are hereby supported, since no differences in the total protein content of kefir and unfermented milk were observed (Table 4). Moreover, utilization of protein nitrogen by bacteria during fermentation is limited, since their preferential energy source are carbohydrates [71]. Contrary results were reported by Vieira et al. [32], showing an increased protein level during fermentation, which were explained by the interaction between stress response proteins and lipid membrane unsaturation in bacterial cells, since fermentation is a stress factor for LAB [32].

Total fat composition of kefir was identical to that of the source milk (Table 4), which is consistent with the literature [32,36], and also no difference was observed during refrigerated storage [32,36,40]. However, the fatty acid profile of freshly made and refrigerated kefir differs (Table 5). Kefir at t0 presented a decrease of 2% in SFA and an increase of 5% MUFA, these variations being identically reflected in the content of palmitic acid (C16:0) and oleic acid (C18:1n-9), respectively. These results are in line with the literature [32,72] and are useful in order to consolidate the potential health benefits of kefir [73]. Vieira et al. [32], justified the change in SFA and PUFA with the increase of desaturase activity of LAB during fermentation [74] since the conversion ratio of saturated into unsaturated fatty acids can be attributed to desaturase activity [75]. Even though, in our data, PUFA content

showed an increase after fermentation, the difference was not statistically significant. PUFAs are known to affect the aroma profile of kefir, and since an increase of PUFA would lead to a loss of the typical scent [76], it is confirmed that in our particular setting conditions the olfactive characteristics of kefir are maintained. In the first 24 h of refrigerated storage no change in fatty acids profile was noted, and after 48 h storage, only a slightly decrease in MUFA was observed. Contrary results were found by Vieira et al. [32], who reported higher MUFA and lower SFA content during storage, which was attributed to the ability of LAB to increase the production of free fatty acids by lipolysis of milk fat during the cold storage [77]. The differences observed in kefir´s fatty acids profiles, according to other authors, may be justified by the different origin of the grains since each bacterial community may present a unique fatty acids production [21,32].

5. Conclusions

Our results showed that the kefir produced under home use conditions using UHT milk is able to fulfill the *Codex Alimentarius* requirements and maintains its characteristics with respect to the physicochemical composition, both after fermentation, as well as during 48 h of refrigerated storage. Whereas fat, protein; and carbohydrate content suffered no significant changes over fermentation, lactic acid increased, and lactose decreased, as expected. The fatty acids profile of the milk and kefir samples changed during fermentation revealing a decrease in SFA, an increase in MUFA, and no change in PUFA. Refrigerated storage did not significantly impact nutritional composition and fatty acids profile, thus attesting for the stability of kefir under these conditions.

To the best of our knowledge, this is the first study to aggregate information on detailed composition, homogeneity; and stability after refrigeration, of kefir produced using CIDCA AGK1 grains in a traditional in use setting. This work further contributes to the characterization of this food that is so widely consumed around the world by focusing on kefir that was produced in typical home use conditions.

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Article 3

Acceptability of Kefir Produced by Fermentation of Portuguese Milk with CIDCA AGK1 Grains in a Sample of Portuguese Consumers

Emilia Alves [1,2], Patricia Rijo [1,3], Luis Monteiro Rodrigues [1],Catarina Rosado [1]

[1]CBIOS - Universidade Lusófona's Research Center for Biosciences and Health Technologies, Av. Campo Grande 376, 1749- 024 Lisbon, Portugal;
[2]Department of Biomedical Sciences, Faculty of Pharmacy, University of Alcalá, Campus Universitario, 28871 Alcalá de Henares, Spain;
[3]Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisbon, Portugal

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Acceptability of kefir produced by fermentation of Portuguese milk with CIDCA AGK1 grains in a sample of Portuguese consumers

Aceitabilidade do kefir produzido pela fermentação do leite português com grãos CIDCA AGK1 numa amostra de consumidores portugueses

Emília Alves, Patrícia Rijo, Luís Monteiro Rodrigues, Catarina Rosado*

CBIOS – Universidade Lusófona's Research Center for Biosciences and Health Technologies, Av. Campo Grande 376, 1749-024 Lisbon, Portugal

*corresponding author: catarina.rosado@ulusofona.pt

Abstract

Fermented foods, such as kefir, tend to be characterized by their unique flavor and aroma. Sensory perception of this type of food or beverage is a key factor for the general consumer acceptance of the product, which can be assessed through sensory tests. Conventional sensory tests include acceptance tests where consumers, not trained panelists, are asked to express their degree of like on a hedonic scale, where the level of acceptability of foods does not require a choice between alternatives. The most commonly used scale for testing consumer acceptability of foods is the 9-point hedonic scale. An average score of 7 or higher on the acceptability test indicates a high sensory quality and represents a good acceptance of the product by the panel. This study aimed to evaluate the acceptance of a kefir drink in a sample of Portuguese consumers. The acceptability test of the kefir obtained by fermentation of Portuguese milk with CIDCA AGK1 kefir grains was conducted in a group of 19 consumers using a 9-point hedonic scale and produced a mean score of 7.00 ± 1.15, which correlates with a qualitative rating of "Like moderately." This work is part of an ongoing study, designated DermapBio, conducted by our research center, with an aim to evaluate the benefits of kefir ingestion for cutaneous health.

Keywords: Kefir, fermented dairy, sensory perception, acceptability test, hedonic scale

Resumo

Alimentos fermentados, como o kefir, tendem a ser caracterizados pelo seu sabor e aroma únicos. A percepção sensorial deste tipo de alimento ou bebida é um fator chave para a aceitação geral do produto pelo consumidor, e pode ser avaliada por testes sensoriais. Testes sensoriais convencionais incluem testes de aceitação, onde os consumidores, provadores não treinados, são solicitados a expressar o grau de preferência numa escala hedónica que não exige uma escolha entre alternativas. A escala mais vulgarmente usada para testar a aceitação de alimentos pelo consumidor é a escala hedónica de 9 pontos. Uma pontuação média igual ou superior a 7 no teste de aceitabilidade indica uma elevada qualidade sensorial e representa uma boa aceitação do produto pelo painel. Este estudo teve como objetivo avaliar a aceitação da bebida kefir numa amostra de consumidores portugueses. O teste de aceitabilidade do kefir, obtido pela fermentação de um leite português com grãos de kefir CIDCA AGK1, foi realizado num grupo de 19 consumidores usando uma escala hedónica de 9 pontos, produziu uma pontuação média de 7,00 ± 1,15, o que se correlaciona com uma avaliação qualitativa de "Moderadamente agradável". Este trabalho enquadra-se num estudo in-use que pretende avaliar os benefícios da ingestão de kefir para a saúde cutânea, conduzido pelo nosso centro de investigação e codificado como DermapBio.

Palavras-chave: Kefir, leite fermentado, percepção sensorial, teste de aceitabilidade, escala hedónica

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Introduction

Kefir is a unique fermented, slightly carbonated, dairy beverage known for its organoleptic characteristics. It originated from the Caucasus, and has been traditionally consumed for centuries in several countries in Eastern Europe (1). Traditional production uses kefir grains as a natural starter, which differentiates this beverage from other fermented milk (2). Kefir can be made from different kinds of milk (cow, goat, sheep, camel, buffalo) and is characterized by an acid and slightly yeasty taste which, combined with the carbon dioxide produced by the yeast flora, confers a prickly sensation that can be considered as its typical flavor (3). Fermented foods, such as kefir, tend to be characterized by their unique flavor and aroma (3). Sensory perception of this type of food is a key factor for the general consumer acceptance of such products, and can be assessed by sensory tests (4,5). In the food industry, sensory evaluation methods used in dairy products include affective or consumers tests, among others (6). By definition, these tests are applied only to a naïve (untrained) panel, since trained panels are potentially more critical and more sensitive than the average consumer, thus ceasing to be considered a typical consumer assessment (4). Affective tests are used to assess consumer likes and dislikes, and the most typical are preference tests and acceptance tests (5,7). In preference tests, consumers are presented with several samples and are required to choose between them, that is, a preference must be indicated. In acceptance tests, also called degree of liking, consumers are asked to indicate the degree of liking on a scale where the degree of acceptability of foods does not require a choice between alternatives (4,7). The most commonly used scale for testing consumer acceptability of foods is the 9-point hedonic scale (8-12). This scale can be presented numerically or verbally, horizontally or vertically, although such structural variations have no critical effect on the results (13). It is a bipolar scale with four positive and four negative categories on each side of a neutral center. The hedonic scale uses the anchors like and dislike, thus assuming a continuum degree of affection in the consumers' preferences which can be categorized based on the like/dislike answers. This approach provides information on the product in a broader sense then a simple choice of yes or no (7,13). In rating scales, the selection of the anchor words must be meaningful and clear to the participants, must be related to the specific scale, and must prevent misinterpretation. Scoring in these scales is used with the main purpose of determining the magnitudes of the differences identified (14). In the 9-point hedonic scale, answers are usually assigned values between 1 and 9,

Introdução

Kefir é uma bebida láctea fermentada, um pouco efervescente, conhecida pelas suas características organolépticas únicas, originária do Cáucaso e tradicionalmente consumida desde há séculos em vários países do Leste Europeu (1). A sua produção tradicional usa grãos de kefir como iniciador, o que diferencia esta bebida de outros leites fermentados (2). O kefir pode ser feito a partir de diferentes tipos de leite (vaca, cabra, ovelha, camelo, búfalo) e é caracterizado por um sabor ácido e levemente fermentado que, combinado com o dióxido de carbono produzido pelas leveduras, lhe confere uma sensação de efervescência e que pode ser considerado como o seu sabor típico (3). Alimentos fermentados, como o kefir, tendem a ser caracterizados pelo seu sabor e aroma únicos (3). A percepção sensorial, desde tipo de alimento é um fator chave para a aceitação destes produtos pelo consumidor em geral e pode ser avaliada por testes sensoriais (4,5). Na indústria alimentar, os métodos de avaliação sensorial usados em laticínios incluem, entre outros, testes afetivos ou de consumo. Por definição, estes são aplicados apenas a um painel ingénuo (não treinado), uma vez um painel treinado é potencialmente mais crítico e mais sensível do que o consumidor médio, deixando assim de ser considerado avaliação típica de consumidor (4). Testes afetivos são usados para avaliar o gosto/não gosto do consumidor, sendo os mais comuns, os testes de preferência e os testes de aceitação (5,7). Nos testes de preferência os consumidores são apresentados a várias amostras e são obrigados a escolher entre elas, ou seja, deve ser indicada uma preferência. Nos testes de aceitação, também denominados grau de gosto, os consumidores são solicitados a indicar o grau de gosto numa escala em que o grau de aceitabilidade dos alimentos não requer uma escolha entre alternativas (4,7). A escala mais comummente usada para testar a aceitação de alimentos pelo consumidor é a escala hedónica de 9 pontos (8-12). Esta escala pode ser apresentada numérica ou verbalmente, horizontal ou verticalmente embora tais variações estruturais não tenham efeito crítico sobre os resultados (13). É uma escala bipolar com quatro categorias positivas e quatro negativas de cada um dos lados de um centro neutro. A escala hedónica usa as palavras âncora gosto e não gosto, assumindo assim um grau contínuo de afeto das preferências do consumidor que pode ser categorizado com base nas respostas gosto/não gosto, fornecendo informações sobre o produto num sentido mais amplo, em vez de uma simples escolha de sim ou não (7,13). Nas escalas de avaliação, a seleção das palavras âncora deve ser significativa

where a value of 1 correlates with "dislike extremely" and a value of 9 with "like extremely". Using this score, a mean value of 7 or higher is usually indicative of a high sensory quality, resulting in a good acceptance of the product (7, 15). When compared to other scaling methods, this scale is a robust way of estimating consumer like due to its simple yet sensitive categories of discriminating power. Its limited number of options also makes it suitable and easy to use for both trained or untrained panelists, thus justifying its wide acceptance (8,14,15). Therefore, when the primary objective of a study is to predict consumer acceptance, the 9-point hedonic scale has proven to be a simple and effective measuring device (8). Based on the fact that the consumption of kefir is not a typical food habit of the Portuguese population, this study aimed to evaluate the acceptance of this beverage in a sample of Portuguese consumers. This work is framed by a study regarding the cutaneous health benefits of kefir intake currently being conducted by our research team and designated as DermapBio .

Materials and Methods

Kefir beverage was prepared by fermenting semi-skim ULHT Portuguese cow's milk (purchased at a local supermarket) using CIDCA AGK1 kefir grains for 24 hours at 20 °C. CIDCA AGK1 kefir grains were obtained from the Centro de Investigacíon y Desarrollo en Criotecnologia de Alimentos (CIDCA), La Plata, Argentina. The microbiological characterization of these grains has been described elsewhere (16-18). All volunteers were recruited from the DermapBio study conducted at our research centre, thus being a convenience sampling. Due to the fact that this work is part of an in-use study where kefir was consumed, we considered this an exploratory study. The study protocol was submitted to and approved by the ethics committee of the School of Sciences and Health Technologies at Lusofona's University (Nº1/2018, 15th May 2018)

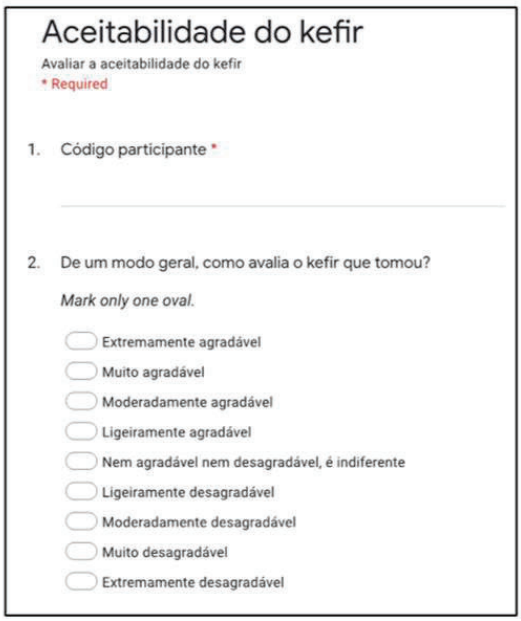
e clara para os participantes, deve estar relacionada com a escala específica e deve evitar interpretações erradas. A pontuação nessas escalas é utilizada com o objetivo principal de determinar as magnitudes das diferenças identificadas (14). Na escala hedónica de 9 pontos, atribuem-se geralmente às respostas valores entre 1 e 9, em que 1 se correlaciona com a categoria "extremamente desagradável" e 9 com a categoria "extremamente agradável". Com esta pontuação, um valor médio de 7 ou superior é geralmente indicativo de uma qualidade sensorial elevada resultando numa boa aceitação do produto (7,15). Quando comparada com outros métodos de escalonamento, esta escala é uma forma robusta de estimar o gosto do consumidor devido às suas categorias simples, porém sensíveis, em termos de poder de discriminação. Também o seu número limitado de opções a tornam adequada e fácil de usar, seja por provadores treinados ou não treinados, justificando assim a sua ampla aceitação (8,13,15). Portanto, quando o objetivo principal de um estudo é predizer a aceitação do consumidor, a escala hedónica de 9 pontos tem mostrado ser um instrumento de medição simples e eficaz (8). Atendendo ao facto do consumo de kefir não ser um hábito alimentar típico da população portuguesa, este estudo teve como objetivo avaliar a aceitação desta bebida numa amostra de consumidores portugueses. Este trabalho enquadra-se num estudo in-use que pretende avaliar os benefícios da ingestão de kefir para a saúde cutânea, conduzido pela nosso centro de investigação e codificado como DermapBio.

Material e Métodos

A bebida kefir foi preparada por fermentação de leite de vaca semidesnatado ULHT português (comprado num supermercado local) usando grãos de kefir CIDCA AGK1, durante 24 horas a 20 °C. Os grãos de kefir CIDCAAGK1 foram obtidos do Centro de Investigacíon y Desarrollo en Criotecnologia de Alimentos (CIDCA), La Plata, Argentina. A caracterização microbiológica desses grãos foi descrita em outro lugar (16-18). Todos os voluntários foram recrutados a partir do estudo DermapBio realizado no nosso centro de investigação, sendo portanto, uma amostra de conveniência. Devido ao fato deste trabalho fazer parte de um estudo in-use, onde o kefir é consumido, este estudo foi considerado um estudo exploratório. O protocolo do estudo foi submetido e aprovado pela comissão de ética da Escola de Ciências e Tecnologias da Saúde da Universidade Lusófona (Nº1 / 2018, 15 de maio de 2018) e foi conduzido de acordo com os princípios da Declaração

and was conducted according to the principles of the Helsinki Declaration. The prepared kefir was evaluated by a consumer panel using 9-point hedonic scale. The consumer group consisted of 19 volunteers, 94.7% women and 5.3% men, aged 19 to 55 years (mean age 29.11 ± 11.61 years). The volunteers had no prior experience with kefir consumption. All volunteers were given a white plastic cup with a sample (100 mL) of freshly made kefir, under controlled conditions, and then were asked to grade the beverage in a 9-point hedonic scale, where the acceptability of the product was evaluated within a score of 1 (dislike extremely) to 9 (like extremely), according to overall acceptability of the product. The scale was presented to the volunteers as a Google form questionnaire (Figure 1). Results were expressed as mean ± standard deviation (SD) or as relative frequency (%), and association between variables was performed with Pearson's Chi-Square Test using SPSS statistical package version 25 (SPSS Inc., Chicago, IL, USA).

de Helsínquia. O kefir preparado foi avaliado por um painel de consumidores usando uma escala hedónica de 9 pontos. O grupo de consumidores foi constituído por 19 voluntários, 94,7% mulheres e 5,3% homens, com idades entre 19 a 55 anos (média de idades 29,11 ± 11,61 anos). Nenhum dos voluntários tinha provado kefir antes de participar neste estudo. Todos os voluntários receberam um copo de plástico branco com uma amostra (100 mL) de kefir acabado de fazer, sob condições controladas, e seguidamente foram solicitados a classificar a bebida numa escala hedónica de 9 pontos, onde a aceitabilidade do produto foi avaliada dentro de uma pontuação de 1 (extremamente desagradável) a 9 (extremamente agradável) de acordo com a aceitabilidade geral do produto. A escala foi apresentada aos voluntários na forma de questionário Google (Figura 1). Os resultados foram expressos como média ± desvio padrão (DP) ou como frequência relativa (%), e a associação entre variáveis foi realizada com Teste Qui-quadrado de Pearson usando o pacote estatístico SPSS versão 25 (SPSS Inc., Chicago, IL, EUA).


Aceitabilidade do kefir

Avaliar a aceitabilidade do kefir

* Required

1. Código participante *

2. De um modo geral, como avalia o kefir que tomou?

Mark only one oval.

- Extremamente agradável
- Muito agradável
- Moderadamente agradável
- Ligeiramente agradável
- Nem agradável nem desagradável, é indiferente
- Ligeiramente desagradável
- Moderadamente desagradável
- Muito desagradável
- Extremamente desagradável

Figure 1 - 9-point hedonic scale translated in Portuguese language, presented as a questionnaire. The kefir consumed was evaluated by each (coded) participant as: dislike extremely [1], dislike very much [2], dislike moderately [3], dislike slightly [4], neither like nor dislike [5], like slightly [6], like moderately [7], like very much [8], or like extremely [9].

Figura 1 - Escala hedónica de 9 pontos traduzida para a língua portuguesa, apresentada na forma de questionário. O kefir consumido foi avaliado por cada participante (codificado) como: extremement desagradável [1], muito desagradável [2], moderadamente desagradável [3], ligeiramente desagradável [4], indiferente [5], ligeiramente agradável [6], moderadamente agradável [7], muito agradável [8], ou extremement agradável [9].

Results

The group of volunteers who participated in the study was characterized socio-demographically (Table 1). Within the study group, 78.9% were university students, 68.4% lived in an urban area, and the majority had no smoking habits (78.9%). The average Body Mass Index (BMI) of the sample was 21.82 ± 2.89 kg/m^2. The consumption of dairy products within the panel was mainly through the consumption of natural yogurt (84.2%), and 57.9% of the participants drank cow's milk regularly (Table 1). The results of the acceptability test using this 9-point hedonic scale showed that 58% of the volunteers assessed the kefir drink as "Like moderately", 32% as "Like very much" and only 11% assigned the rating "Dislike slightly" (Figure 2). The average score given by this sample of volunteers to our kefir was 7.00 ± 1.15, which is correlates with a "Like moderately" rating, and the acceptability of kefir was not related to the consumption of dairy products, namely, consumption of milk or yogurt (p = 0.310 and p = 0.568, respectively).

Discussion

Our results, correlated with a "Like moderately" rating, seem to indicate a reasonable acceptance of kefir by our panel. A classification such as ours can be an indicator of a high quality sensory product (13). Our results were consistent with those obtained by Moretti *et al.* (14), in which the acceptability of kefir made with CIDCA AGK1 grains, was also tested using untrained panelists (n=93) and a 9-point hedonic scale. In their study, the average score obtained was 7.88 ± 0.35 and this result was correlated with a qualitative grade of "Like very much", which indicated a good acceptance of the product by the panel (14).

The use of a 9-point hedonic scale has been widely accepted as a good tool to infer consumer acceptability as it is able to provide internal validity to the test (14). The use of this type of scale for assessment of consumer acceptability of dairy has been confirmed by other researchers (6,10,14). Nevertheless, some weak points have been identified, namely the small number of categories available, the lack of equal intervals between categories, the presence of the neutral category "neither like or dislike" that lessens the scale efficiency, and the general consumer tendency to avoid using the extreme categories, which may increase the scale vulnerability to ceiling effects (7,11,19). A consideration must be

Resultados

O grupo de voluntários que participaram no estudo foi caracterizado sócio-demograficamente (Tabela 1). Cerca de 78,9 % eram estudantes universitários, 68,4 % viviam em área urbana e a maioria não tinha hábitos tabágicos (78,9 %). O Índice de Massa Corporal (IMC) médio da amostra foi de 21.82 ± 2.89 kg/m^2 e o consumo de lacticínios, no painel, foi principalmente devido ao consumo de iogurte natural (84,2 %) e 57,9 % dos participantes bebiam leite de vaca regularmente (Tabela 1). Os resultados do teste de aceitabilidade utilizando esta escala hedónica de 9 pontos mostraram que 58 % dos voluntários consideraram a bebida kefir como "Moderadamente agradável", 32 % como "Muito agradável" e apenas 11 % atribuíram a classificação de "Ligeiramente desagradável" (Figura 2). A pontuação média dada por esta amostra de voluntários ao nosso kefir foi de 7,00 ± 1,15 o que equivale a uma classificação de "Moderadamente agradável," e a aceitabilidade do kefir não se relacionou com o consumo de laticínios, nomeadamente, consumo de leite ou iogurte (p = 0,310 e p = 0,568, respectivamente).

Discussão

Os nossos resultados, correlacionados com uma classificação de "Moderadamente agradável", parecem indicar uma aceitação razoável do kefir pelo nosso painel. Uma classificação como a nossa pode ser indicador de um produto sensorial de alta qualidade (13). Os nossos resultados foram consistentes com os obtidos por Moretti *et al.* (14), que testou a aceitabilidade de kefir feito a partir de grãos CIDCA AGK1, usando também provadores não treinados (n=93) e uma escala hedónica de 9 pontos. No seu estudo, o score médio obtido foi de 7,88 ± 0,35 e esse resultado foi correlacionado com uma classificação qualitativa de "Gosto muito", o que indicou uma boa aceitação do produto por parte dos consumidores (14).

O uso de uma escala hedónica de 9 pontos tem sido amplamente aceite como uma boa ferramenta para inferir sobre a aceitabilidade dos consumidores, uma vez que é capaz de fornecer validade interna ao teste (15). O uso deste tipo de escala para avaliação da aceitabilidade do consumidor de produtos lácteos foi confirmado por outros investigadores (6,10,14). No entanto, foram identificados alguns pontos fracos, nomeadamente o pequeno número de categorias disponíveis, a falta de intervalos iguais entre as categorias, a presença

Table 1 - Sociodemographic characteristics of consumers sample
Tabela 1 - Características sociodemográficas da amostra de consumidores.

Sociodemographic Characteristics / Características sociodemográficas	Portuguese consumers sample / Amostra de consumidores Portugueses (n=19)
Gender / Género	
Female / Feminino, n (%)	18 (94.7)
Male / Masculino, n (%)	1 (5.3)
Age / Idade, mean (SD) / média (DP), years / anos	29.11 (11.61)
Scholarity / Escolaridade	
High School (12th grade) / Ensino Secundário (12ºano), n (%)	16 (84.2)
Doctorate / Doutoramento, n (%)	3 (15.8)
Career / Profissão	
Professor / Professor, n (%)	3 (15.8)
University student / Estudante universitário, n (%)	15 (78.9)
Entrepreneur / Empresário, n (%)	1 (5.3)
Residence / Residência	
Urban / Urbano, n (%)	13 (68.4)
Rural / Rural, n (%)	6 (31.6)
Smoking habits / Hábitos tabágicos	
Non smoker / Não fumador, n (%)	15 (78.9)
Occasional smoker / Fumador ocasional, n (%)	3 (21.4)
Smoker / Fumador, n (%)	1 (7.1)
BMI / IMC, mean (SD) / média (DP), kg/m^2	21.82 (2.89)
Dairy consumption / Consumo de laticínios	
Cow milk / Leite de vaca, n (%)	11 (57.9)
Natural yogurt / Iogurte natural, n (%)	16 (84.2)
Vegetable drink / Bebida vegetal, n (%)	5 (26.3)

SD – Standard Deviation / DP – Desvio Padrão; BMI – Body mass Index / IMC – Índice de Massa Corporal.

made about the use of a scale that was translated to Portuguese language. As demonstrated by Curia *et al*. (20) regarding the Spanish language, the use of the 9-point hedonic scale in languages different from English must be done with caution, as the general population may fail to perceive the translations with the same meaning as they have in English, especially with regard to the extreme categories of the scale (20). To the best of our knowledge, the translation of this scale into Portuguese has not yet been validated and, as such, care should be taken in generalizing the conclusions drawn. It should be noted that acceptance tests (consumer liking tests) should preferably be carried out with a larger number of individuals (6). Some authors recommend 50 consumers as the minimum desirable to guarantee the accuracy of the statistical analysis and to be able to draw conclusions product acceptance (not applicable to

da categoria neutra "nem gosto, nem não gosto" que diminui a eficiência da escala e a tendência geral do consumidor em evitar usar as categorias extremas, que podem aumentar a vulnerabilidade da escala aos efeitos de teto (7,11,19). Devemos ainda ter em consideração o facto de termos usado uma escala que foi traduzida para a língua portuguesa. Conforme demonstrado por Curia *et al*. (20) para a língua espanhola, o uso da escala hedónica de 9 pontos em outras línguas diferentes do inglês deve ser feito com cautela, pois a população em geral pode não perceber as traduções com o mesmo significado que elas possuem em inglês, especialmente no que diz respeito às categorias extremas da escala (20). Até onde sabemos, a tradução desta escala para a língua portuguesa ainda não foi validada e, como tal, devemos ter alguma precaução na generalização das conclusões tiradas. De notar que os testes de

trained tasters) (5,6,8). Furthermore, because we used a convenience sample of volunteers already willing to participate in a study involving kefir consumption for eight weeks, the main disadvantage of which is the lack of clear generalization (21), we cannot extrapolate our results to the general population. Therefore, due to the type of sampling and the limited number of consumers used in our study, we cannot fully infer about the acceptability of kefir for Portuguese consumers. Nevertheless, these results can provide an indication of how the product is viewed by consumers with no previous contact with this beverage. Hedonic opinions, such as food choice, are affected by environmental context and individual expectations (22,23). Both the intra- and the inter-variability of consumers may influence the product acceptance throughout time (7,22,23). In general, the individual like/dislike stimuli may be influenced by environment, for example, type of meal, time of day, number of times the food has been consumed recently or temperature of the food, thus increasing the difficulty of measuring a stable attitude toward a certain food (7,24). As stated by Lawless & Heymann (2010), habits, experiences, contexts and attitudes are important contributors to the actual consumption of a food in a specific situation (7). Since the participants in our study were all part of the same group and so, were not blind to kefir consumption, this

aceitação (testes de gosto do consumidor) devem ser realizados preferencialmente com um grande número de indivíduos (6). Alguns autores recomendam que 50 consumidores como o mínimo desejável para garantir a precisão da análise estatística, podendo, assim, concluir sobre a aceitação do produto (não aplicável a provadores treinados) (5,6,8). Além disso, porque utilizámos uma amostra de conveniência, com voluntários já dispostos a participar num estudo envolvendo o consumo de kefir por oito semanas, cuja principal desvantagem é a falta de generalização clara (21), não podemos extrapolar os nossos resultados para a população em geral. Portanto, devido ao tipo de amostragem e ao número limitado de consumidores usados no nosso estudo, não podemos inferir completamente sobre a aceitabilidade do kefir para o consumidor português. No entanto, estes resultados podem fornecer uma indicação de como o produto é visto por consumidores sem contato prévio com este bebida. As opiniões hedónicas, como a escolha alimentar, são afetadas pelo contexto ambiental e pelas expectativas individuais (22,23). A intra e a intervariabilidade dos consumidores podem, ambas, influenciar a aceitação do produto ao longo do tempo (7,22,23). Em geral, os estímulos individuais de gosto/ não gosto podem ser influenciados pelo contexto ambiental, como por exemplo, pelo tipo de refeição, hora do dia, número de vezes que o alimento foi

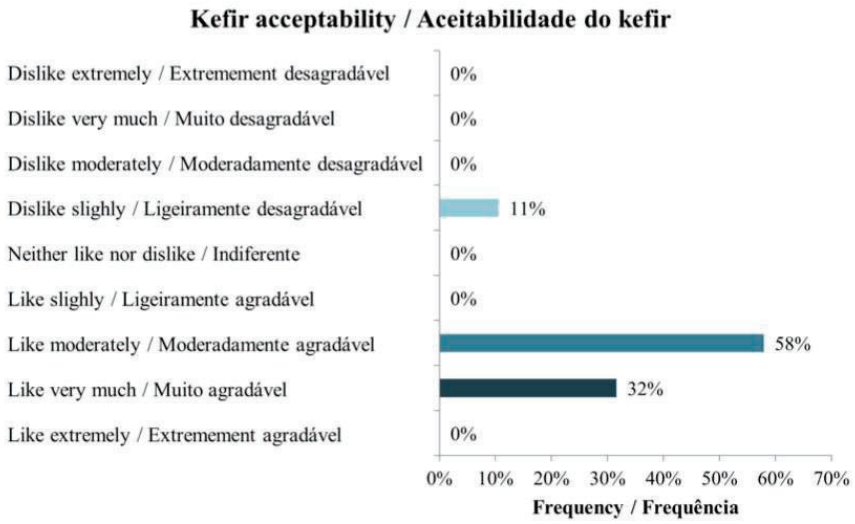


Figure 2 - Kefir acceptability in a sample of Portuguese consumers based on the 9-point hedonic scale (frequency (%), n=19)
Figura 2.- Aceitabilidade do kefir numa amostra de consumidores portugueses baseada na escala hedónica de 9 pontos (frequência (%), n=19).

factor may have influenced the individual choices of the volunteers, despite the fact that the scale was presented to each participant individually. Nevertheless, these results support the adoption of the protocol applied to our in-use "DermapBio" study of the impact on the skin health of kefir consumption conducted by our research team, as during the study this beverage must be taken daily.

Conclusion

General consumer acceptance of fermented foods, such as kefir, that are characterized by their unique flavor and aroma are mainly dependent of their sensory perception. Although kefir is not traditionally consumed in Portugal, our kefir drink showed a good acceptance in this sample of consumers and supports the protocol adopted in a posterior study.

Author Contributions Statement

PR, CR, LMR, and EA conceptualization and study design; EA experimental implementation; EA and CR data analysis; EA illustrations; EA, PR and CR drafting editing and reviewing

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consumido recentemente ou temperatura do alimento, aumentando assim a dificuldade de medir uma atitude estável em relação a um determinado alimento (7,24). Como afirmam Lawless & Heymann (2010), hábitos, experiências, contextos e atitudes são importantes contribuintes para o consumo real de um alimento numa situação específica (7). Dado que os participantes no nosso estudo faziam parte do mesmo grupo e, portanto, não eram cegos ao consumo de kefir, esse fator pode ter influenciado nas escolhas individuais dos voluntários, apesar de a escala ter sido apresentada a cada participante individualmente. No entanto, estes resultados apoiam a adoção do protocolo aplicado a nossa estudo in-use "DermapBio" do impacto do consumo de kefir na saúde da pele, conduzido pela nossa equipa de investigaçãom, á que este bebida deve ser tomado diariamente.

Conclusões

A aceitação pelo consumidor em geral de alimentos fermentados, como o kefir, que são caracterizados pelo seu sabor e aroma únicos, depende principalmente da sua percepção sensorial. Embora o kefir não seja tradicionalmente consumido em Portugal, a nossa bebida kefir mostrou uma boa aceitação nesta amostra de consumidores e apoia o protocolo adotado num estudo posterior.

Declaração sobre as contribuições do autor

PR, EA, LMR e CR, Conceptualização e desenho de estudos; EA implementação experimental; EA e CR Análise de dados; EA ilustrações;EA PR e CR, edição e revisão da redação

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Conflito de Interesses

Os editores senior envolvidos na autoria deste manuscrito não tiveram qualquer participação no processo de revisão ou de decisão.

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Chapter II

Article 4

Homemade Kefir Consumption Improves Skin Condition—A Study Conducted in Healthy and Atopic Volunteers

Emília Alves [1,2], João Gregório [1], André Rolim Baby [3], Patrícia Rijo [1,4], Luis M. Rodrigues [1,*] and Catarina Rosado [1,*]

[1] CBIOS—Universidade Lusófona's Research Center for Biosciences & Health Technologies, Campo Grande 376, 1749-024 Lisboa, Portugal; emilia.alves@ulusofona.pt (E.A.); joao.gregorio@ulusofona.pt (J.G.); patricia.rijo@ulusofona.pt (P.R.)
[2] Department of Biomedical Sciences, Faculty of Pharmacy, University of Alcalá, Carretera Madrid-Barcelona, Km 33.100, 28805 Alcalá de Henares, Spain
[3] Department of Pharmacy, Faculty of Pharmaceutical Pharmacy, University of São Paulo, São Paulo 05508-000, Brazil; andrerb@usp.br
[4] Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisboa, Portugal

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Emília Alves [1,2], João Gregório [1], André Rolim Baby [3], Patrícia Rijo [1,4], Luis M. Rodrigues [1,*] and Catarina Rosado [1,*]

[1] CBIOS—Universidade Lusófona's Research Center for Biosciences & Health Technologies, Campo Grande 376, 1749-024 Lisboa, Portugal; emilia.alves@ulusofona.pt (E.A.); joao.gregorio@ulusofona.pt (J.G.); patricia.rijo@ulusofona.pt (P.R.)
[2] Department of Biomedical Sciences, Faculty of Pharmacy, University of Alcalá, Carretera Madrid-Barcelona, Km 33.100, 28805 Alcalá de Henares, Spain
[3] Department of Pharmacy, Faculty of Pharmaceutical Pharmacy, University of São Paulo, São Paulo 05508-000, Brazil; andrerb@usp.br
[4] Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisboa, Portugal
* Correspondence: monteiro.rodrigues@ulusofona.pt (L.M.R.); catarina.rosado@ulusofona.pt (C.R.)

Abstract: Diet has a fundamental role in the homeostasis of bodily functions, including the skin, which, as an essential protective barrier, plays a crucial role in this balance. The skin and intestine appear to share a series of indirect metabolic pathways, in a dual relationship known as the "gut-skin axis". Hence, the gut-skin axis might be receptive to modulation via dietary modification, where probiotics can be included, thus representing a potential therapeutic target in inflammatory skin diseases, such as atopic dermatitis (AD), in order to control and/or ameliorate symptoms. Kefir is one of the most ancient fermented foods, with probiotic characteristics that have been associated with a wide variety of health-promoting benefits, and it presents a microbiological diversity that makes its application as a probiotic in the gut-skin relationship of the utmost interest. However, the impact of a diet containing kefir on skin health has yet to be reported in scientific literature. This study aimed to assess the impact of the intake of homemade kefir in the skin of healthy and atopic volunteers. The intervention resulted in a boost on barrier function in both skin types verified only in the respective kefir intake groups. An improvement in the degree of severity of AD was also confirmed for the kefir intake group. Atopic individuals may benefit from kefir intake, especially in regard to their skin hydration. Finally, the effects observed on skin barrier function in this study probably culminate from the effects of all the ingredients in kefir, including the complex microbiota, its metabolites and macro- and micronutrients resulting from the fermentation. This work opens the way for more advanced research on the impact of the probiotic kefir on cutaneous health, further clarifying its mechanism of action namely via gut-skin axis.

Keywords: kefir; cutaneous health; atopic dermatitis; transepidermal water loss (TEWL); hydration; skin barrier; scoring of atopic dermatitis (SCORAD)

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1. Introduction

Diet has a fundamental role in the homeostasis of bodily functions, including the functions of the skin, which, as an essential protective barrier, plays a crucial role in this balance [1–3]. The skin and intestine appear to share a series of indirect metabolic pathways in a dual relationship known as the "gut-skin axis" [4–6]. On the one hand, the impairment of the intestinal microbiota is linked to the development of allergic diseases, and the intestinal microbiota and/or dietary metabolites can be detected in the skin. On the other hand, skin health has been linked to the integrity of the intestinal barrier and/or suppression of pro-inflammatory mediators, e.g., via vitamin D [4,7–9].

In recent years, growing research in these areas of interest within human nutrition has led to the expansion of probiotics as health promoters [10,11]. Probiotics are live microorganisms that, by definition, must confer a health benefit on the host. Probiotics may act via numerous mechanisms, including the restoration of intestinal microbial balance, prevention of pathogen invasion by competitive binding to epithelial cells, suppression of pathogen growth by bacteriocin secretion, and restoration of impaired intestinal barrier function [10,12]. Given that the gastrointestinal mucosa and gut-associated lymphoid tissue harbor more than 70% of the body's immune cells, this may explain the growth in research data linking these organs to multiple disease mechanisms. This seems to be the case in atopic dermatitis (AD), one of the most prevalent inflammatory skin diseases [6,7,13].

AD has been associated with an exacerbated skin response to environmental agents, characterized by relevant symptoms including pruritic lesions with typical morphology, pain, and sleep disturbances [1,14]. The onset of AD points towards a complex interaction between skin barrier dysfunction, immune dysregulation, environmental risk factors, and (intestinal and skin) dysbiosis [1,5,6,15]. Intestinal dysbiosis seems to increase epithelial permeability via pro-inflammatory cytokines, promote immune dysregulation, and intensify the chronic systemic inflammation in AD [4–7,9,16]. Conversely, this also suggests that the gut-skin axis would be receptive to modulation via dietary modification, wherein probiotics can be included, thus representing a potential therapeutic target in AD to control and/or ameliorate AD symptoms [11,17–19].

Kefir is one of the most ancestral fermented foods with probiotic characteristics [20–23]. Traditionally prepared by the fermentation of milk with kefir grains and most popular in northeastern Europe and Asia, it consists of a symbiotic mixture of lactic acid bacteria (LAB) and yeasts that, in addition to acting synergistically, also produce several bioactive compounds [24–27]. A wide variety of health-promoting benefits have been associated with its use [28–33] and have expanded its popularity beyond its traditional borders within northeaster Europe and Asia. Anti-inflammatory effects [34], antimicrobial activity [35], strengthening of the immune system [36], antioxidant activity [37], and the inhibition of pathogenic microorganisms [24,38] have been reported as a result of kefir consumption. In addition, the topical application of a gel made from a non-microbial fraction of kefir showed an improvement in the wound healing capacity [39]. These properties have been attributed both to the presence of a complex microbiota, with high resistance to passage through the gastrointestinal tract and high adhesion capacity to the intestinal mucus, and to the action of metabolites released during fermentation, namely organic acids and short chain fatty acids (SCFA) [24,37,40–42]. Nevertheless, studies demonstrating its therapeutic interest in specific conditions are very limited, and its potential skin benefits and applicability in the management of AD have yet to be explored.

This study aimed to assess the impact of the regular consumption of kefir prepared in homemade conditions in the skin of healthy and atopic volunteers.

2. Materials and Methods

2.1. Study Design

A controlled intervention study, coded DermapBio, was conducted according to the principles of the Helsinki Declaration and after informed consent. The study protocol was approved by the ethics committee of the School of Sciences and Health Technologies at Lusofona University (N°1/2018, 15 May 2018). Study subjects were recruited by convenience sampling between October 2019 and December 2020. Subjects were asked to answer a questionnaire that examined sociodemographic and lifestyle conditions, as well as specific inclusion/exclusion criteria. These criteria are summarized in Table 1.

Table 1. General inclusion and exclusion criteria and atopic group specific inclusion criteria.

General Inclusion criteria	
1.	Volunteers of both genders aged between 18 and 64 years old
Atopic inclusion criteria	
1.	Eczema/atopic dermatitis diagnosis
2.	Rhinitis or allergic conjunctivitis diagnosis
3.	Asthma diagnosis
General Non-inclusion/Exclusion criteria	
1.	Regular consumption of kefir or any probiotic strains (as supplements or pharmaceuticals) in the three months prior to the study or during the study
2.	Oncologic disease
3.	Women who were pregnant or breastfeeding
4.	Gastrointestinal disease diagnostic affecting bowel movement (such as Irritable Bowel Syndrome or Crohn's Disease)
5.	Retinoid treatment in the three months prior to the study or during the study
6.	Antibiotic treatment in the 30 days prior to the study or during the study
7.	Topical treatment with corticosteroids/anti-inflammatories in the study area in the eight days prior to the study or during the study
8.	Chronic illness that involves taking regular (daily) medications such as insulin, oral antidiabetics, anti-inflammatories, or immunosuppressants
9.	Skin disease in the study areas
10.	Cosmetic treatment of the skin, scrubbing, or depilation at the study areas in the 30 days prior to the study, or during the study period
11.	Failure to comply with the guidelines of the study

Subjects were assigned to the different groups according to the inclusion criteria. The atopic group (n = 19) included 1 male and 18 females, aged between 19 and 56 years (mean age 31.7 $\pm$ 11.9 years), wherein 47% were under 30 years old. Within this group, all subjects along with presented with AD; 14 (74%) of the subjects within this group also reported rhinitis, and 6 (32%) reported asthma diagnosis. All other subjects who fulfilled the eligibility criteria, excluding the atopic criteria, and were free of skin diseases, including AD, psoriasis, and other systemic diseases that may impact skin condition, were assigned to the healthy group. These subjects (n = 33) included 6 males (18%) and 27 females (82%), aged between 20 and 60 years (mean age 27.0 $\pm$ 10.1 years), wherein 61% were under 30 years old. Within each group, volunteers were assigned to either the kefir intake or the control (without intake) group, according to their preference.

This research aimed to compare, for each skin type evaluated, the effect of kefir ingestion between the intervention groups and respective controls, thus using a parallel group design. However, this design is unable to distinguish between changes induced by food ingestion and those induced by differences between individuals at baseline [43]. Therefore, in order to minimize baseline individual variability, especially in studies involving dietary interventions, a crossover design is recommended as individuals are used as their own controls (paired comparisons) [43,44]. Hence, a crossover design was then sequentially applied to each study group, since, for each individual, a comparison was made between the parameters measured before and after the intervention (Figure 1).

All subjects were instructed to proceed as follows during the study period: avoid overexercising and major lifestyle changes; not consume dietary supplements or fermented foods; not change their usual dietary intake of food fiber or food containing oligosaccharides; refrain from using laxatives; refrain from changing type and frequency of regularly used skin-care agents; avoid travelling abroad.

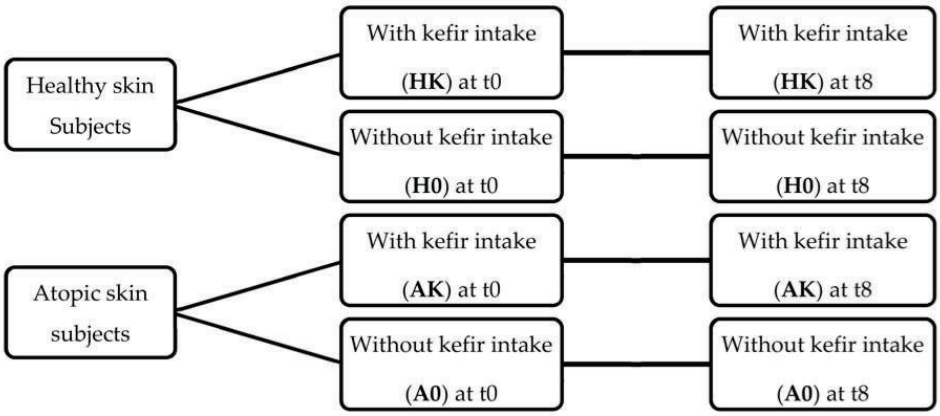

Figure 1. Study design regarding skin conditions and kefir intake during the eight-week intervention period: HK—healthy skin with kefir intake; H0—healthy skin without kefir intake; AK—atopic skin with kefir intake; A0—atopic skin without kefir intake.

Physiological conditions, skin phototype, and anthropometric measurements (weight, height, and waist circumference) were obtained from all the participants. The skin phototype was assessed (by a single researcher) using the Fitzpatrick phototype classification [45]. Height was self-reported, weight was measured using a digital weight scale Tanita® BC601 (Tanita Europe BV, Amsterdam, the Netherlands), and waist circumference (WC) was measured using a Kern® MSW circumference tape measure (KERN & SOHN GmbH, Balingen, Germany). Body Mass Index (BMI) was calculated as weight (kg)/(height (m))^2 [46].

The control groups, A0 and H0, did not consume kefir. The intervention in groups AK (atopic skin with kefir intake) and HK (healthy skin with kefir intake) consisted of the daily consumption of kefir for eight weeks. This period has been adopted in similar trials [47–52], and is supported by the fact that approximately two weeks are required for the development of a consistent probiotic gut colonisation, i.e., stable detection in faecal content, and approximately one month to observe a significant change in cytokines at the gut level. Thus, the period of eight weeks, being sufficient to impact the bowel, would also be long enough for a putative effect on the skin.

The primary endpoints in this study were a decrease in transepidermal water loss (TEWL) and an increase in *stratum corneum* (SC) hydration for all subjects, and a decrease in the SCORAD Index for atopic subjects.

2.2. Assessment of Dietary Intake

Dietary intake was assessed at baseline, for all subjects, through a three-day dietary record (two weekdays and one weekend day) [53,54]. Detailed instructions for record-keeping were provided in writing to all subjects.

2.3. Kefir Intervention

Kefir grains CIDCA AGK1 were obtained from the *Centro de Investigación y Desarrollo en Criotecnología de Alimentos* (CIDCA), La Plata, Argentina. Microbiological characterization, preservation, and storage of these grains have been described elsewhere [55–57]. Kefir was produced by fermentation of a commercial ultra-high temperature pasteurized (UHT) semi-skimmed cow milk of Portuguese provenance (Nova Açores®, S. Miguel, Portugal), with CIDCA AGK1 kefir grains using a grain inoculum of 10% (*w*/*v*), for 24 h, at a temperature of 20 ± 1 °C. The fermentation conditions were designed to be representative of Portuguese household conditions, as described elsewhere [27]. In order to assure the daily intake of kefir for eight consecutive weeks, each subject of both intake groups (AK or HK) visited the research center three times a week (Monday, Wednesday and Friday). During the

visit, the subjects drank 100 mL of kefir and were given white plastic sterile containers with the kefir doses of the following days. The subjects were instructed to store these kefir samples in their household refrigerator to maintain their characteristics [27]. It was determined that 100 mL of the prepared kefir had a nutritional composition of 1.28 ± 0.04 g of fat, 3.15 ± 0.19 g of protein and 4.91 ± 0.19 g of carbohydrates and 0.6 g of lactic acid. Microbiologically, it provided 7×10^9 colony-forming units (CFU) of LAB and 2×10^8 CFU of yeast [27], which is consistent with the literature for the daily ingestion of probiotic bacteria capable of surviving passage through the gastrointestinal tract and thus reaching the necessary sites to exercise their positive physiological functions, both intestinal and immunological [58,59].

2.4. Skin Measurements

The skin condition was quantitatively evaluated by non-invasive bioengineering equipment, including those assessing TEWL, SC hydration, and erythema, which is a sign of exacerbation in AD [60–62]. TEWL, a measure of the rate of water lost through the skin, reflects barrier dysfunction directly, thus being a parameter of interest to evaluate skin barrier function in both healthy and diseased skin [60,63]. It was measured using a Tewameter® TM300 (Courage + Khazaka Electronic GmbH, Köln, Germany) in accordance with the published guidelines [64], and measurements were expressed as $g/m^2/h$. Skin hydration is indicative of the water content of the SC, which is also a parameter of interest in both healthy and atopic skin [61,62]. It was measured using a Corneometer® CM825 (Courage + Khazaka Electronic GmbH, Köln, Germany) and was assessed as skin conductance given by the reactive capacitance of skin, using the stratum corneum as a dielectric membrane [65]. Measurements were expressed in arbitrary units (AU).

All participants were advised to refrain from using moisturizers or other cosmetic products in the tested areas 48 h before the measurements. Measurement areas were assigned in the ventral forearm (10 cm below the inner elbow crease), leg (outer side, 10 cm below the knee), and forehead (mid area). Measurements were taken in all subjects before and after the eight weeks of intervention, t0 and t8, respectively, and were performed by the same researcher using identical standards. Measurements were performed under controlled temperature (21 ± 1 °C) and humidity conditions (relative humidity, 50 ± 10%) after a period of acclimatization of 20 min.

As shown in a previous study, the use of a stress test to assess the skin barrier function after a probiotic intervention represents a novel approach in this field [63]. Therefore, a sodium lauryl sulfate (SLS)-induced skin lesion model was applied at baseline (t0) and after the intervention period (t8). This test, consisting of the application of a 1% solution of SLS under occlusion for 24 h, was conducted in the forearm and only on volunteers with healthy skin, as the application of SLS would be detrimental to the volunteers in the atopic group, potentially causing excessive discomfort. The extent of the impact of SLS was assessed by evaluation of TEWL combined with measurement of erythema as described elsewhere [63] using a Chroma Meter® CR300 (Konica Minolta, Tokyo, Japan) and expressed as a* in the L*a*b* system color [66].

2.5. SCORAD Index Assessment

The standard scoring system of Atopic Dermatitis—SCORAD Index, developed by the European Task Force Group on Atopic Dermatitis (ETFAD), considered the best validated scoring system to assess AD clinical severity, was applied in this study [67,68]. This severity classification system contemplates two distinct scores: the objective SCORAD score (intensity and extent of the lesions), which ranges from 0 to 83; and the subjective SCORAD score (pruritus and sleep loss), which extends the SCORAD total score to a maximum of 103. The objective SCORAD score is divided into part A, consisting of the interpretation of the extent of the disorder, which represents the affected body sites, and part B representing the intensity of the lesions. The subjective SCORAD score is given by

part C, consisting of symptoms such as itching and sleep loss during the three days prior, and is scored by the patients. SCORAD Index is determined by [68]:

$$SCORAD\ Index = \frac{A}{5} + \frac{7B}{2} + C$$

The SCORAD Index was assessed at t0 and t8 (only) in the atopic group by the same researcher using identical criteria. Considering the ETFAD recommendation, the AD severity was classified as mild for SCORAD Index < 25, as moderate for SCORAD Index between 25–50, and as severe for SCORAD Index > 50 [67].

2.6. Statistical Analysis

Results were expressed as mean ± standard deviation (SD), as relative frequencies, or as median and first and third quartiles. Since the data were not normally distributed (normality assessed by the Shapiro–Wilk test), non-parametric tests were chosen to test different hypotheses. For continuous variables, differences within individuals were identified by Wilcoxon signed rank test and differences between kefir intake and control groups by Mann-Whitney U test. The Chi-square test was used to test associations between categorical variables. Pearson's correlation was used to evaluate possible relations between skin barrier function parameters and the severity of AD. Linear regressions were used to evaluate the association between kefir intake and skin improvements and their potential confounding factors. All analyses were performed using the SPSS statistical package version 25 (SPSS Inc., Chicago, IL, USA) with a significance level of 0.05.

3. Results

3.1. Study Groups Characteristics

Before the beginning of the intervention, socio-demographic characteristics were assessed (Supplementary Table S1). Data concerning daily food intake were also collected (Supplementary Table S2). The physiological characteristics are presented in Table 2.

Table 2. Physiological characteristics of study participants (relative frequency (%); mean ± SD).

Physiological Characteristics	Healthy Group (n = 33)			Atopic Group (n = 19)		
	HK	H0	p-Value	AK	A0	p-Value
Gender						
Female, n (%)	12 (92.3)	15 (75.0)	0.208	9 (100)	9 (90.0)	0.330
Male, n (%)	1 (7.7)	5 (25.0)		0	1 (10.0)	
Age, mean (SD), years	28.9 (13.0)	25.8 (7.71)	0.739 *	30.4 (12.3)	32.9 (12.1)	0.538 *
Skin Phototype						
Type II, n (%)	6 (46.2)	7 (35.0)	0.388	4 (44.4)	2 (20.0)	0.252
Type III, n (%)	5 (38.5)	12 (60.0)		5 (55.6)	8 (80.0)	
Type IV n (%)	2 (15.3)	1 (5.0)		0	0	
BMI, mean (SD), kg/m^2	22.6 (3.68)	23.3 (4.19)	0.439 *	22.7 (3.40)	22.8 (2.18)	0.540 *
Waist circumference, mean (SD), cm	72.4 (9.19)	77.5 (13.6)	0.328 *	77.2 (8.67)	78.6 (6.02)	0.653 *

SD—standard deviation. BMI—Body Mass Index. HK—healthy skin with kefir intake; H0—healthy skin without kefir intake; AK—atopic skin with kefir intake; A0—atopic skin without kefir intake. Groups compared by Chi-square test, except (*) where Mann-Whitney U test was applied, with $p < 0.05$ for statistical significance.

Despite the different sample sizes of the groups, subjects who were given kefir, either healthy or atopic, showed no differences in physiological characteristics, regarding the respective control groups, at baseline, as shown in Table 2. In addition, no differences were found for lifestyle indicators, such as cigarettes and alcohol consumption, nor dairy intake (Supplementary Table S1). All groups presented a mean BMI below 24.9 kg/m^2, representative of normal weight, and a mean WC below 80 cm, considered within the

normal range for both men and women, thus indicating low risk of metabolic diseases [69]. Regarding the dietary intake, no differences were observed for energy, macronutrients, and water, between subjects who drank kefir, either healthy or atopic, and their respective controls (Supplementary Table S2). Although all macronutrients (assessed as a percentage of the energy intake) were within the recommended range, fiber intake was found to be lower than the recommendation [70]. These data indicate identical baseline characteristics and conditions for the intervention among kefir intake and control for both healthy and atopic groups.

3.2. Skin Measurements

Skin condition was assessed by measuring TEWL, SC hydration, and erythema, at t0 and at t8.

An analysis of the variation of skin parameters after eight weeks was conducted, comparing kefir intake and control groups in both healthy and atopic volunteers (Table 3). In order to minimize the impact of interindividual variability, for this comparison, variation on skin parameters was computed as a deviation from baseline, calculated as:

$$Deviation\ (variable) = [(variable\ at\ t8) - (variable\ at\ t0)]/(variable\ at\ t0)$$

Table 3. Comparison of skin parameters variation, between kefir intake and control groups, for both healthy and atopic volunteers, after eight weeks of kefir ingestion (median (Q1, Q3)).

Deviation of Skin Parameters	Healthy Group (n = 33)			Atopic Group (n = 19)		
	HK	H0	p-Value	AK	A0	p-Value
TEWL						
Forearm	−0.302 (−0.489, 0.0149)	0.0058 (−0.12, 0.081)	0.018	−0.529 (−0.601, −0.428)	0.148 (−0.571, 0.578)	<0.001
Leg	−0.0976 (−0.321, 0.244)	0.0143 (−0.248, 0.116)	0.854	−0.288 (−0.333, −0.176)	0.507 (0.0656, 1.36)	<0.001
Forehead	−0.220 (−0.375, −0.0448)	−0.0128 (−0.196, 0.152)	0.036	−0.457 (−0.612, −0.243)	0.150 (−0.0571, 0.636)	<0.001
Hydration						
Forearm	−0.0196 (−0.0784, 0.0959)	−0.184 (−0.256, −0.0132)	0.034	0.452 (0.300, 0.560)	−0.0810 (−0.282, 0.119)	0.001
Leg	0.143 (−0.134, 0.212)	−0.0270 (−0.246, 0.0896)	0.320	0.250 (0.213, 0.522)	0.0470 (−0.0894, 0.220)	0.034
Forehead	0.128 (−0.0447, 0.373)	−0.127 (−0.325, 0.0356)	0.012	0.244 (0.146, 0.537)	0.0450 (−0.297, 0.466)	0.086
Erythema						
Forearm	−0.0556 (−0.186, −0.0170)	−0.0745 (−0.111, 0.0119)	0.685	−0.133 (−0.185, −0.0692)	−0.0404 (−0.191, 0.129)	0.221

Q1—first quartile; Q3—third quartile. HK—healthy skin with kefir intake; H0—healthy skin without kefir intake; AK—atopic skin with kefir intake; A0—atopic skin without kefir intake. TEWL—transepidermal water loss. Groups compared by Mann-Whitney U test, with $p < 0.05$ for statistical significance.

Deviation variables must be interpreted as follows: for TEWL and erythema, negative values represent an improvement in skin condition after the intervention, while for hydration, an improvement is only observed when the deviation value is positive.

As shown in Table 3, on the healthy skin volunteers, forearm and forehead TEWL decreased in the HK group compared to H0 group ($p = 0.018$ and $p = 0.036$, respectively). Moreover, the kefir-supplemented group showed increased forearm and forehead hydration, compared to the control ($p = 0.034$ and $p = 0.012$, respectively). No differences were observed between HK and H0 for erythema ($p = 0.685$). These results are supported by those obtained in individual paired comparisons that showed that after eight weeks of kefir ingestion, on healthy subjects, forearm and forehead TEWL and erythema decreased significantly compared to t0 ($p = 0.016$, $p = 0.019$, and $p = 0.023$, respectively) (Supplementary Table S3), thus confirming the effective improvement in skin conditions.

Furthermore, results from application of the SLS induction lesion model on healthy skin, performed at t0 and t8, are shown in Table 4.

Table 4. Variation of skin parameters, after lesion induction with SLS, for the healthy group (median (Q1, Q3)).

Deviation of Skin Parameters at Forearm	Healthy Group (n = 33)		
	HK	H0	p-Value
TEWL SLS	−0.2931 (−0.510, −0.180)	0.0878 (−0.0924, 0.243)	<0.001
Hydration SLS	0.0000 (−0.133, 0.106)	0.0065 (−0.0889, 0.138)	0.347
Erythema SLS	−0.0287 (−0.0371, 0.0644)	0.0144 (−0.0775, 0.135)	0.825

Q1—first quartile; Q3—third quartile. Deviation (variable) = [(variable at t8) − (variable at t0)]/(variable at t0). HK—healthy skin with kefir intake; H0—healthy skin without kefir intake; TEWL—transepidermal water loss. SLS—sodium lauryl sulphate. Groups compared by Mann-Whitney U test, with $p < 0.05$ for statistical significance.

The results from Table 4 showed a significant decrease in TEWL on the HK group compared to control ($p < 0.001$), thus corroborating the above-mentioned results for forearm TEWL, shown in Table 3.

For atopic skin subjects, variations on skin parameters were noted in the AK group after eight weeks (Table 3). TEWL decreased in the forearm (the more significant change), forehead, and leg, compared to the A0 group ($p < 0.001$, for all cases). Regarding hydration, the AK group showed an increase in forearm and leg compared to control ($p = 0.001$ and $p = 0.034$, respectively). No differences were observed for erythema ($p = 0.221$) between these groups (Table 3). These results were reinforced by those from individual paired comparisons that showed that at t8, the atopic subjects who drank kefir presented a significantly lower TEWL and erythema and a significantly higher hydration compared to t0 in all anatomical study areas ($p < 0.05$, for all parameters), while in the control group, no differences were observed (Supplementary Table S3), thus confirming the effective change in skin conditions, despite individual baseline conditions.

3.3. SCORAD Index Assessment

The SCORAD Index was evaluated at t0 and t8 for all subjects from the atopic group. Variation on the SCORAD Index was assessed as a deviation and was computed using the previously explained approach; thus, negative values represent an improvement in AD symptoms after the intervention. The results are shown in Table 5.

Table 5. Comparison of SCORAD Index variation, between kefir intake and control, for atopic group, after intervention (median (Q1, Q3)).

Deviation of SCORAD Index	Atopic Group (n = 19)		
	AK	A0	p-Value
SCORAD	−0.626 (−0.758, −0.491)	0.0402 (−0.0293, 0.273)	<0.001

Q1—first quartile; Q3—third quartile. SCORAD—SCORing of Atopic Dermatitis. AK—atopic skin with kefir intake; A0—atopic skin without kefir intake. Groups compared by Mann-Whitney U test, with $p < 0.05$ for statistical significance.

As shown in Table 5, after eight weeks of kefir intake, the AK group showed a significant decrease in the SCORAD Index, compared to control ($p < 0.001$). These results were corroborated by those of paired individual comparisons in which at t8 atopic individuals who drank kefir had a significantly lower SCORAD index compared to t0 ($p < 0.05$), whereas for control individuals, no differences were observed (see Supplementary Table S3), thus confirming the effective change in skin conditions despite individual baseline conditions.

It is noteworthy that, at the beginning of the study, the AK group presented a median SCORAD Index value of 61.9 (41.2, 72.2), a range classified as severe AD, while after the intervention with kefir, the median SCORAD Index was 16.2 (12.1, 32.9), a range classified

as mild AD, according to the ETFAD recommendation [67]. As for group A0, the median SCORAD Index value was 33.4 (23.1, 52.1) at t0 and 33.9 (26.8, 69.4) at t8, thus classifying the severity of AD as moderate both at baseline and after the intervention period.

Furthermore, in atopic volunteers, a possible relationship between cutaneous parameters and the AD severity was also assessed using Pearson's correlation. We observed a significant correlation between the improvement of AD severity given by the deviation of the SCORAD Index and skin barrier improvement given by the deviation of TEWL on the forearm, leg, and forehead ($r = 0.630$, $p = 0.004$; $r = 0.481$, $p = 0.037$; $r = 0.680$, $p = 0.001$, respectively), and also on the forearm hydration ($r = -0.839$, $p < 0.001$). Although erythema, a well-known sign of skin inflammation, is present in both acute and chronic stages of AD [60], our results were not able to detect a relation between erythema and the improvement of AD severity ($r = 0.286$, $p = 0.236$).

3.4. Adjusted Models for Skin Parameters

To assess the effect of different independent variables on the outcomes of skin parameters, multiple linear regression models were performed. All socio-demographic variables, food intake variables, kefir intake, and skin status were considered as possible predictors for the influence in deviation of skin parameters. After testing the assumptions for linear regression and collinearity diagnostics, independent variables were excluded from the models if the variance inflation factor (VIF) was superior to 10. Following this step, backward stepwise linear regressions were performed for each outcome variable to identify which variables better explained the outcome variable. Although the climatic conditions (temperature and humidity) were evaluated at t0 and t8, they were not used in the regression models as they did not affect the effect of kefir intake ($p = 0.329$, $p = 0.464$, $p = 0.352$ and $p = 0.363$, respectively), thus not being considered relevant.

The most common variables in the models and so considered as possible predictor variables on skin parameters identified by this method were: kefir status, defined as with or without kefir intake; skin status, defined as belonging to the healthy or the atopic group; gender, defined as male or female; and water intake in liters. New linear regressions with the Enter method were then run, which are presented in Table 6.

Table 6. Multiple linear regression for effect of kefir status on skin parameters (standardized regression coefficient ß (p-value), $n = 52$).

Deviation of Skin Parameters	β for Kefir Intake (p-Value)		
	Model 1	**Model 2**	**Model 3**
TEWL			
Forearm	−0.596 (<0.001)	−0.597 (<0.001)	−0.625 (<0.001)
Leg	−0.304 (0.029)	−0.323 (0.018)	−0.332 (0.020)
Forehead	−0.501 (<0.001)	−0.502 (<0.001)	−0.524 (<0.001)
Hydration			
Forearm	0.481 (<0.001)	0.458 (<0.001)	0.539 (<0.001) a
Leg	0.294 (0.034)	0.267 (0.042)	0.347 (0.006) a
Forehead	0.362 (0.008)	0.346 (0.011)	0.358 (0.012)
SCORAD Index (**)	−0.910 (<0.001)	n.a.	−0.866 (<0.001)

β—standardized regression coefficient (reference category: without kefir intake), $p < 0.05$ for statistical significance. Model 1—kefir Status; Model 2—kefir status, skin status; Model 3—kefir status, skin status, gender; water intake. (**) Variable skin status was excluded from the models. n.a.—not applicable. a—gender and water contribution showed $p < 0.05$. TEWL—transepidermal water loss. SCORAD—SCORing of Atopic Dermatitis.

The results from Table 6 show that drinking kefir for eight weeks is associated with a significant improvement in TEWL and in SC hydration, in all study areas (Model 1). In the adjusted models for skin status (Model 2) and skin status, gender, and water intake (Model 3), the effect of kefir intake remained significant, continuing to show an improvement in TEWL and hydration, with the best results obtained for the forearm. The SCORAD Index clearly improved with kefir intake.

4. Discussion

New insights in the field of nutrition increasingly support the evidence of a close relationship between diet and health, namely skin health [2,71]. Diet is a major regulator of the intestinal microbiota, and short-term changes in the diet have the ability to rapidly alter gut bacteria [44,72]. The use of probiotics presents itself as one of the most common interventions to beneficially regulate the gut microbiota [2,10,71]. Probiotics are beginning to be recognized as being able to beneficially impact skin health by modifying its microbiota, preventing pathogen invasion and contributing to the restoration of impaired barrier function [13,73–75].

The consumption of kefir has been reported to positively impact the gut microbiota and overall condition of the digestive system [33,76–78]. Additionally, an in vitro study suggests that kefir's passage through the human gastrointestinal tract, and its consequent digestion, can improve its nutritional profile and bioactivity [79]. However, to date, most studies aiming to establish the benefits to human health of kefir consumption have been based in animal models, or in cell culture systems wherein the digestion of kefir does not occur, thus providing limited information [28,79]. Of note, none of the in vivo human studies found in the literature observed the skin impact of a diet containing traditionally homemade kefir as the probiotic, neither in healthy nor atopic subjects.

In this study, kefir intake for eight weeks caused an improvement in the skin condition of healthy subjects, quantitatively demonstrated by a significant decrease in TEWL and increase in hydration on the forearm and forehead, compared to the control. Similar results were found in other in vivo studies evaluating the effect of ingested specific probiotic strains (*Lactobacillus* and *Bifidobacterium* species) in human adults with healthy skin [48,49,80–82]. Kano et al. and Mori et al. evaluated the effect of ingesting fermented milk containing one strain of *Bifidobacterium* species for eight weeks. They both found a significant improvement on SC hydration in the probiotic ingestion group, and attributed their results to an improvement in intestinal conditions, as the levels of toxic metabolites excreted by intestinal bacteria such as phenol decreased [81,82]. In the study by Gueniche et al., a significant decrease in TEWL was observed after eight weeks of probiotic intervention [48]. Moreover, Ogawa et al. found a significant decrease in TEWL and an increase in SC hydration after twelve weeks of probiotic intake [49], and Lee et al. observed identical results after twelve weeks of probiotic intake [80]. However, not all skin studies using probiotics have been able to demonstrate this type of outcome. Saito et al. tested the ingestion of one probiotic strain (*Lactobacillus* species) by healthy volunteers and found a decrease in TEWL at the arm, but not the face, and was not able to detect changes in skin hydration [47].

An innovative note in our approach is the use of the SLS irritation induction model to further demonstrate the beneficial impact of kefir consumption in barrier function in healthy skin. These results are supported by previous research by the authors [63]. Other studies using similar approaches but conducted in animal models exposed to irritants (ex vivo and in vivo) observed a decrease in TEWL after ingesting probiotics [83,84].

Atopic dermatitis (AD), the most common form of eczema, is a chronic inflammatory skin disease characterized by symptoms such as pruritic lesions, pain, and sleep disturbances [1,15]. AD onset points towards a complex interaction between skin barrier dysfunction, immune dysregulation, environmental risk factors, and dysbiosis of the intestinal and skin microbiota, which correlates with the clinical severity of AD [1,14,15,61]. Through the gut-skin axis, intestinal dysbiosis has been shown to negatively impact skin function either through an increase of epithelial permeability, via pro-inflammatory cytokines, thus promoting immune dysregulation and contributing to the chronic systemic inflammation in AD, as by perpetuating pruritus via secretion of neuroendocrine itch mediators, leading to a chronic itch–scratch cycle, thus further disrupting the skin barrier [1,5,6,9].

In AD, the presence of an impaired epidermal skin barrier is demonstrated by both a defective inside–outside barrier (increased TEWL) as well as a defective outside–inside barrier (increased penetration of environmental substances triggering immunological

mechanisms), along with decreased hydration of the SC [1,14,15]. A lower content in SC ceramides, unsaturated fatty acids, and structural proteins such as filaggrin (involved in SC barrier formation and hydration) underlies the cutaneous barrier dysfunction in AD [1,74,85]. Traditional therapy used in AD is based on topical treatments, often corticosteroids, thus being focused on treating symptoms rather than the underlying causes, therefore mainly resulting in a short-term repair of the defective barrier [1,85,86].

Although to date several studies have explored the potential efficacy of probiotics in the prevention and treatment of AD, the results are not consistent, thus contributing to the lack of evidence for the use of probiotics in skin health [11,73,75,85–87]. The variation in types of strains used, both in diversity and in doses, different types of formulation (supplement or food), duration of the ingestion period, as well as the type of parameters used to assess skin conditions can somehow justify this lack of consistency in the results obtained [86].

In our study, a significant decrease in TEWL and increase in hydration was observed in subjects with AD who drank kefir for eight weeks in all the anatomical areas of study, which was not observed in the controls. Furthermore, our data also showed a significant decrease in the SCORAD index in the kefir ingestion group compared to controls, with the level of AD severity changing from severe to mild, which reflects a notable clinical improvement. These results are in agreement with similar in vivo studies conducted on other probiotics [50,51,88,89]. In a randomized cross-over study using a combination of the probiotics (*Lactobacillus* and *Bifidobacterium* species) delivered as food (yogurt) for eight weeks, Roessler et al. observed a non-significant decrease in SCORAD in the atopic group only [50]. Similarly, Yoshida et al. supplemented adults with AD for eight weeks using a capsule formulation with one probiotic strain (*Bifidobacterium* species) and found a significant decrease in SCORAD only in the probiotic intake group, which was attributed to changes in intestinal microflora [51]. In another study, Iemoli et al. found an improvement in SCORAD in adults with AD after a twelve-week intake of a freeze-dried powder mixture of two probiotics (*Lactobacillus* and *Bifidobacterium* species), and justified this by an improvement in the immune response, namely, by the increased production of T-helper cell type-2 (Th2) and regulatory T cells, and by the reduction of microbial translocation in the intestine [90]. Drago et al. observed identical results after a 16 week intervention with sachets containing one *Lactobacillus* species, in AD volunteers, and attributed them to a significant decrease of T-helper cell type-1 (Th1) inflammatory cytokines and Th1/Th2 ratio [88]. These studies highlight the microbiota's ability to impact the lymphoid tissue associated with the intestine, via microbial–mucosal interaction [6,13]. To date, the only meta-analysis performed evaluating the effect of oral probiotics in adults with AD found an overall improvement in the SCORAD index (−8.26, 95% CI: −13.28, −3.25) favoring probiotics [87]. Moreover, a minimum intervention time of eight weeks has also been shown to be adequate to assess the impact of probiotics on AD [87]. Finally, and in contrast to our study, Matsumuto et al. were not able to find any differences in AD severity between probiotic and control groups using one strain of *Bifidobacterium* delivered in the form of capsules to AD patients for eight weeks [52].

Furthermore, in studies that assess the impact of probiotics on AD, typically, only clinical parameters are evaluated, usually severity using the SCORAD Index or equivalents [11,75,86,90]. Our approach, combining clinical and skin barrier function assessment, revealed a strong correlation between the improvement in both the severity of skin lesions and TEWL, thus confirming previous reports on the relationship between TEWL and the clinical status of patients with AD [60,89].

Additionally, no differences in erythema were observed between the study groups in our study, which can be explained by the fact that although erythema is particularly associated with acute skin inflammation, it can also be present in both the acute and chronic stages of the disease; similar results were found in the literature [60]. This may be indicative that erythema measurement is not as sensitive as the measurement of TEWL and so it may not be useful to detect subclinical lesions.

The set of results obtained showed that for both skin types, subjects who drank kefir for eight weeks presented a significant improvement in skin barrier function. Among volunteers who consumed kefir, it is noteworthy that the greatest improvement in both TEWL and hydration was observed in atopic individuals, especially in the forearm. These results also show the relevance of evaluating different anatomical areas in skin studies. Among all anatomical study areas, the forearm showed to be the most sensitive area in obtaining skin variations, for both TEWL and hydration. Such variations observed in the cutaneous parameters in different anatomical regions may be related to differences in thickness and also at the SC level, namely in ceramides and filaggrin; as well as differences in the cutaneous microcirculation [91]. Probiotics may positively impact the skin by enabling the production of bioactive bacterial compounds such as lactic acid, hyaluronic acid, and SCFA [42,74]. We have previously demonstrated that the kefir produced under home use conditions used in this study fulfills the lactic acid requirement for a fermented product [27].

The concept of hormesis can also contribute to justify our results, since it is a biphasic dose/concentration response, characterized by a low-dose stimulation and a high-dose inhibition, based on adaptive responses of biological systems to moderate or self-imposed environmental challenges, whereby the system improves its functionality and/or tolerance to more severe challenges [92]. Calabrese et al. found that while normal and high-risk groups generally exhibit hormonal dose responses to the same inducing agent, high-risk groups tend to respond better to lower doses [93].

Exposure of probiotic LAB to stressors, both during fermentation and in the gastrointestinal tract, affects its survival, as well as its proliferation and gastrointestinal functionality [94]. In the intestine, the probiotic LAB exhibits substantial antioxidant activity, promoting the production of antioxidant enzymes, thereby helping to remove ROS and alleviating oxidative stress [94]. Furthermore, improved survival of the probiotic LAB during fermentation is achieved by co-culture with initial strains. Given that yeasts have greater antioxidant activity than LAB, when used in co-culture, there is an increase in antioxidant activity, growth rate, and protective effect against oxidative damage [95]. Exposure of a probiotic strain to a sublethal level of oxidative stress will induce an adaptive response and improve the strain's resistance to potentially higher levels of oxidative stress. Probiotic bacteria can exert their antioxidant activity through the scavenging of free radicals, chelation of metal ions, enzymatic regulation, and modulation of the intestinal microbiota [37,94].

It can be highlighted that in AD, chronic skin inflammation is associated with the overproduction of reactive oxygen species (ROS), such as superoxide (O^{2-}) and hydrogen peroxide (H_2O_2), thus generating an oxidative stress condition [96,97]. It is known that mitochondria play an essential role in both homeostasis and inflammatory conditions of the skin [98]; thus, the mitochondrial dysfunction of the skin, caused by the production of ROS, is a potential contributer to the mechanism of AD initiation [99].

The effects observed on skin barrier function in this study can likely be attributed to the combined effects of all kefir ingredients, including its complex microbiota, its metabolites, and macro- and micronutrients resulting from the fermentation, as eating a food promotes a whole-body effect [38,40,41,100].

However, although the kefir grain microbial composition is very stable [55,100], the concentration of metabolites and inhibitory compounds that interact with each other may differ in every fermentation process, thus in part justifying the different results reported in the literature. Of note, no adverse effects were reported during the kefir intake period in this study. In addition, unlike many previous reports, our work was conducted in vivo in humans, which probably highlights the effect of kefir digestion in putative health benefits.

Despite all the positive outcomes found in our study, some limitations must be acknowledged. First, this was not a double-blind, placebo-controlled study. Furthermore, although the study design was intended to minimize the effect of individual variability and the small number of participants, individual changes can occur over time, influencing the dynamics of the gut-skin axis and thus impacting the results [44]. However, these

challenges can be mitigated by introducing a washout period and collecting new baseline samples before starting a second sequential intervention.

Moreover, although we did not identify any relationship between nutrient intake and the measured skin parameters, that influence is expected to exist due to the impact of food in the gut, particularly fiber and water intake [2,44].

Along with the proven utility of the determination of TEWL and SC hydration, evaluation of the skin barrier function should also include assessment of the content of other relevant components of SC, with a focus on the ceramides profile, as well as a determination of the impact of the probiotic intake in the skin microbiota [5,14,74,87].

The ability of probiotics to modulate the gut microbiota and the immune status suggests that systemic immunomodulation occurs following ingestion [4,10]. Although all probiotics must present common properties such as low pathogenicity, resistance to gastric acid and bile salt digestion, and adherence to intestinal mucosa, their clinical effects may be species-dependent [10,11]. Therefore, monitoring changes in the human gut microbiome after ingesting a multi-strain probiotic, such as kefir, can provide a better understanding of the mechanisms underlying its many health benefits. Finally, conditions affecting the kefir production, such as the fermentation conditions or origin of the grains, should be considered for in-depth analyses regarding the impact of kefir on health [27].

5. Conclusions

We investigated the effects of ingestion of homemade kefir on the skin condition, as well as on the SCORAD Index of the atopic individuals. Our results showed a significant improvement on all skin outcomes and suggest that atopical individuals may benefit from kefir intake, especially regarding their skin hydration. The nutritional and microbial richness of kefir makes its application highly relevant within many sectors of health care.

To the best of our knowledge, this was the first study to provide information regarding the cutaneous impact of the intake of kefir produced in household representative conditions. Furthermore, the simultaneous improvement seen in all skin parameters observed in this study is considered a new finding.

This work opens the possibility of continuing the research of the impact of the probiotic kefir on cutaneous health and its mechanism of action via the gut-skin axis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10112794/s1; Table S1: Socio-demographic characteristics of study groups. Table S2: Regular food intake characteristics of study groups. Table S3: Individual variation in skin parameters, between t0 and t8.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are available from the corresponding author upon reasonable request.

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Supplementary Material

Supplemental Table S1 – Socio-demographic characteristics of study groups (relative frequency (%)).

Sociodemographic characteristics	Healthy group (n = 33)			Atopic group (n = 19)		
	HK	H0	*p* - value	AK	A0	*p* - value
Scholarity						
High School (12th grade), n (%)	0	0	0.225	4 (44.5)	0	0.167
Graduate, n (%)	12 (92.3)	15 (75.0)		3 (33.3)	5 (50.0)	
Master, n (%)	0	4 (20.0)		1 (11.1)	1 (10.0)	
Doctorate, n (%)	1 (7.7)	1 (5.0)		1 (11.1)	3 (30.0)	
Professional School, n (%)	0	0		0	1 (10.0)	
Career						
Employed, n (%)	1 (7.7)	2 (10.0)	0.822	4 (44.4)	5 (50.0)	0.809
University student, n (%)	12 (92.3)	18 (90.0)		5 (55.6)	5 (50.0)	
Residence area						
Urban, n (%)	8 (61.5)	18 (90.0)	0.051	6 (66.7)	9 (90.0)	0.213
Rural, n (%)	5 (38.5)	2 (10.0)		3 (33.3)	1 (10.0)	
Smoking habits						
Smoker, n (%)	4 (30.8)	2 (10.0)	0.121	1 (11.1)	1 (10.0)	0.622
Occasional smoker, n (%)	1 (7.7)	0		0	1 (10.0)	
Non smoker, n (%)	8 (61.5)	18 (90.0)		8 (88.9)	8 (80.0)	
Dairy consumption or substitutes						
Cow milk, n (%)	6 (46.2)	8 (40.0)	0.727	5 (55.6)	3 (30.0)	0.260
Natural yogurt, n (%)	11 (84.6)	17 (85.0)	0.976	6 (66.7)	8 (80.0)	0.510
Vegetable drink, n (%)	7 (53.8)	7 (35.0)	0.284	4 (44.4)	6 (60.0)	0.498
Alcohol consumption						
Never, n (%)	5 (38.5)	9 (45.0)	0.445	5 (66.7)	2 (20.0)	0.276
1 to 2 times/week, n (%)	7 (53.8)	11 (55.0)		3 (22.2)	6 (60.0)	
3 to 6 times/week, n (%)	1 (7.7)	0		1 (11.1)	2 (20.0)	

Groups were compared by Chi-Square test,with $p<0.05$ for statistical significance.

Supplemental Table S2 – Daily dietary intake characteristics of study groups (mean ± SD).

Daily dietary intake characteristics	Healthy group (n = 33)			Atopic group (n = 19)		
	HK	H0	*p* - value	AK	A0	*p* - value
Energy, kcal	1624 ± 469.1	1634 ± 592.5	0.941	1684 ± 315.8	1670 ± 344.0	0.870
Carbohydrates, %	43.7 ± 6.78	47.2 ± 5.97	0.224	50.4 ± 6.69	47.2 ± 5.07	0.191
Protein, %	23.7 ± 5.52	21.1 ± 4.87	0.197	22.5 ± 3.74	22.9 ± 3.98	0.806
Fat, %	32.3 ± 6.57	31.5 ± 5.24	0.912	27.1 ± 4.47	29.9 ± 4.45	0.165
Fiber, g	16.9 ± 4.78	16.4 ± 5.69	0.631	21.9 ± 4.80	18.8 ± 5.68	0.288

1

Water, L/day	2.18 ± 0.423	2.41 ± 0.459	0.071	2.39 ± 0.429	2.20 ± 0.286	0.347

SD – Standard deviation. HK – Healthy skin with kefir intake; H0 - Healthy skin without kefir intake; AK – Atopic skin with kefir intake; A0 – Atopic skin without kefir intake. Groups were compared using Mann-Whitney U-test, with $p < 0.05$ for statistical significance.

Supplemental Table S3 – Individual variation in skin parameters, between t0 and t8 (Wilcoxon standardized (Z) test statistic (*p*-value)).

Skin parameters	Healthy group (n=33)		Atopic group (n = 19)	
	HK	H0	AK	A0
TEWL (g/m²/h)				
Forearm	-2.412 (**0.016**)a	-0.597 (0.550)a	-2.666 (**0.008**)a	-1.274 (0.203)b
Leg	-1.014 (0.311)a	-0.784 (0.433)a	-2.666 (**0.008**)a	-2.701 (0.007)b
Forehead	-2.341 (**0.019**)a	-0.411 (0.681)a	-2.666 (**0.008**)a	-1.580 (0.114)b
Hydration (a.u.)				
Forearm	-0.039 (0.969)b	-2.380 (0.017)a	-2.675 (**0.007**)b	-1.429 (0.153)a
Leg	-0.774 (0.439)b	-1.069 (0.285)a	-2.668 (**0.008**)b	-0.358 (0.721)b
Forehead	-1.575 (0.115)b	-2.524 (0.012)a	-2.670 (**0.008**)b	-0.408 (0.683)b
Erythema (a*)				
Forearm	-2.271 (**0.023**)a	-2.782 (0.005)a	-2.310 (**0.021**)a	-0.561 (0.575)a
SCORAD Index	n.a.	n.a.	-2.666 (**0.008**)a	-1.682 (0.092)b

HK – Healthy skin with kefir intake; H0 - Healthy skin without kefir intake; AK – Atopic skin with kefir intake; A0 – Atopic skin without kefir intake. TEWL – Transepidermal Water Loss. Individuals were compared by Wilcoxon signed rank test, with $p<0.05$ for statistical significance. a - based on positive ranks (variable at t0 > variable at t8); b – based on negative ranks (variable at t0 < variable at t8).

2

Supplementary Information

This work, considered an exploratory study, was crucial for the training with non invasive bioengineering methods used for skin barrier function analysis and the additional use of a challenge method in the evaluation of healthy and atopic skin.

Article 5

Determination of relevant endpoints to evaluate the *in vivo* barrier function incutaneous health

Emília Alves[1], Patrícia Rijo[1], Luís Monteiro Rodrigues[1,2], Catarina Rosado[1]

[1]CBIOS – Universidade Lusófona's Research Center for Health Science and Technologies, Campo Grande, 376, 1749-024, Lisboa, Portugal
[2]Dep. Pharmacol Sciences – Universidade de Lisboa (Faculty of Pharmacy)

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Determination of relevant endpoints to evaluate the *in vivo* barrier function in cutaneous health

Determinação de "endpoints" relevantes para a avaliação in vivo da função "barreira" na saúde cutânea

Emília Alves[1], Patrícia Rijo[1], Luís Monteiro Rodrigues[1,2], Catarina Rosado[1]
[1]CBIOS – Universidade Lusófona's Research Center for Health Science and Technologies,
Campo Grande, 376, 1749-024, Lisboa, Portugal
[2]Dep. Pharmacol Sciences – Universidade de Lisboa (Faculty of Pharmacy)
Email: catarina.rosado@ulusofona.pt

Abstract

This work aims to identify endpoints to evaluate the in vivo barrier function of the skin by noninvasive methods in healthy and atopic individuals, thus contributing to the consolidation of methodologies that can be employed in later studies of the action on skin health of different health products and food supplements. In this context, the cutaneous aggression induction model using sodium lauryl sulphate (SLS) was used, followed by evaluation of transepidermal water loss (TEWL) and vascular blood flow of the dermis by colorimetry and laser Doppler flowmetry (LDF), in a group of healthy volunteers (n = 15) and in a group with atopic dermatitis (n = 5). The healthy individuals presented a basal TEWL slightly superior to the atopic group. In skin without intervention, baseline values did not change, 24 hours after induction of irritation, in both groups ($p < 0.05$). In the aggressed skin, in the same period, values presented a higher variation than that of the control zone ($p < 0.05$), being greater in the atopic than in the healthy group. Results confirm that measurement of TEWL after SLS aggression allows a good assessement of skin barrier function in both healthy and atopic subjects and suggest that erythema may be a measure of support for the robustness of the results.

Keywords: cutaneous health, TEWL, sodium lauryl sulphate

Resumo

Este estudo pretende identificar endpoints para avaliar in vivo a função de barreira da pele, por métodos não invasivos, em indivíduos com atopia e em saudáveis, contribuindo assim para a consolidação de metodologias de estudo que possam ser depois aplicadas na avaliação da ação na saúde da pele de diferentes produtos de saúde e suplementos alimentares. Neste contexto, usou-se o modelo de indução de agressão cutânea com lauril sufato de sódio (LSS) seguido de avaliação da perda transepidérmica de água (PTEA) e do fluxo sanguíneo vascular da derme, por colorimetria e por fluxometria de laser Doppler (FLD), num grupo de voluntários saudáveis (n=15) e num grupo de atópicos (n=5). Os voluntários saudáveis apresentaram uma PTEA basal ligeiramente superior aos atópicos. Na pele sem intervenção, os valores basais não sofreram alteração, 24h após indução da irritação, em ambos os grupos ($p < 0,05$). Na pele tratada com LSS, no mesmo período, os valores apresentaram uma variação maior do que na zona controle ($p < 0,05$), sendo maior no grupo atópico do que no grupo saudável. Os resultados confirmam que a avaliação das alterações cutâneas após agressão com LSS, por medição da PTEA permite uma boa apreciação da função de barreira da pele, quer em voluntários saudáveis quer em atópicos, e sugerem que a medição do eritema por colorimetria pode ser uma avaliação de suporte à robustez deses resultados.

Palavras-Chave: barreira cutânea, PTEA, lauril sulfato de sódio

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Introduction

The skin is a functionally complex organ made up of three interdependent layers, the epidermis, the dermis and the hypodermis [1]. Its relationship with other organs contributes to both balance with the external environment and internal balance, enhancing its function beyond the coating and protective properties of the body [2].

The epidermis provides a physical and functional barrier to the human body and its outermost layer, the stratum corneum (SC), ensures the integrity and hydration of the skin [2]. The SC acts as a homogeneous membrane for the diffusion of water and is involved in the regulation of the loss of water from the body to the atmosphere, termed transepidermal water loss (TEWL). TEWL is defined as the passive diffusion of water through the epidermis, i.e., the constitutive loss of skin water vapor in the absence of sweat glandular activity [3]. A low TEWL is characteristic of intact and healthy skin, while an elevated TEWL indicates that the skin barrier function is compromised [4,5].

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with an exacerbated skin response to environmental agents and characterized by pruritic lesions with typical dryness and morphology. Defects in the cutaneous barrier are considered as an initial step in the development of AD [6]. When the barrier is compromised, the penetration of substances may stimulate keratinocytes and Langerhans cells to produce mediators that are involved in the inflammatory response [7].

As mentioned, TEWL can be measured non-invasively and is considered a parameter of interest to evaluate skin barrier function [4]. In some studies conducted in the context of the evaluation of cutaneous conditions, such as AD, the affected skin regions show higher TEWL compared to normal skin, meaning lower water retention capacity [2,4,5]. However, not all papers report these types of results . In addition, the limitations of the methodology are known, especially since a tenuous correlation was found between skin damage and TEWL variations [3,8]. Thus, the competence of the barrier can be assessed by measuring the basal TEWL and/or by observing the recovery of TEWL after rupture of the cutaneous barrier [9].

Contact dermatitis is the inflammatory reaction of the skin, whose main clinical manifestation is eczema. Substances that can cause eczema in any skin type are considered primary irritants [10]. These may be used experimentally in certain circumstances, but it should be ensured that they do not cause systemic toxicity, sensitization, carcinogenesis, or cosmetic inconvenience.

Introdução

A pele é um órgão funcionalmente complexo constituído por três camadas interdependentes, a epiderme, a derme e a hipoderme [1]. A sua relação com outros órgãos, contribui quer para o equilíbrio com o ambiente externo quer para o equilíbrio interno, elevando a sua função para além das propriedades de revestimento e proteção do corpo [2].

A epiderme proporciona uma barreira física e funcional ao corpo humano e a sua camada mais externa, o estrato córneo (EC), assegura a integridade e a hidratação da pele [2]. O EC está envolvido na regulação da perda de água do organismo para a atmosfera, designada por perda transepidérmica de água (PTEA) que se define como a difusão passiva da água através da epiderme, i.e., a perda constitutiva de vapor de água da pele na ausência de atividade glandular sudorípara [3]. Uma PTEA baixa é característica de uma pele intacta e saudável, enquanto uma PTEA elevada indica que a função de barreira está comprometida [4,5].

A dermatite atópica (DA) é uma doença inflamatória crónica da pele associada a uma resposta cutânea exacerbada aos agentes ambientais e caracterizada por lesões pruríticas com secura e morfologia típica. Defeitos na barreira cutânea são considerados como um passo inicial no desenvolvimento de DA [6]. Quando a barreira está comprometida, a penetração de substâncias pode estimular os queratinócitos e as células de Langerhans para produzir mediadores que estão envolvidos na resposta inflamatória [7].

Como referido, a PTEA pode ser medida de forma não invasiva sendo considerada um parâmetro interessante para avaliar a função de barreira cutânea [4]. Em alguns estudos efectuados no contexto da avaliação de patologias cutâneas, como a DA, as regiões da pele afetadas mostram maior PTEA em comparação com a pele normal, ou seja, menor capacidade de retenção de água [2,4,5]. No entanto, nem todos os trabalhos mostram este tipo de resultados. Adicionalmente, são conhecidas as limitações da metodologia, nomeadamente por ter sido encontrada uma correlação ténue entre danos infligidos à pele e variações da PTEA [3,8]. Assim, a competência da barreira poderá ser avaliada pela medição da PTEA basal e/ou através da observação da recuperação de PTEA após a ruptura da barreira cutânea [9].

A dermatite de contacto é uma reação inflamatória da pele, cuja principal manifestação clínica é o eczema. As substâncias que podem causar eczema em qualquer tipo de pele, são considerados irritantes primários [10]. Estes podem ser usados experimentalmente em determinadas circunstâncias, devendo no entanto garantir-se que não

Additionally, such substance should have no extreme pH and be well defined chemically [11].

The surfactant Sodium Lauryl Sulfate (SLS) is the most commonly used substance in the experimental induction of contact dermatitis by primary irritancy. Although most clinically observed cases of irritant contact dermatitis occur in the hands, it has been shown that the forearm provides reliable and reproducible results and is therefore a good alternative for experimental tests [12].

Different non-invasive methods can be used to evaluate the cutaneous response to the irritant, distinguished by the type of physiological phenomenon analyzed, or by the quantification technique. In assessing the skin barrier function experimentally induced by irritation, the combination of TEWL measurements and another non-invasive method is recommended to obtain more robust results [13]. Skin exposure to SLS causes changes in the skin barrier and hemodynamic changes that are manifested by differentiated red blood cell perfusion and increased local microvascular blood flow. Two dermal bioengineering methodologies allow the quantitative evaluation of vascular dermis blood flow: laser Doppler flowmetry (LDF) and colorimetry [14-17].

The majority of the studies conducted on the action of health products and food supplements in skin condition are based only on scoring rates of lesions in the form of eczema. This work aims to identify endpoints to evaluate *in vivo* in healthy and atopic individuals the cutaneous health using noninvasive methods, enabling a later study to the consolidation of methodologies for efficacy assessment of these products. Thus, an assessment was made of the applicability in this context of the model of induction of cutaneous aggression using SLS followed by evaluation by cutaneous bioengineering methodologies.

causam toxicidade sistémica, sensibilização, carcinogénese ou inconveniência cosmética, que não possuem pH extremo e que são bem definidos quimicamente [11].

O surfactante Lauril Sulfato de Sódio (LSS) é a substância utilizada com mais frequência na indução experimental da dermatite de contacto por irritante primário. Embora a maioria dos casos clinicamente observados de dermatite de contato por agente irritante ocorra nas mãos, foi demonstrado que o antebraço fornece resultados confiáveis e reprodutíveis sendo, portanto, uma boa alternativa para estudos experimentais [12].

Podem ser utilizados diferentes métodos não invasivos para avaliar a resposta cutânea ao irritante, distinguindo-se quer pelo tipo de fenómeno fisiológico analisado, quer pela técnica de quantificação. Na avaliação da função de barreira da pele induzida experimentalmente por irritação, a combinação das medições de PTEA e de outro método não invasivo é recomendada para obter resultados mais robustos [13]. A exposição da pele ao LSS provoca alterações à barreira cutânea e alterações hemodinâmicas que se expressam pela perfusão diferenciada dos eritrócitos e aumento do fluxo sanguíneo microvascular local. Duas metodologias de bioengenharia cutânea permitem avaliar quantitativamente variações do fluxo sanguíneo vascular da derme: a fluxometria por laser Doppler (FLD) e a colorimetria [14-17].

A maioria dos estudos da ação de suplementos alimentares e produtos de saúde sobre a pele baseiam-se em sistemas de scoring da severidade de eczema. Este trabalho pretende identificar endpoints para avaliar *in vivo* em indivíduos com atopia e em saudáveis o impacto na saúde cutânea, por métodos não invasivos, permitindo um estudo posterior para a consolidação de metodologias de avaliação da eficácia destes produtos. Assim, foi feita uma avaliação da aplicabilidade neste contexto do modelo de indução de agressão cutânea recorrendo ao LSS seguido de avaliação por metodologias de bioengenharia cutânea.

Materials and methods

A convenience sample was used in this study. The work was conducted in accordance with the principles of the Helsinki Declaration. After informed consent, 20 participants of both sexes were included in the study. Of these participants, 15 were considered healthy with no history of cutaneous disease, aged between 18 and 45 years (mean age 25.33 ± 7.29 years), and 5 were with self-reported atopy, aged between 18 and 50 years (mean age 26.20 ± 13.50 years).

Healthy participants had no visible cutaneous lesions and no past or present record of dermatological disease

Materiais e Métodos

Foi utilizada neste estudo uma amostra de conveniência. O trabalho foi conduzido de acordo com os princípios da Declaração de Helsínquia. Após consentimento informado, 20 participantes de ambos os sexos, 15 dos quais saudáveis sem historial de doença cutânea, com idade entre os 18 e os 45 anos (idade média 25,33 ± 7,29 anos) e 5 dos quais com atopia autoreportada, com idade entre os 18 e os 50 anos (idade média 26,20 ± 13,50 anos), foram incluídos no estudo.

Os participantes saudáveis, não apresentavam lesões cutâneas visíveis e nenhum registo passado ou presen-

or atopy. Atopic participants had no visible skin lesions in the study area. All participants were advised not to use moisturizers or other cosmetic products in the test area, 48 hours before the start of the test and during the test period (2 days).

The impact on the skin barrier function caused by SLS application was evaluated by the measurement of transepidermal water loss (TEWL) and vascular blood perfusion of the dermis. To quantify the latter, measurements of erythema by colorimetry and blood perfusion using laser Doppler flowmetry (LDF) were used.

TEWL measurements ($g/m^2/h$) were performed using a Tewameter® TM300 (CK Electronics, Germany). Measurements of erythema were performed with a Chroma Meter CR300 (Minolta, Japan). Color was expressed in the L*a*b system, where skin erythema is indicated by a* [16]. Measurements of laser Doppler flowmetry were performed with a Laser Doppler Flowmeter® PF5010 (Perimed, Denmark). Blood flow measurements were expressed in arbitrary perfusion units (PU). For each parameter, two consecutive measurements were performed, by test zone, under controlled temperature and humidity conditions (ambient temperature 21 ± 1 °C, relative humidity of 40-60%), according to the guideline recommendations [15,16,18]. The volunteers rested for at least 20 minutes in these conditions before measurements.

Two zones – a control zone and a SLS zone - were evaluated in the ventral part of the forearm, randomly distributed, in each participant..

The basal measurements were performed at time t0 in both zones, after which a occlusve chamber adhesive (Finn Chambers, USA) was applied to the treated zone with 100 µl SLS (purity> 99%; Sigma Chemical Co., St. Louis , MO, USA) in 1% aqueous solution. This adhesive was kept in contact with the skin for 24 hours. After this period (t24h) the measurements were repeated, and the SLS zone was evaluated 1 hour after removal of the adhesive.

To reduce the impact of intra and interindividual variability the results were analyzed as the ratio between the value obtained at the end of the study (t24) and the baseline value (t0).

For the statistical analysis of the continuous variables a non-parametric test was used, Mann Whitney test. The significance level was established at $p<0.05$.

te de doença dermatológica ou atopia. Os participantes atópicos, não apresentavam lesões cutâneas visíveis na área de estudo. Todos os participantes foram aconselhados a não utilizar cremes hidratantes ou outros produtos cosméticos na zona de teste, 48h antes do inicio do ensaio e durante o período de realização do mesmo (2 dias).

O impacto na função de barreira cutânea causado pela aplicação de LSS foi avaliado pela medição da perda transepidérmica de água (PTEA) e pelo fluxo sanguíneo vascular da derme. Para quantificação deste último foram usadas medições de eritema por colorimetria e da perfusão sanguínea por fluxometria por laser Doppler (FLD).

As medições de PTEA ($g/m^2/h$), foram efetuadas usando um Tewameter® TM300 (CK Electronics, Alemanha). As medições de eritema foram realizadas com um Chroma Meter CR300 (Minolta, Japão) usando o sistema CIE L*a*b, onde o eritema da pele é indicado por a* [16]. As medições FLD foram realizadas com um Laser Doppler Flowmeter® PF5010 (Perimed, Dinamarca) e expressas em unidades arbitrárias de perfusão (UP). Para cada parâmetro, foram realizadas 2 medições consecutivas, por zona de teste, sob condições de temperatura e humidade controladas (temperatura ambiente 21±1°C; humidade relativa de 40-60%), de acordo com as recomendações das guidelines [15,16,18]. Os voluntários descansaram pelo menos 20 minutos, nestas condições, antes das medições.

Foram avaliadas duas zonas na parte ventral do antebraço, distribuídas de forma aleatória, em cada participante: Zona controlo e Zona LSS.

As medições basais foram efetuadas no tempo t0, em ambas as zonas, após o que foi aplicado na zona tratada um adesivo com câmara oclusiva (Finn Chambers, USA) com 100µl de LSS (pureza> 99%; Sigma Chemical Co., St Louis, MO, EUA) em solução aquosa a 1%. Este adesivo foi mantido em contacto com a pele por 24 horas. Após esse periodo (t24h) repetiram-se as medições, sendo a zona LSS avaliada 1 hora após remoção do adesivo.

Para diminuir o impacto da variabilidade intra e interindividual os resultados foram analisados como o rácio entre o valor obtido no final do estudo (t24) e o valor basal (t0).

Para a análise estatística das variáveis continuas utilizou-se um teste não paramétrico, teste de Mann Whitney. Um nível de significância inferior a 0,05 foi considerado.

Results

Baseline TEWL values obtained in the forearm were compared, and the healthy group presented a slightly higher baseline TEWL than the atopic group, but with a greater dispersion. The differences found were not statistically significant (p = 0.553) (Figure 1).

Resultados

Compararam-se os valores de PTEA basal do antebraço, verificando-se que o grupo de saudáveis apresentou uma PTEA basal ligeiramente superior ao grupo dos atópicos, mas com uma dispersão maior. As diferenças encontradas não foram estatisticamente significativas (*p*=0,553) (Fig.1).

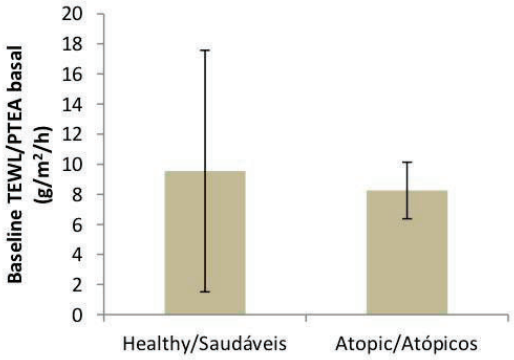

Figure 1 / Figura 1 – Baseline TEWL, in the healthy group and in the atopic group (mean ± SD), p = 0.553 / PTEA basal, no grupo de saudáveis e no grupo de atópicos (média±DP), p=0,553.

The mean values of the variation between t0 and t24 for TEWL, a* and blood perfusion in the two test zones were determined in healthy volunteers and in the atopic group (Table 1 and Figure 2).
As expected, in the skin without intervention (control zone) baseline values of TEWL, a* and LDF did not change after 24 hours in both the healthy and atopic volunteers, with values close to unity. These results indicate the suitability of the methodology.
In the SLS zone, 24 hours after induction of irritation, the variation of TEWL, a* and LDF presented values significantly higher than those of the control zone. This variation was greater in atopic than healthy volunteers and that the differences recorded between the two zones were statistically significant for each group. The blood perfusion values presented a very high dispersion in both groups.

Foram determinados os valores médios da variação entre t0 e t24 para PTEA, a* e perfusão sanguínea nas duas zonas de teste, no grupo de voluntários saudáveis e no grupo atópico (Tabela 1 e Figura 2).
Como esperado, na pele sem intervenção (zona de controlo) os valores basais de PTEA, a* e FLD não sofreram alteração após 24h, tanto no grupo de voluntários saudáveis, como no dos atópicos, tendo por isso sido obtidos valores de rácio próximos da unidade. Estes resultados são indicadores da adequabilidade da metodologia desenvolvida.
Na zona LSS, 24h após indução da irritação, os resultados de PTEA, a* e UP de FLD apresentaram valores significativamente mais elevados do que na zona de controlo (Tabela 1). Deve ser notado que essa variação foi um pouco maior nos voluntários atópicos do que em saudáveis embora as diferenças registadas entre as duas zonas não tenham sido estatisticamente significativas. Os valores de perfusão sanguínea apresentaram uma dispersão muito elevada, em ambos os grupos.

Discussion and Conclusion

AD is a cutaneous condition of etiology not yet fully understood. Numerous approaches to this pathology, both therapeutic and non-therapeutic, have been studied, one

Discussão e Conclusão

A DA é uma patologia cutânea de etiologia ainda não completamente esclarecida. Têm sido estudadas inúmeras abordagens para esta patologia, terapêuticas e não

Table 1/ Tabela 1 – Variation (t24 / t0) in TEWL, a* and LDF, in atopic and healthy subjects / Variação (t24/t0) em PTEA, a* e FLD, em atópicos e saudáveis.

		Control zone/ Zona Controlo Mean ± SD/ média ± dp	**SLS zone/ Zona LSS** Mean ± SD/ média ± dp	***p-value***
Atopic/Atópicos (n=5)	TEWL/PTEA	0.99 ± 0.21	3.90 ± 0.41	0.008
	Erythema/Eritema (a*)	1.01 ± 0.08	1.51 ± 0.30	0.008
	LDF/FLD	0.82 ± 0.22	4.71 ± 3.10	0.008
Healthy/Saudáveis (n=15)	TEWL/PTEA	0.90 ± 0.26	3.41 ± 1.10	0.000
	Erythema/Eritema (a*)	1.00 ± 0.12	1.29 ± 0.26	0.001
	LDF/FLD	0.75 ± 0.17	3.03 ± 3.25	0.000

Significance level <0.05 / Nível de significância <0,05

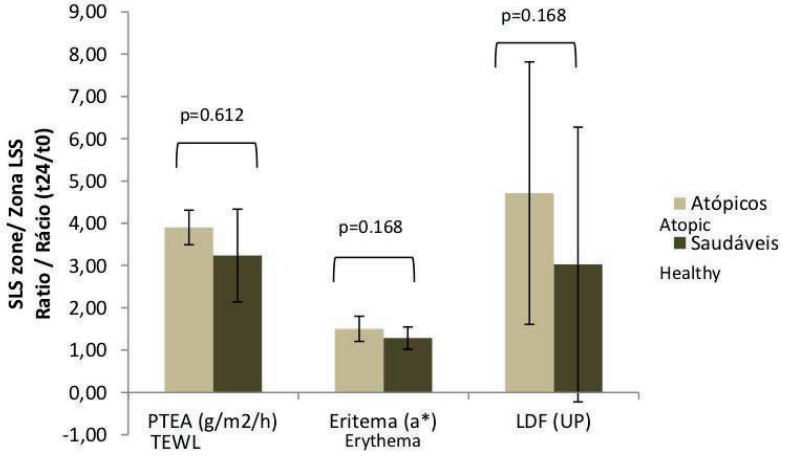

Figure 2 / Figura 2 – Variation (t24/t0), at SLS zone, in TEWL, a* and LDF, between atopic and healthy volunteers (mean±SD) / Variação (t24/t0), na zona LSS, em PTEA, a* e FLD, entre voluntários atópicos e saudáveis (média±DP)

of the most recent being intervention by probiotic supplementation. There are numerous reports in the literature of a compromised molecular composition in the epidermis of individuals with AD, especially in a protein critical for the integrity of the stratum corneum - filaggrin [19]. However, most of the studies conducted on the efficacy of this strategy have been based only on improved scoring rates of lesions in the form of eczema [20], which is perhaps behind the poor correlations found between the use of probiotics and improvements in skin health.

There are different methods to evaluate skin barrier function *in vivo* as a quantitative parameter applied to the characterization of the evolution of cutaneous pathologies. The most commonly used is the direct measurement of the TEWL, but the evaluation of the impact on the TEWL by action of primary irritant can be an interesting approach [9], especially considering the limitations of the methodology previously described.

The results obtained in this study for the baseline TEWL in atopic and healthy volunteers confirm the interest of this approach, since no statistically significant differences were found between them. In addition, the average values recorded in the healthy group are slightly higher, contrary to what would be expected. It should, however, be noted that these results were obtained in groups with quite different numbers of volunteers. In this study we found statistically significant differences between the basal state of the skin and after intervention with a primary irritant, both in healthy volunteers and in atopics, in all endpoints tested. However, the LDF values obtained, following the same trend as the other parameters, showed a high dispersion, which suggests a greater interference of the intra and intervariability effects on the participants, as well as the limitations of the methodology itself. It is worth noting the much lower variability of the results obtained with colorimetry, which indicates that this methodology may be more advantageous in the evaluation of erythema.

In a study comparing the results obtained with LDF and TEWL in healthy volunteers, it was observed that only with higher concentrations of SLS did the increase of LFD values occur [21]. This fact was justified by the fact that the damage caused by the surfactant in the epidermal barrier, by altering the lipids and proteins of the stratum corneum, occurs faster than the inflammatory and vascular response. However, when the barrier is more severely damaged by high concentrations of SLS, the inflammatory response is more intense, and it is then possible to verify the changes by flowmetry [21].

In the present study, the comparison between the mean values obtained in the present study of TEWL, erythema quantified by colorimetry (a *) and LDF allows us

terapêuticas, sendo uma das mais recentes a intervenção por suplementação com probióticos. Encontram-se inúmeros relatos na literatura de uma composição molecular comprometida na epiderme de indivíduos com DA, especialmente numa proteína crítica para a integridade da barreira do estrato córneo- a filagrina [19].No entanto, a maioria dos estudos realizados sobre a eficácia desta estratégia tem sido baseada apenas na melhoria de índices de scoring das lesões sob a forma de eczema [20], o que talvez esteja por trás das pobres correlações encontradas entre o uso de probióticos e melhorias da saúde cutânea.

Existem diferentes métodos para avaliar *in vivo* a função de barreira da pele como parâmetro quantitativo aplicado à caracterização da evolução de patologias cutâneas. O mais usado consiste na medição directa da PTEA, mas a avaliação do impacto na PTEA por ação de agente irritante primário pode ser uma abordagem interessante [9], sobretudo tendo em conta as limitações da metodologia descritas anteriormente. Os resultados obtidos neste estudo para a PTEA basal nos voluntários atópicos e sem esta patologia confirmam o interesse desta abordagem, já que não se encontraram diferenças estatisticamente significativas entre estes. Adicionalmente, a média de valores registados no grupo saudável é ligeiramente superior, contrariamente ao que seria de esperar. Deve, no entanto, ser notado que estes resultados foram obtidos em grupos com números bastante diferentes de voluntários.

Neste estudo foram encontradas diferenças estatisticamente significativas entre o estado basal da pele e após intervenção com um irritante primário, quer em voluntários saudáveis quer em atópicos, em todos os endpoints testados. No entanto, os valores de FLD obtidos, tendo seguido a mesma tendência dos outros parâmetros, apresentaram uma elevada dispersão, o que sugere uma maior interferência dos efeitos da intra e intervariabilidade nos participantes, bem como das limitações da própria metodologia. É de salientar a muito menor variabilidade dos resultados obtidos com a colorimetria, o que indica que esta metodologia pode ser mais vantajosa na avaliação do eritema.

Num estudo que comparou os resultados obtidos com FLD e com a PTEA em voluntários saudáveis observou-se que apenas com concentrações mais elevadas de LSS se verificou o aumento dos valores de FLD [21]. Este facto foi justificado pelo facto de os danos causados pelo surfactante na barreira epidérmica, pela alteração dos lipídos e proteínas do estrato córneo, ocorrerem mais rapidamente que a resposta inflamatória e vascular. No entanto, quando a barreira é danificada de forma mais severa por concentrações elevadas de LSS, a res-

to observe some differences in the competence of the cutaneous barrier of both groups, since the impact of SLS on these variables was higher in atopic volunteers. There appears to be an increase in cutaneous permeability in the presence of atopic dermal disposition, resulting in the penetration of larger amounts of SLS through SC, and hence a greater inflammatory response [22]. However, the differences found did not reach statistical significance, likely due to the lower number of volunteers in the atopic group. It would be desirable to confirm this trend by increasing the size of both participant groups, particularly the atopic.

A study with similar objectives using LDF and TEWL was performed by Bandier et al. [23]. As in our study, no differences were found in the baseline TEWL of the volunteers with and without AD. The cutaneous response was compared to the SLS application of 20 healthy volunteers and 38 volunteers with AD and different types of filaggrin gene mutation. Only statistically significant differences were found after 24 hours of application between healthy volunteers and those with AD and a filaggrin mutation in the LDF results, whereas for the TEWL there were differences in the groups of volunteers with and without mutation.

In an investigation carried out by Angelova-Fischer et al. [24], the susceptibility to cumulative application (during five days) of SLS and / or aqueous solution of NaOH was observed by colorimetry and TEWL. Twenty volunteers with AD and 20 healthy volunteers participated in this study. After five days of exposure to the different agents, no differences in a * results were observed in volunteers with and without AD. For the TEWL, although higher values were recorded in each day in the patients with AD, only statistically significant differences were reached on Day 5.

Although based on a small number of atopic volunteers, this study confirms the interest of the SLS-based model as a good way to assess skin barrier function in both healthy and atopic subjects and that measurement of erythema by colorimetry can be used as a measure to support the robustness of TEWL results.

posta inflamatória é mais intensa, sendo então possível constatar as mudanças pela fluxometria [21].

No presente estudo, a comparação entre os valores médios de PTEA, de eritema quantificado por colorimetria (a*) e de FLD permite observar algumas diferenças na competência da barreira cutânea dos dois grupos, já que o impacto do LSS nestas variáveis foi maior nos voluntários atópicos. Parece haver um aumento da permeabilidade cutânea na presença de disposição cutânea atópica, resultando na penetração de maiores quantidades de LSS através do SC, e logo numa maior resposta inflamatória [22]. No entanto, as diferenças encontradas não atingiram significância estatística, muito provavelmente devido à menor quantidade de voluntários no grupo de atópicos Seria desejável confirmar esta tendência alargando os resultados dos dois grupos, particularmente dos atópicos.

Um estudo com objectivos semelhantes usando FLD e PTEA foi realizado por Bandier et al [23]. Também não foram encontradas diferenças na PTEA basal dos volutários com e sem DA. Foi comparada a resposta cutânea à aplicação de LSS de 20 voluntários saudáveis e de 38 voluntários com DA e diferentes tipos de mutação do gene da filagrina. Apenas foram encontradas diferenças estatisticamente significativas após 24h de aplicação entre os volutários saudáveis e aqueles que tinham DA e uma mutação da filagrina nos resultados de LDF, enquanto que para a PTEA observaram-se diferenças nos grupos de volutários com DA com e sem mutação.

Na investigação realizada por Angelova-Fischer et al [24], foi observada através de colorimetria e PTEA a susceptibilidade à aplicação cumulativa (durante 5 dias) de LSS e/ou solução aquosa de NaOH. Colaboraram neste trabalho 20 voluntários com DA e 20 saudáveis. Após 5 dias de exposição aos diferentes agentes, não foram observadas diferenças nos resultados de a* obtidos nos voluntários com e sem DA. Para a PTEA, embora tenham sido registados em cada dia do estudo valores superiores nos voluntários com DA, apenas se atingiram diferenças estatisticamente significativas no dia 5.

Embora baseado num pequeno número de voluntários atópicos, este estudo confirma o interesse do modelo baseado na agressão com LSS, como uma boa forma de avaliar o estado da função de barreira da pele quer em indivíduos saudáveis quer em atópicos e que a medição do eritema, por colorimetria pode ser usada como medida de suporte à robustez dos resultados de PTEA.

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Conflict of interest

The authors declare that there is no financial or personal relationship that might be perceived as posing a potential conflict of interest

Conflito de Interesses

Os autores declaram não existir qualquer relação pessoal ou financeira que possa ser entendida como representando um potencial conflito de interesses.

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CHAPTER III

This chapter is based on the following article:
Kefir as a modulator of the gut-skin axis: a study conducted in healthy and atopic volunteers. 2021. *Foods*. (*In submission*)

Kefir as a modulator of the gut-skin axis: a study conducted in healthy and atopic volunteers

Emília Alves[1,2], João Gregório[1], Patrícia Rijo[1,3], L.M Rodrigues[1], Catarina Rosado[1]

[1] CBIOS – Universidade Lusófona's Research Center for Biosciences & Health Technologies, Campo Grande 376, 1749-024 Lisboa, Portugal; emilia.alves@ulusofona.pt (E.A.); joao.gregorio@ulusofona.pt (J.G.); patricia.rijo@ulusofona.pt (P.R.); monteiro.rodrigues@ulusofona.pt (L.M.R.).
[2] Department of Biomedical Sciences, Faculty of Pharmacy, University of Alcalá, Carretera Madrid-Barcelona, Km 33.100, 28805 Alcalá de Henares, Madrid, Spain.
[3] Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisboa, Portugal.

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Abstract
The human gastrointestinal tract is a dynamic system that is influenced by various environmental factors, such as diet and exposure to ingested probiotics. Functional gastrointestinal disorders (FGID) are classified primarily in terms of symptoms, which is an experience perceived as different from normal. Atopic dermatitis (AD) is a chronic inflammatory skin disease, but its pathogenesis seems to be associated with factors such as intestinal microbial imbalance and immunological dysfunction, thus evidencing a gut-skin relationship. Therefore, disruption of the intestinal microbial balance, known as gut dysbiosis, has the ability to negatively impact skin function by increasing the intestinal permeability. Consequently, the gut-skin axis may be receptive to modulation via dietary modification, namely via probiotics ingestion, thus representing a potential alternative in AD therapy. Kefir, one of the most ancient foods known to present health benefits attributed to its microbial and nutritional richesness, has already demonstrated to positively impact the general condition of the digestive system, including the intestinal microbiota. However, the literature is still scarce on the impact on the gut-skin relationship of a diet containing kefir. This study aimed to explore the impact of the ingestion of kefir on gastrointestinal symptoms of healthy and AD skin subjects. Results showed a significant improvement on FGID, namely in constipation, abdominal pain intensity and abdominal distension, thus supporting the hypothesis that kefir intake is positively associated with improvement in FGID. The existence of a relationship between the improvement in skin parameters and the improvement in FGID after kefir consumption was established, thus reinforcing the role of homemade kefir as a potential modulator of the gut-skin axis, both on healthy and atopic individuals. Further studies should be conducted on the mechanisms underlying this action.

Keywords: kefir, intestinal health, skin health, gut microbiota, probiotics, functional gastrointestinal disorders, atopic dermatitis

1. Introduction

The human gastrointestinal tract is a dynamic system that is influenced by several environmental factors, such as diet, where the ingestion of probiotics can be included [1]. Probiotics are, by definition, live microorganisms that when administered in adequate amounts confer a health benefit to the host [2]. They have the ability to increase the microbial diversity in the gut, as well as impact the host metabolism via immune system and inflammatory response, thereby promoting health and preventing disease [1,3,4].

Functional gastrointestinal disorders (FGID) are classified primarily in terms of symptoms, such as constipation, diarrhea, flatulence and associated pain, and are based on the patients' interpretation and reporting of their experience of the disease. This symptom-based classification allows the identification of underlying pathophysiological determinants, whether related to motility, hypersensitivity or intestinal dysfunction [5]. From the Rome IV criteria, a new concept emerged: gut-brain interaction disorders, which are defined as a group of gastrointestinal symptoms related to any combination of motility disorders, visceral hypersensitivity, changes in mucosal, immune function and intestinal microbiota and/or involving the central nervous system [5]. However, for the scope of this study, the term FGID was adopted when referring to this type of disorders.

The intestinal microbiota can present itself in a state of balance, known as eubiosis, where the microbiota tolerates small changes resulting from the environment, diet or water consumed, or in a state of imbalance, known as dysbiosis, resulting from changes such as the growth of specific bacterial groups, colonization by pathogenic bacteria, use of antibiotics or major dietary changes [6,7]. Regardless of the state, the gut microbiota affects both physiological processes and other organs, such as the skin, suggesting that the modulation of the gut microbiota may be a key event for the maintenance of health [6]. Nowadays, modulation of the intestinal microbiota is a reality, whether due to technological advances or through foods, where probiotics play an essential role [8–11].

Foods with probiotic characteristics, typically resulting from fermentation processes, contain bioactive metabolites such as organic acids and short-chain fatty acids (SCFAs) which, in addition to being used as preferential sources of energy by intestinal cells, also have the ability to modulate the host's immune function, wherein the consumption of probiotics via fermented foods is associated with protection from metabolic and immune-mediated diseases [6,9,12]. The beneficial impact on host-microbiota interactions resulting from the use of probiotics may underlie a possible mechanism by which this type of food can positively impact human health, hence leading to the hypothesis of their potential action in the control of diseases associated with the intestinal microbiome, such as inflammatory diseases [1,4,9,13,14].

Kefir, one of the most ancient foods known to present health benefits commonly attributed to its microbial and nutritional richesness, has already demonstrated to positively impact the general condition of the digestive system, including the intestinal microbiota [10,15–22]. The nutritional and microbiological value of kefir, in addition to its increasing popularity, make its application as a probiotic in the gut-skin axis regulation of the utmost interest. Regular consumption of kefir has been able to reduce gut dysbiosis, which opens the possibility that by modulation of the gut, inflammatory skin diseases may be better controlled [6,9,21,23], however, to date, the literature is still scarce on the impact of a diet containing kefir on the gut-skin relationship.

Atopic dermatitis (AD) is a chronic inflammatory skin disease, but its pathogenesis appears to be associated not only with immune dysfunction but also with intestinal dysbiosis, thus evidencing an intestine-skin relationship [13,14,24,25]. This intestinal dysbiosis has the ability to negatively impact skin function, since by increasing intestinal permeability via pro-inflammatory cytokines it promotes immune dysregulation, thus contributing to chronic systemic inflammation [4,24,26].

Consequently, the gut-skin axis may be receptive to modulation via dietary modification, namely via probiotics ingestion, thus representing a potencial complementary alternative in AD therapy [27–30]. This study aimed to explore the impact of the ingestion of kefir on gastrointestinal symptoms, not only of healthy individuals, but also of those with AD. This investigation gives continuity to a previous study, where the relation between kefir intake and skin health improvement was found [31], therefore allowing to further support the evidence of a gut-skin axis not only in atopic, but also in healthy individuals.

2. Materials and Methods

2.1. Study design

A controlled intervention study, coded DermapBio, was conducted according to the principles of the Helsinki Declaration and after informed consent. The study protocol was approved by the ethics committee of the School of Sciences and Health Technologies at Lusofona's University (Nº1/2018, May 15th 2018). Study subjects were recruited by convenience sampling between October 2019 and December 2020 among a university population (students and academic staff). Subjects were assigned to the different groups according to the inclusion criteria described elsewhere [31]. The atopic group (*n* = 19) included 1 male and 18 females, aged between 19 and 56 years (mean age 31.7 ± 11.9 years), wherein 47% were under 30 years old. Within this group, all subjects reported a diagnosis of AD, 14 (74%) of the subjects also reported rhinitis, and 6 (32%) reported a asthma diagnosis. All other subjects who fulfilled the eligibility criteria, excluding the atopic criteria, and were free of skin diseases, including AD, psoriasis, and other systemic diseases that may impact skin condition, were assigned to the healthy group. These subjects (*n* = 33) included 6 males (18%) and 27 females (82%), aged between 20 and 60 years (mean age 27.0 ± 10.1 years), wherein 61% were under 30 years old. Within the healthy (H) and the atopic (A) groups, volunteers were assigned to either the kefir intake (HK and AK, respectively) or the control (H0 and A0, respectively) group, according to their preference.

The primary endpoint in this study was the improvement of gastrointestinal symptoms after kefir intake.

2.1.1.Baseline conditions

Sociodemographic and lifestyle conditions, as well as dietary intake profiles were assessed, at baseline, before the beginning of the intervention, as previously described by the authors [31].

2.2. Kefir intervention

The intervention consisted on the daily consumption of kefir, during 8 weeks, produced by fermentation of a commercial ultra-high temperature pasteurized (UHT) semi-skimmed cow milk of Portuguese provenance (Nova Açores®, S. Miguel, Portugal), with CIDCA AGK1 kefir grains using a grain inoculum of 10 % (w/v), for 24 hours, at a temperature of 20 ± 1 °C, replicating Portuguese household representative conditions. Prior to the intervention, the kefir beverage consumed during this study was characterized by the authors, including the description of the preparation, storage, and intake conditions [32].

2.3. FGID assessment

Participants' gastrointestinal symptoms were assessed at baseline (t0) and at the end of the 8 weeks intervention (t8) using a self-completed questionnaire, adapted from the recommendations of the ROMA IV criteria [5] (Supplemental Figure S1).

2.4. Statistical analysis

Results were expressed as relative frequencies. Since the data were not normally distributed (normality assessed by the Shapiro-Wilk test), non-parametric tests were chosen to test different hypotheses. Differences within individuals were identified by Wilcoxon signed rank test and differences between kefir intake and control groups by Chi-Square test. Logistic regressions were used to evaluate the association between kefir intake and FGID improvements and their potential confounding factors. All analyses were performed using the SPSS statistical package version 25 (SPSS Inc., Chicago, IL, USA) with a significance level of 0.05.

3. Results

3.1. Baseline characteristics

As mentioned in a previous work by Alves and coworkers, neither healthy nor atopic subjects who were given kefir, showed any differences in baseline characteristics, either physiological, sociodemographic and regarding dietary intake, when compared to the respective control groups [31].

3.2. FGID assessment

FGID were assessed through the above mentioned questionnaire, at t0 and at t8, for all subjects. Baseline FGID showed no differences between kefir intake groups and the respective controls, for both healthy and atopic groups (p>0.05, for all parameters, Supplemental Table S1).

An analysis of the variation of FGID after 8 weeks of intervention was conducted, comparing kefir intake and control groups in both healthy and atopic volunteers (Table 1). Comparisons were made using an outcome variable “Improved FGID”, that must be interpreted as follows: FGID at t8 < FGID at t0.

Table 1 – FGID improvement, after 8 weeks of kefir ingestion, between kefir intake and control groups, for both healthy and atopic volunteers (relative frequency).

Improved FGID	**Healthy group (n = 33)**			**Atopic group (n = 19)**		
	HK	**H0**	***p* – value**	**AK**	**A0**	***p* – value**
Constipation, % (n)	38.5 (5)	0.0 (0)	**0.003**	55.6 (5)	10.0 (1)	**0.033**
Laxative use, % (n)	15.4 (2)	5.0 (1)	0.311	n.a.*	n.a.*	--
Diarrhea, % (n)	30.8 (4)	0.0 (0)	**0.008**	11.1 (1)	20.0 (2)	0.596
Dejection frequency, % (n)	23.1 (3)	0.0 (0)	**0.024**	22.2 (2)	20.0 (2)	0.906
Abdominal pain, % (n)	23.1(3)	0.0 (0)	**0.024**	44.4 (4)	30.0 (3)	0.515
Pain intensity, % (n)	30.8 (4)	0.0 (0)	**0.008**	55.6 (5)	30.0 (3)	0.260
Abdominal distension, % (n)	53.8 (7)	0.0 (0)	**<0.001**	66.7 (6)	20.0 (2)	**0.040**
Flatulence, % (n)	38.5 (5)	10.0 (2)	0.051	55.6 (5)	40.0 (4)	0.498

Table 1 (*Continued)*

Improved FGID	Healthy group (n = 33)			Atopic group (n = 19)		
	HK	**H0**	***p* – value**	**AK**	**A0**	***p* – value**
Associated pain, % (n)	23.1 (3)	0.0 (0)	**0.024**	77.8 (7)	60.0 (6)	0.405
Belching, % (n)	15.4 (2)	0.0 (0)	0.070	44.4 (4)	20.0 (2)	0.252
Fullness sensation, % (n)	n.a.*	n.a.*	--	22.2(2)	30.0 (3)	0.701
Headache, % (n)	30.8 (4)	0	**0.008**	33.3 (3)	20.0 (2)	0.510

HK – Healthy skin with kefir intake; H0 - Healthy skin without kefir intake; AK – Atopic skin with kefir intake; A0 – Atopic skin without kefir intake. Groups were compared by Chi-Square test, $p < 0.05$ for statistical significance. n.a. – not applicable, *Outcome is constant.

As shown in Table 1, data revealed that in healthy skin subjects, kefir ingestion for 8 weeks improved constipation (p=0.003), diarrhea as well as dejections frequency (p=0.008 and p=0.024, respectively), abdominal pain occurrence as well as intensity of the pain (p=0.024 and p=0.008, respectively), abdominal distension *(*p<0.001), pain associated to flatulence (p=0.024) and headache (p=0.008), when compared to control. Moreover, an improvement trend was also observed for flatulence and belching, although not significant (p=0.051 and p=0.070, respectively), compared to control.

Furthermore, because FGID can also be influenced by intrinsic individual variation, an individual paired comparison, between t0 and t8, was performed. The results obtained support the data on Table 1, since it was observed that healthy individuals who drank kefir for 8 weeks significantly improved constipation, diarrhea, abdominal distension and flatulence *(*p=0.025, p=0.046, p=0.008 and p=0.025, respectively) and an improvement trend for headache (p=0.063), while in those who did not drink, no differences were found after the intervention (p>0.05, for all FGID) (Supplemental Table S2).

A similar analysis regarding atopic subjects revealed significant improvements in constipation and abdominal distension *(*p=0.033 and p=0.040, respectively), for the intake group compared to control (Table 1). These results were also reinforced by those from individual paired comparisons that showed that at t8, the atopic subjects who drank kefir, presented a significant improvement in constipation, abdominal distension and pain associated to flatulence (p=0.038, p=0.023 and p=0.015, respectively). Additionally, an improvement trend was observed for flatulence and belching (p=0.052 and p=0.059, respectively). In controls, no differences were observed after the intervention (p>0.05, for all FGID) (Supplemental Table S2). Notably, these results confirming the effective improvement in FGID, represent an important contribution to support the role of kefir as a gut modulator.

3.3. Adjusted models for FGID

To assess the effect of different independent variables on the outcomes of FGID, logistic regression models were performed. All socio-demographic variables, food intake variables, kefir intake and skin status were considered as possible predictors for the influence in variation of the FGID outcomes. After testing the assumptions for collinearity diagnostics, independent variables were excluded from the models if the variance inflation factor (VIF) was superior to 10. After this step, logistic regressions were performed for each outcome variable, in order to identify which variables better explained it. The most common variables in the models and so considered as possible

predictor variables on FGID outcomes identified by this method were: kefir status, defined as with or without kefir intake, water intake in liters and age, defined as less than 30 years old or greater than or equal to 30 years old. Final logistic regressions were then run, which are

Table 2 – Association between kefir intake and FGID improvement (Odds Ratio (*p*-value), n = 52).

	Odds Ratio (*p*-value)		
Improved FGID	**Crude OR**	**aOR1**	**aOR2**
Constipation	24.17 (**0.004**)	33.93 (**0.003**)	32.22 (**0.003**)
Laxative use	2.900 (0.389)	2.671 (0.439)	2.707 (0.433)
Diarrhea	4.118 (0.112)	3.901 (0.138)	4.150 (0.128)
Dejection frequency	4.118 (0.112)	3.869 (0.137)	3.868 (0.137)
Abdominal pain	4.200 (0.059)	4.102 (0.065)	4.083 (0.066)
Pain intensity	6.231 (**0.014**)	6.208 (**0.015**)	6.153 (**0.016**)
Abdominal distension	20.22 (**<0.001**)	27.74 (**<0.001**)	30.29 (**<0.001**)
Flatulence	3.333 (0.054)	3.326 (0.056)	3.994 (**0.040**)
Associated pain	3.333 (0.054)	3.321 (0.056)	3.446 (0.052)
Belching	5.250 (0.058)	6.103 (**0.046**)	8.125 (**0.033**)
Fullness sensation	0.900 (0.913)	0.920 (0.931)	0.836 (0.854)
Headache	6.533 (**0.030**)	6.719 (**0.028**)	6.635 (**0.031**)

OR – Odds ratio for kefir status (Reference category: without kefir intake), p<0.05 for statistical significance. aOR1 – Odds ratio for kefir status adjusted for water intake; aOR2 - Odds ratio for kefir status adjusted for water intake and cut-off age 25 years old.

Results from Table 2 showed that drinking kefir for 8 weeks is associated with a significant improvement in constipation, abdominal pain intensity, abdominal distension and headache (crude OR). In the model adjusted for water intake (aOR1), the effect of kefir intake remained significant for the same results, with an additional significant improvement for belching. Further adjustments for water intake and age (aOR2) showed that the effect of kefir intake remained significant for the same results, with an additional significant improvement for flatulence. Although not significant, abdominal pain and pain associated with flatulence showed a trend towards improvement. Noteworthy, these results allow us to conclude that, in this study, kefir intake was positively associated with FGID improvement particularly for constipation and abdominal distension, with the water intake having a higher impact in constipation, as expected due to the well known impact of water in the gut [30,33].

3.4. Comparison between FGID improvement and skin parameters modification

The DermapBio Study aimed to explore potential relationships between the gut and the skin, assessed by improvement of skin barrier function and functional gastrointestinal disorders (FGID). The set of results regarding the skin, obtained by the authors in a previous study, showed that both

healthy and atopic skin individuals who ingested kefir for 8 weeks (n = 22) presented an improvement in the following skin parameters: forearm and forehead TEWL and forearm hydration. Additionally, atopic individuals who ingested kefir also showed an improvement in the SCORAD index [31]. The set of results regarding the FGID obtained in this study allow us to conclude that kefir intake was positively associated with FGID improvement particularly for constipation and abdominal distension (Table 2). Therefore, a further analysis of the existence of a relationship between the improvement in skin parameters and the improvement in FGID, for those who drank kefir, was performed (Table 3). These relations were not evaluated in the control groups due to the fact that no differences were found after the 8 weeks, for both skin parameters [31] and FGID.

Table 3 – Comparison between FGID improvement and skin parameters modification, after 8 weeks of kefir intake (relative frequency (%) (*p* – value), n = 22).

	Improved FGID						
Modification of skin parameters	**Functional Constipation**	**Functional Diarrhea**	**Dejection frequency**	**Abdominal pain**	**Abdominal pain intensity**	**Functional abdominal distention**	**Pain associated to flatulence**
TEWL							
Forearm	10 (45.4 %) (0.644)	5 (22.7 %) (0.557)	5 (22.7 %) (0.906)	7 (31.8 %) (0.378)	9 (40.9 %) (0.301)	13 (59.1 %) (0.271)	10 (45.4 %) (0.235)
Forehead	10 (45.4 %) (0.510)	5 (22.7 %) (0.327)	5 (22.7 %) (0.225)	7 (31.8 %) (0.217)	9 (40.9 %) (0.271)	13 (59.1 %) (0.815)	10 (45.4 %) (**0.041**)
Hydration Forearm	10 (45.4 %) (0.668)	5 (22.7 %) (**0.038**)	5 (22.7 %) (0.196)	7 (31.8 %) (0.672)	9 (40.9 %) (0.894)	13 (59.1 %) (0.229)	10 (45.4 %) (0.070)
SCORAD Index*	5 (55.6 %) (1.000)	1 (11.1 %) (0.439)	2 (22.2 %) (0.558)	4 (44.4 %) (0.221)	5 (55.6 %) (0.327)	6 (66.7 %) (0.197)	7 (77.8 %) (0.380)

TEWL – Transepidermal Water Loss. SCORAD - SCORing of Atopic Dermatitis. * - only on atopics. Groups compared by Mann-Whitney U-test, with $p < 0.05$ for statistical significance.

From Table 3, it can be seen that after 8 weeks of kefir ingestion, among those who showed improvement in forearm hydration, 22.7% (p = 0.038) reported a significant improvement in functional diarrhea. In addition, in those who showed improvement in forehead TEWL, 45.5% (p = 0.041) of the individuals showed significant improvement in pain associated with flatulence. Furthermore, despite the lack of statistical significance of the results, in more than 50 % of atopics that showed a decrease in the SCORAD index, improvements were recorded in terms of constipation, abdominal pain intensity, abdominal distension and pain associated with flatulence.

4. Discussion

Kefir is an ancient fermented food that has recently gained popularity due to its putative role as a healthy food [6,34]. The consumption of kefir has been reported to positively impact the gut microbiota and the overall condition of the digestive system [6,9,10,21,34]. The literature contains research on the effect of kefir consumption on gastrointestinal functionality [8,10], but specific information on the effects of kefir on functional bowel disorders is still scarce.

Despite the wide range of health-promoting benefits potentially ascertained to kefir [15,34–37], namely anti-inflammatory effects [38], antimicrobial activity [39], strengthening of the immune system [40], antioxidant activity [41], and the inhibition of pathogenic microorganisms [42], most of them have only been demonstrated in vitro or in animal models. Regarding the cutaneous effect of kefir, the topical application of a gel made from a non-microbial fraction of kefir showed an improvement in the wound healing capacity, using animal models [43]. Notably, although in inflammatory skin diseases, such as AD, the onset points to a gut-skin relationship may be potentially receptive to the dietary modulation pathway [1,27,30], none of the *in vivo* human studies found in the literature observed the impact of a diet containing traditionally homemade kefir as the probiotic, on both the skin and on the FGID.

In this study, drinking kefir for 8 weeks was associated with a significant improvement in constipation, abdominal pain intensity, abdominal distension, fullness sensation, headache and flatulence, on healthy skin volunteers. Additionally, although not statistically significant, abdominal pain and pain associated with flatulence showed a trend towards improvement. Furthermore, our results showed that healthy skin subjects who drank kefir for 8 weeks, had a significant improvement in eight of the twelve FGID explored, compared to the control group. Similar results were found in other studies carried out in humans regarding the effect of kefir consumption on gastrointestinal function [19,20,44,45]. Maki *et al*., studying 42 hospitalised patients with constipation, observed that lyophilized kefir had no impact on laxative use, stool consistency and stool volume compared to control, however the number of patients not requiring any laxatives was higher 12 weeks following the kefir intervention compared to baseline [20]. Turan *et al*. in an uncontrolled trial with 20 people with functional constipation showed that kefir for 4 weeks significantly increased stool frequency, improved bowel satisfaction score and reduced gut transit time compared to baseline [45]. Additionally, Hertzler *et al*. observed a significant decrease in flatulence severity after a 5 days kefir intervention, but no differences were found for flatulence frequency, abdominal pain and diarrhea, in people with lactose malabsorption [44]. Furthermore, Bekar *et al*. investigated the impact of kefir, on *Helicobacter pylori* eradication rates, in patients with dyspepsia and found a significantly higher rate of eradication in the kefir group compared to the control group, accompanied by a significantly lower occurrence of diarrhea, abdominal pain and nausea in the kefir group compared to control [19].

As for atopic skin subjects, improvements were also only seen in the kefir intake group, however they were only evident on two of the FGID. These differences observed between healthy and atopic subjects may be justified by the fact that because AD is characterized by systemic inflammation and intestinal dysbiosis, the intervention period may not have been sufficient enough to detect more changes at the intestinal level, particularly when only assessing symptoms [26]. Results from other studies that evaluated the effect of kefir on the intestine, including in cases of pathologies associated with low-grade inflammation, enhance the ability of kefir to positively impact the gut [8,12,46]. Bellikci-Koyu *et al*., evaluating the impact of kefir consumption, for 12 weeks, on the gut of 22 patients with metabolic syndrome, was only able to observe a significant increase in Actinobacteria abundance [8]. Praznikar *et al*. assessing the effect of kefir intake for 3 weeks in 28 overweight adults, found an improvement in serum zonulin levels, an important intestinal barrier dysfunction marker which enhanced the kefir probiotic capacity to modulate intestinal microbiota composition and consequent capacity to control the grade chronic inflammation promoted by increased intestinal permeability [12]. St-Onge *et al*., testing the impact of kefir for 4 weeks, in 13 hypercholesterolemic subjects found a significant increase in fecal short chain fatty acids, which evidenced the positive effect of kefir on intestinal regulation [46].

These positive results obtained in the kefir intake groups can be attributed to both kefir's nutritional value and kefir's microbial composition [16,17,32,47]. The microbial fraction of kefir has demonstrated, *in vitro*, to have an impact on the gut microbiota population, with increases in *Lactobacillus*, *Lactococcus* and *Bifidobacterium* and reductions in *Proteobacteria* and *Enterobacteriaceae* concentrations [22,48], which may be enhanced by its *in vitro* capacity to adhere to human enterocyte-like Caco-2 cells, indicating a potential ability to colonise the human gut [49]. In addition, Kim *et al*. found a significantly greater number of stool total yeasts and *Candida kefyr* compared to control following kefir consumption, using a mouse model [50]. Moreover, Maeda *et al*. showed that kefir increased stool weight and moisture in mice, in a dose-responsive manner, compared to control, suggesting a potential beneficial effect in constipation, and reinforcing the role of the non-microbial fraction of kefir in intestinal health [51].

The existence of a relationship between kefir intake and the improvement in skin parameters and in FGID demonstrated in this work is in line with the recent work of Fang and coworkers who found that probiotic intake for 8 weeks ameliorated severity in atopic dermatitis subjects through improvement of gut health, via microbial and immune responses and attributed it to regulatory T cells differentiation, and increased microbial diversity and evenness, thus supporting the hypotheses of recovery from a state of intestinal dysbiosis to a state of healthy balance, promoted by the ingestion of probiotics [52]. In addition, Petersen *et al*., in a recent systematic review investigating the efficacy of probiotics on the severity of AD, verified that although all of studies included showed alterations in the gut microbial composition promoted by the use of probiotics, only 8 of them were able to link those alterations to a positive effect on the severity of AD [25]. Identical results were found by Kim et al. in a previous meta-analysis [53].

Despite all the positive outcomes found in our study, some limitations must be acknowledged. First of all, the fact that this was not a double-blind, placebo-controlled study, may be identified as an important limitation of the study [54]. It is also worth noting the use of a non-validated questionnaire to collect self-reported symptoms. If, on the one hand, self-reported health information depends on the individual's true health status and perception of their own health [55], on the other hand, it is more subject to biases, such as the social desirability bias, the most frequent in self-reported information on women's health [56]. Furthermore, although the study design is intended to minimize the effect of individual variability and the small number of participants, individual changes can happen over time, influencing the gut, and impact on the results [33]. However, these challenges can be mitigated by introducing a washout period and collecting new baseline samples before starting a second sequential intervention. Another limitation noteworthy was the small group sizes, which may have contributed to difficult the detection of small to medium shifts in the gut environment, since inadequate sample size increases the risk of failure to detect a difference when there is one (β-error) [54]. Additionally, besides the proven relevance of using FGID to evaluate changes in the gut, monitoring microbial changes in the human gut microbiome after ingesting a multistrain probiotic, like kefir, can provide a better understanding of its health benefits [47]. Finally, conditions affecting the kefir production, such as fermentation conditions or origin of the grains, must be accounted for when taking conclusions on kefir impact on health [23,32].

5. Conclusions

This work investigated the effects of regular ingestion of homemade kefir on the functional gastrointestinal symptoms of both healthy and atopic subjects. Our results showed a significant improvement on FGID outcomes after kefir intake for 8 weeks, in both healthy and atopic skin individuals. Notably, the observed differences were more easily identified in healthy volunteers who drank kefir than in atopics, which may be justified by the inflammatory and dysbiotic pattern

associated with AD. The correlations established between the improvement in skin parameters and the improvement in FGID, after kefir consumption, indicate that homemade kefir can be a potential modulator of the gut-skin axis, on healthy and AD skin individuals.

To the best of our knowledge, this was the first study to provide information regarding the functional gastrointestinal impact of the intake of kefir produced in household representative conditions. Furthermore, the improvement in FGID of atopic individuals, but not in their controls, observed in this study is considered a new finding.

Further *in vivo* studies in humans are needed to consolidate the effect of kefir on the intestinal microbiota, including functional gastrointestinal disorders, as well as its additional systemic impact. Future studies should be conducted on the mechanisms underlying the gut-skin axis modulation.

In conclusion, our study added a strong contribution to support the hypothesis that ingestion of probiotics in the form of fermented food, namely kefir, improves skin health and promotes the reduction of the severity of AD, due to a possible concomitant change in the gut microbiota.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Baseline FGID parameters for each study group. Table S2: Individual variation in FGID, between t8 and t0. Figure S1 – Functional Gastrointestinal Disorders Questionnaire.

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Supplementary Material

Supplemental Table S1 - Baseline FGID parameters for each study group (relative frequency).

Baseline FGID	Healthy group (n = 33)			Atopic group (n = 19)		
	HK	**H0**	***p* - value**	**AK**	**A0**	***p* - value**
Constipation, % (n)	38.5 (5)	25.0 (5)	0.076	55.6 (5)	60.0 (6)	0.956
Laxant use, % (n)	15.4 (2)	5.0 (1)	0.422	n.a.*	n.a.*	--
Diarrhea, % (n)	30.8 (4)	25.0 (5)	0.452	22.2 (2)	40.0 (4)	0.405
Dejection frequency > 3 times per day, % (n)	30.8 (4)	25.0 (5)	0.178	22.2 (2)	20.0 (2)	0.362
Abdominal pain, % (n)	30.8 (4)	30.0 (6)	0.181	66.7 (6)	60.0 (6)	0.805
Pain intensity ≥ 5, % (n)	23.1 (3)	5.0 (1)	0.438	66.7 (6)	60.0 (6)	0.634
Abdominal distension, % (n)	53.8 (7)	55.0 (11)	0.449	88.9 (8)	90.0 (9)	0.242
Flatulence, % (n)	61.5 (8)	55.0 (11)	0.832	88.9 (8)	90.0 (9)	0.782
Associated pain, % (n)	30.8 (4)	35.0 (7)	0.136	88.9 (8)	70.0 (7)	0.563
Belching, % (n)	15.4 (2)	25.0 (5)	0.784	55.6 (5)	30.0 (3)	0.413
Fullness sensation, % (n)	30.8 (4)	25.0 (5)	0.716	33.3 (3)	50.0 (5)	0.312
Headache, % (n)	92.3 (12)	65.0 (13)	0.076	55.6 (5)	70.0 (7)	0.445

Groups were compared by Chi-Square test, with $p < 0.05$ for statistical significance. n.a.- not aplicable, *outcome is constant.

Supplemental Table S2– Individual variation in FGID, between t8 and t0 (Wilcoxon Standartized (Z) test statistic (*p*-value)).

FGID	Healthy group (n = 33)		Atopic group (n = 19)	
	HK	**H0**	**AK**	**A0**
Constipation	-2.236 (0.025)	0.000 (1.000)	-2.707 (**0.038**)	-1.000 (0.317)
Laxative use	-1.414 (0.157)	-1.000 (0.317)	0.000 (1.000)	0.000 (1.000)
Diarrhea	-2.000 (**0.046**)	0.000 (1.000)	-1.000 (0.317)	-1.414 (0.157)
Dejection frequency > 3 times per day	-1.633 (0.102)	0.000 (1.000)	-1.342 (0.180)	-1.414 (0.157)
Abdominal pain	-1.732 (0.083)	0.000 (1.000)	-1.414 (0.157)	-0.447 (0.655)

Supplemental Table S2 (*Continued)*

FGID	Healthy group (n = 33)		Atopic group (n = 19)	
	HK	**H0**	**AK**	**A0**
Pain intensity ≥ 5	-1.841 (0.066)	0.000 (1.000)	-1.612 (0.107)	-1.214 (0.225)
Abdominal distension	-2.646 (**0.008**)	0.000 (1.000)	-2.271 (**0.023**)	-0.577 (0.564)
Flatulence	-2.236 (**0.025**)	-1.414 (0.157)	-1.947 (0.052)	-0.333 (0.739)
Associated pain	-1.633 (0.102)	0.000 (1.000)	-2.428 (**0.015**)	-1.265 (0.206)
Belching	-1.414 (0.157)	0.000 (1.000)	-1.890 (0.059)	-1.342 (0.180)
Fullness sensation	0.000 (1.000)	0.000 (1.000)	-1.342 (0.180)	-0.447 (0.655)
Headache	-1.857 (0.063)	0.000 (1.000)	-1.633 (0.102)	-0.447 (0.655)*

HK – Healthy skin with kefir intake; H0 - Healthy skin without kefir intake; AK – Atopic skin with kefir intake; A0 – Atopic skin without kefir intake. Individuals were compared by Wilcoxon signed rank test, with $p<0.05$ for statistical significance. * - based on negative ranks (variable at t0 < variable at t8).

Supplemental Figure S1– Functional Gastrointestinal Disorders Questionnaire.

Do you suffer from constipation?
Did you take any laxative in the last 6 months?
Do you suffer from diarrhea?
What is the dejection frequency?
Do you suffer from abdominal pain?
From 1 to 10 what is the intensity of the pain?
Do you suffer from abdominal distension?
Do you suffer from flatulence?
Do you suffer from pain associated to the flatulence?
Do you suffer from belching?
Do you have a sensation of fulness after starting a meal?
Do you suffer from headaches?

CONCLUSIONS

CONCLUSIONS

The experimental work conducted for the production of this thesis aimed to explore the effects of kefir, produced in household conditions, in cutaneous health via a gut-skin axis.

Even though the use of kefir probiotic microorganisms have shown promising beneficial effects in modulating the intestine *in vitro* and in animal models, there is still much to know about the health effects of the regular ingestion of typical homemade kefir in humans, *in vivo*. As such, an extensive review was carried out on the intestinal and systemic effects of probiotics, namely on the skin, with a focus on atopic dermatitis. In this review, kefir was highlighted in relation to its effects on health and its potential mechanisms of action.

This work was based on the daily consumption of kefir, for 8 weeks, produced in conditions representative of the household, with the *in vivo* investigation being preceded by the physicochemical and nutritional characterization of this food. The results obtained allowed the following conclusions:

1. Kefir produced under home use conditions using semi-skimmed cow milk of Portuguese provenance was able to fulfill the *Codex Alimentarius* requirements and maintained its characteristics with respect to the physicochemical and nutritional composition, both after fermentation, as well as during 48 h of refrigerated storage. Additionally, although kefir is not traditionally consumed in Portugal, this kefir drink showed a good acceptance in the sample of consumers used.

2. Daily ingestion of kefir for eight weeks, produced under home use conditions, caused:
   a. an improvement on skin barrier function in both healthy and atopic skin subjects;
   b. an improvement in the degree of severity of atopic dermatitis (AD), reflecting a notable clinical improvement;
   c. a significant improvement in several functional gastrointestinal symptoms such as constipation, abdominal pain intensity, abdominal distension, fullness sensation and headache and flatulence on both healthy and atopic skin subjects;

3. It was also possible to acknowledge the existence of an association between the skin barrier improvements and the improvements on the gastrointestinal symptoms, thus reinforcing the role of kefir as potential modulator of the gut-skin axis.

To our knowledge, this was the first human *in vivo* study to provide information regarding the impact of homemade kefir ingestion on both skin health and functional gastrointestinal symptoms in healthy and atopic individuals. The innovative character of this investigation also lies in the fact that, in order to evaluate atopic skin condition, an approach combining skin barrier function analysis with the severity assessed by Scoring Atopic Dermatitis (SCORAD) Index was used.

In conclusion, this work added a strong contribution to support the hypotesis that ingestion of probiotics in the form of fermented food, namely kefir, improves skin health and promotes the

reduction of the severity of AD, due to a possible concomitant change in the gut health, thus further opening up the possibility to continuing the research on the impact of the probiotic kefir on cutaneous health and its mechanism of action, namely via the gut-skin axis.

ANNEX

Opinion issued by the Research Ethics Committee

Comissão de Ética

PARECER DA COMISSÃO DE ÉTICA DA ESCOLA de CIÊNCIAS e TECNOLOGIAS DA SAÚDE DA ULHT

A Comissão de Ética da ULHT emite o seguinte parecer sobre o protocolo de investigação datado de 15 de Maio de 2018 e registado com o nº1/2018 pela investigadora administradora do projecto Senhora Professora Doutora Catarina Rosado:

1. O protocolo agora presente à CE intitulado "Contribuição para o estudo dos efeitos do kefir e seus subprodutos sobre a saúde da pele".

2. Confirma-se o protocolo submetido a parecer como respeitador das regras de ética exigidas para a sua execução pelo que se aprova desde que executado no estrito respeito das condições apresentadas.

Lisboa, 15 de Junho de 2018

O Presidente da Comissão de Ética

(Amílcar Elizeu Rato da Silva Roberto)