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Bioactive Composition and *Ex Vivo* Cutaneous Effects of *Lactiplantibacillus plantarum* T5-derived Cell-free Supernatants from Tarkhineh

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Abstract

Background and Objective: The skin microbiome preserves cutaneous health and barrier function and its dysbiosis contributes to diverse dermatological disorders. Although probiotics are widely investigated, the composition and cutaneous effects of strain-specific cell-free preparations from traditional fermented foods are poorly characterized. This study characterized selected fermentation components in cell-free supernatant derived from *Lactiplantibacillus plantarum* T5 (T5-CFS) isolated from Iranian traditional food and assessed its protective effects using *ex vivo* human skin model.

Materials and Methods: *Lactiplantibacillus plantarum* T5 was cultured in reconstituted skim milk. The resulting cell-free supernatant was analysed using high-performance liquid chromatography to quantify organic and free amino acids and exopolysaccharides were assessed via the phenol-sulphuric acid method. *Ex vivo* human foreskin explants treated with various T5-CFS concentrations were assessed for viability and sodium lauryl sulphate-induced damages using 3-(4,5-di methyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Morphology was investigated using hematoxylin-eosin staining and barrier-associated gene expression was quantified using quantitative real-time polymerase chain reaction.

Results and Conclusion: The T5-derived cell free supernatant contained lactic acid (2.44 mg/ml), acetic acid (0.97 mg/ml) and exopolysaccharide content (2705 mg/l). In an exploratory amino-acid screening, total free amino acids increased from 2340 to 13130 mg/l after fermentation. Under the *ex vivo* conditions, T5-derived CFS treatment preserved tissue viability, preserved epidermal morphology, decreased sodium lauryl sulphate-associated tissue damages and upregulated the mRNA expression of key barrier-associated genes, including *ZO*1, *CLDN*1 and *FLG*. The *Lactiplantibacillus plantarum* T5-derived CFS contained a complex fermentation-associated composition and was associated with preserved epidermal morphology and increased expression of barrier-associated transcripts in *ex vivo* human skin. Collectively, these findings indicate that the preparation contains a diverse pool of fermentation-derived components that may contribute to the observed barrier-associated responses; however, component-specific causality and functional barrier restoration were not established. These findings support further investigation of T5-derived CFS as a candidate preparation for future topical formulation development.

What is "already known":	• Probiotic metabolites are recognized for their capacity to modulate skin homeostasis and strengthen barrier function. • Most dermatological postbiotic research focuses on commercial strains or isolated single-component actives. • The functional and protective potential of metabolites derived from unique, traditional fermented food matrices remains largely unexplored.

What this article adds:	• Identifies *L. plantarum* T5, isolated from traditional Iranian fermented Tarkhineh, as a potent source of functional postbiotic metabolites. • Demonstrates that T5 cell-free supernatant effectively attenuates surfactant-induced cytotoxicity and maintains epidermal structural integrity. • Reveals that T5 metabolites actively drive the transcriptional upregulation of key skin barrier-related genes, accelerating cutaneous repair and homeostasis.

1. INTRODUCTION

The skin microbiota contributes to cutaneous homeostasis, immune response regulation and epidermal integrity preservation [1,2]. Disturbance of this microbial balance by environmental, chemical or immunological stressors increases skin permeability and contributes to inflammatory conditions such as atopic dermatitis, acne and psoriasis [3]. Accordingly, strategies aimed at preserving or restoring epidermal barrier homeostasis are important in dermatological research and topical product development. Recent microbiome research has increased interests in probiotic and postbiotic preparations as potential tools for supporting intestinal and cutaneous health. Although the skin and gastrointestinal tract differ substantially in their anatomy and microbial ecology, the two rely on interactions within microbial, epithelial and immune components to respond to environmental challenges [4,5]. Fermented foods represent valuable sources for isolating novel probiotic strains and evidence shows that the origin and strain-specific characteristics of probiotics greatly affect their functional and therapeutic characteristics [6]. Probiotic strains belonging to genera such as *Lactiplantibacillus* and *Bifidobacterium* have been reported to affect skin barrier-associated outcomes, inflammatory skin conditions [4,7] and wound healing processes [8]. However, the topical use of live probiotics presents formulation challenges linked to preserving viability, ensuring product stability and controlling potential safety risks [9].

Based on the International Scientific Association for Pro-biotics and Prebiotics consensus, a postbiotic is a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host [10]. Such preparations may offer practical advantages over live microorganisms, including improved formulation stability and decreased concerns linked to microbial viability; however, their composition and biological effects depend on the processing method and the components in the final preparation [11,10]. Depending on their composition and biological context, fermentation-associated components e.g. organic acids, EPSs and free amino acids (FAA) have been reported to exhibit antioxidant, anti-inflamm-atory or immunomodulatory activities in previous studies [8,9, 12,13]. These reports provide a rationale for investigating their potential relevance to cutaneous biology but such activities were not directly assessed in the present study.

Previous studies suggest that selected non-viable microbial preparations or their components may affect epithelial and dermal cell responses, including the expression of genes or proteins involved in tissue homeostasis [14,15]. Within probiotic candidates, *Lactiplantibacillus* (*L.*) *plantarum* has shown strain-dependent functional characteristics in diverse biological systems, including reported antimic-robial and immunomodulatory activities [14,16,17]. For example, the strain *L. plantarum* GMNL6 has been reported to affect collagen-linked responses, melanin production and skin microbiome-associated outcomes in previous studies [15]. In particular, *L. plantarum* T5, an indigenous strain isolated from a traditional Iranian fermented foods [18], was selected because of its complementary strain-specific bioactive and bioprocessing attributes. Previous studies have demonstrated antimuta-genic activity in its cellular fraction and cell-free super-natant, supporting the presence of biologically active extracellular fermentation products [19]. In addition, T5 has been reported to promote cutaneous wound healing in an animal model, accompanied by decreased inflammation and accelerated tissue repair, thereby providing a relevant rationale for its investigation in skin-linked uses [20]. The strain has shown EPS-production capacity [18] and robust biomass production under optimized laboratory-scale fermentation conditions [21], making it a promising candidate for generating a bioactive, metabolite-enriched cell-free preparation for cutaneous assessment. So, isolated or characterized EPS preparations from *L. plantarum* have been reported to promote human dermal fibroblast prolif-eration and migration in previous studies [22,23]. These findings provide a literature-based

rationale for considering EPS as one possible contributor to the biological characteristics of complex fermentation-derived preparations; however, EPS-specific activity was not assessed in the present study. Despite these reports, the fermentation-associated composition and cutaneous effects of *L. plantarum* strains isolated from traditional Iranian fermented foods are insufficiently characterized.

This study characterized selected fermentation-associated components of the T5-derived CFS and assessed its effects using *ex vivo* human skin model. The authors hypothesized that T5-derived CFS was well tolerated by *ex vivo* human skin explants and attenuated sodium lauryl sulphate (SLS)-associated tissue damages while preserving epidermal morphology and influencing the expression of barrier-associated genes. To assess this hypothesis, the authors characterized selected components of T5-derived CFS, assessed tissue viability under baseline conditions, investigated its effects on SLS-associated tissue damages and epidermal morphology in *ex vivo* human skin explants and assessed changes in the expression of selected barrier-associated genes.

2. MATERIALS AND METHODS

2.1. Bacterial Strain and Culture Conditions

Briefly, *L. plantarum* T5, previously isolated from Tarkhineh (a traditional Iranian fermented food product) described by Noori et al. [21], was used. The partial 16S rRNA gene sequence is deposited in GenBank under accession number of JQ301796. Cryopreserved stocks were preserved at -80 °C in 25% (v/v) glycerol. Working cultures were prepared by inoculating 1% (v/v) of the thawed suspension into 10 ml of de Man, Rogosaand Sharpe (MRS) broth (Sigma-Aldrich, USA) supplemented with 0.15% (w/v) L-cysteine hydrochloride, followed by incubation at 37 °C for 24 h.

2.2. Preparation of *Lactiplantibacillus plantarum* T5-derived Cell-free Supernatant

The T5-derived CFS was prepared by dissolving skimmed milk powder (Scharlau, Spain) in distilled water (DW) to a final concentration of 8% (w/v) with 0.15% (w/v) L-cysteine hydrochloride and inoculating with 1% (v/v) *L. plantarum* T5. The cultures were incubated at 37 °C with shaking at 100 rpm for up to 24 h. Bacterial growth was monitored at 0, 4, 8, 12, 16, 20 and 24 h using viable plate counting. At the stationary phase (20 h), cultures were centrifuged at 10,000× g for 20 min and the supernatant was filtered through 0.22-µm syringe filters (Sartorius, Germany). The T5-derived CFS was assessed in its native, non-neutralized state to preserve the complete physico-chemical composition of the preparation, including its naturally occurring organic acids. Unfermented reconstituted skim milk (RSM) (pH 6.50 ±0.14), prepared using the identical media composition, served as the vehicle-matrix control. The sterile T5-derived CFS was aliquoted and stored at -20 °C until compositional analysis and *ex vivo* tissue assessments [24].

2.3. Assessment of Growth Kinetics and Acidification Dynamics

Viable cell counts (VCC) were assessed using standard serial dilution and plating on MRS agar supplemented with 0.15% (w/v) L-cysteine hydrochloride [16]. Briefly, samples were collected at the designated time points, serially diluted in a sterile 0.9% (w/v) NaCl solution and 100-µl aliquots were spread-plated in triplicate. The plates were incubated at 37 °C for 24–48 h and then colony-forming units (CFUs) were enumerated. Parallel pH values were assessed at each sampling point using calibrated pH meter (Model PHS 550, INESA, China), standardized with buffer solutions at pH 4.0 and 7.0 (Merck, Germany).

2.4. Analysis of Organic Acids, Exopolysaccharides and Free Amino Acids in T5-derived CFS

2.4.1. Organic acid quantification via high-performance liquid chromatography

The organic acids, specifically lactic acid and acetic acid, were quantified using high-performance liquid chromatography (HPLC) system (Shimadzu, Japan). Concentrations were assessed by comparing retention times with those of standard solutions of acetic acid and lactic acid (Sigma-Aldrich, Germany). The HPLC system was equipped with a UV absorbance detector set at 220 nm, a C18 column (250 × 4.6 mm, 5 µm particle size; V.D.S., Germany), a mobile phase consisting of 25 mM KH_2PO_4 (pH 2.5) with a flow rate of 0.6 ml/min under isocratic elution conditions with a sample injection volume of 20 µl and a column temperature of 45 °C [16,17].

2.4.2. Exopolysaccharides isolation and quantification

The EPS were isolated and quantified from *L. plantarum* T5 fermentation broth using modified version of the method described by Kim et al. [25]. Briefly, proteins were precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10% (w/v), followed by incubation at 4 °C for 30 min and centrifugation at 12,000× g for 15 min at 4 °C. The EPS-containing supernatant was mixed with two volumes of ice-cold absolute ethanol and incubated at 4 °C for 24 h to precipitate the EPS fraction. The resulting pellet was harvested by centrifugation (6,000× g, 20 min) and washed twice with 70% (v/v) ethanol. To remove residual milk sugars, the pellet was dissolved in DW and dialyzed against running DW for 48 h using 12–14 kDa molecular weight cut-off membrane (Sigma-Aldrich, USA). Total EPS content was quantified using phenol-sulphuric acid colorimetric method [26] and D-glucose as the standard calibration reference.

2.4.3. Amino acid profile

The FAA profile of the T5-derived CFS was analysed using HPLC system (Agilent Technologies, USA) equipped with a diode array detector (DAD). Chromatographic separation was achieved using Agilent Zorbax Eclipse AAA reversed-phase column (150 × 46 mm, 3.5 µm) incubated at 40 °C. Online pre-column derivatization was automatically carried out via automated reaction of primary amino acids with *o*-phthalaldehyde (OPA) and secondary amino acids with 9-fluorenylmethyl chloroformate (FMOC) (Merck, Germany) prior to injection. Sarcosine and norvaline (Sigma-Aldrich, USA) were used as internal standards. Derivatized amino acids were detected at 338 nm for *o*-phthalaldehyde derivatives and 262 nm for FMOC derivatives. Absolute concentrations were calculated using linear regression calibration curves generated from certified amino acid standards (Sigma-Aldrich, USA) [27]. The FAA profiling was carried out as an exploratory quantitative screening to characterize the free amino acid composition of the fermented skim milk-derived preparation after 20 h of culture. This analysis was carried out using a pooled fermentation sample and was therefore reported as a descriptive $n = 1$ dataset. Accordingly, the results are presented as absolute concentrations of individual FAAs (mg/L), and no variance estimates [mean ± standard deviation (SD)] or inferential statistical analyses were applied.

2.5. *Ex Vivo* Foreskin Explant Culture and Treatment

Foreskin tissue segments (approximately 1 × 2 cm^2) were initially rinsed three times with a 1% (v/v) penicillin/streptomycin solution (Gibco, USA). Subcutaneous fat was meticulously excised from the lower dermal layer using surgical scalpel and stereomicroscope. The prepared samples were cultured in DMEM (Gibco BRL, France). This media was supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco BRL, France), 200 µg/ml L-glutamine (Gibco BRL, France) and 2% foetal bovine serum (FBS). Samples were cultured at 37 °C for 20–24 h under 96% humidity and 5% CO_2 using incubator (Memmert, Germany). After the pre-incubation, each tissue sample was treated with 100 µl/cm^2 of the T5-derived CFS. The explants were harvested for downstream analysis after short-term (24 and 48 h) or long-term (8 d) exposures. Control wells received an equal volume of sterile phosphate-buffered saline (PBS). The basal culture media were refreshed every 48 h throughout the experiment [14]. Human foreskin tissue explants were achieved as discarded surgical tissue after routine, elective paediatric circumcisions in accordance with the ethical principles of the Declaration of Helsinki. Prior to sample collection, written informed consents were collected from the legal guardians for the scientific use of discarded surgical materials. All tissues were rendered fully anonymous and de-identified at the source. The procurement and handling of discarded biological waste complied with standard institutional safety and bioethics guidelines for anonymized clinical materials.

2.6. *in vitro* Cytotoxicity and Biocompatibility Assessment

Tissue viability and metabolic activity were assessed using 3-(4,5-di methyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric method for assessing cellular metabolic activity [28]. To assess the cytocompatibility of the T5-derived CFS, *ex vivo* skin explants were exposed topically to various concentrations of the T5-derived CFS (25, 50, 75 and 100% v/v) for 24 h. Each explant was topically treated with 100 µl/cm^2 of the

assigned treatment solution. Explants receiving an equivalent volume of sterile PBS (100 µl/cm²) under similar culture conditions served as the PBS vehicle control. After the incubation, the treatment solutions were removed and the skin explants were washed three times with sterile PBS to remove residual treatment solution. The explants were then submerged in culture media containing 0.5 mg/ml of MTT reagent (Sigma-Aldrich, USA) and incubated at 37 °C for 3 h under humidified atmosphere containing 5% CO_2, setting mitochondrial dehydrogenases of viable cells to reduce the tetrazolium salt to insoluble formazan crystals. Then, the tissue pieces were transferred into microcentrifuge tubes containing 1 ml of dimethyl sulfoxide (DMSO) (Sigma, Germany) and incubated under constant agitation at room temperature (RT) for 4 h to extract the intracellular formazan crystals from the tissue matrix. The optical density (OD) of the resulting solutions was assessed using enzyme-linked immunosorbent assay (ELISA) microplate reader (BioTek Instruments, USA) at a primary wavelength of 570 nm, with background turbidity corrected by subtracting the absorbance at a reference wavelength of 630 nm. Relative tissue viability was calculated as a percentage relative to the untreated control group based on the Eq. 1:

$$\text{Tissue Viability (\%)} = \left(\frac{\text{OD}_{570-630\text{ nm}} \text{ of Treated Explant}}{\text{OD}_{570-630\text{ nm}} \text{ of Untreated Control}}\right) \times 100 \quad \text{Eq. 1}$$

2.7. Cytoprotective Efficacy against Sodium Lauryl Sulphate-induced Damages

To assess whether pretreatment with T5-derived CFS affected tissue responses to surfactant-induced stress, an *ex vivo* SLS-challenge assay was carried out. The *ex vivo* skin explants were topically pretreated with T5-derived CFS at 50 or 100% (v/v) for 8 d, with the basal culture media refreshed every 48 h. Control explants were similarly treated and received a similar volume of PBS as the vehicle control. After 8-d pretreatment time, the explants were exposed to SLS (0, 0.1, 0.3, 0.5 and 1% w/v; 99% purity; Sigma-Aldrich, Germany) for 3 h at 37 °C. After SLS exposure, the explants were rinsed with PBS to remove residual chemical traces. The rest tissue metabolic activity and relative cell rescue rates were immediately quantified via the solid-tissue MTT reduction protocol and chemical formazan extraction method as explicitly detailed in Section 2.6. Explants receiving PBS pretreatment and exposed to 0% SLS served as the reference group for normalization of relative tissue metabolic activity.

2.8. Histological Assessment of Epidermal Architecture

After 8-d pretreatment and then 3-h exposure to 0.3% (w/v) SLS (with corresponding control and vehicle-treated groups), foreskin explants were collected for morphological analysis. Samples were fixed in 4% (w/v) neutral buffered formalin (NBF) for 24 h at RT, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. Tissues were sectioned into 4–5 µm slices using microtome (Leica Biosystems, Germany). Sections were mounted on glass slides and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, Germany). After staining, sections were dehydrated, cleared and mounted with Entellan (Merck, Germany) under glass coverslips. Morphological characteristics of the epidermis and dermo-epidermal junction were assessed using light microscope equipped with a digital imaging system (LABOMED, USA) by an observer blinded to the treatment group [29].

2.9. Total RNA Extraction, cDNA Synthesis and Quantitative Real-time PCR

Total RNA was extracted from harvested skin explants using Qiazol lysis reagent (Qiagen, Germany) following the manufacturer's protocol. To eliminate potential genomic DNA contamination, isolated RNA was treated with RNase-free DNase I (Sinaclone, Iran) at 37 °C for 30 min, followed by thermal inactivation with EDTA at 65 °C for 10 min. Purified RNA concentration and purity were assessed spectrophotometrically using Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Similar quantities of purified RNA (1µg) were reverse-transcribed into complementary DNA (cDNA) using RevertAid first strand cDNA synthesis kit (Thermo Scientific, USA) based on the thermal profile of 25 °C for 5 min (priming) and 42 °C for 60 min (reverse transcription), followed by termination at 70 °C for 5 min. Quantitative real-time PCR (qRT-PCR) was carried out using ABI StepOne real-time PCR system (Applied Biosystems, USA) and RealQ Plus 2× master mix green (HIGH ROX; Amplicon, Denmark). Target-specific primers were synthesized by Sinaclone, Iran (Table 1). The $10-\mu$l reaction contained 5 μl of Master Mix, 2 μl of cDNA, 0.5 μl of each primer (10 pmol)

and 2 μl of nuclease-free water. The cycling conditions included initial activation and denaturation at 95 °C for 15 min and then 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A final melting curve analysis (60 to 95 °C) verified amplicon specificity. All reactions were run in technical duplicates in independent biological replicates ($n = 3$). Glyceraldehyde-3-phosphate dehydrogenaseserved as the endogenous reference gene, for normalization and relative fold changes in gene expression were calculated using comparative $2^{-\Delta\Delta C_T}$ method.

Table 1. Primer sequences used for quantitative real-time PCR analysis of target genes

Target Gene	Primer Name	Sequence (5′→3′)
Zonula occludens-1 (ZO-1)	h-ZO-1-F‡	TCGTCCCAAACCCACTTCTC
	h-ZO-1-R*	GAGGTGGCTTCAGTTGAGGT
Filaggrin (*FLG*)	h-Filaggrin-F	CACTCAGCATCCCAATACGGTCA
	h-Filaggrin-R	AGTGTCCAGAGCTATCTACCGAA
Claudin-1 (*CLDN1*)	h-Claudin1-F	CTGAACAAAACCTACACACGTACC
	h-Claudin1-R	AGAGAGGAAGGCACTGAACCAC
Loricrin (LOR)	h-Loricrin-F	GAGGCGAAGGAGTTGGAGGTG
	h-Loricrin-R	CACTGGGGTTGGGAGGTAGTT
GAPDH† (Reference)	h-GAPDH-F	GCAGGGATGATGTTCTGG
	h-GAPDH-R	CTTGGTATCGTGGAAGGAC

‡ F, Forward; *R, Reverse, † Glyceraldehyde-3-phosphate dehydrogenase

2.10. Statistical Analysis

All biological assays (growth kinetics, organic acids /EPS quantification, MTT viability, cytoprotection assay, histological analysis and RT-qPCR gene expression) were carried out using three independent biological replicates ($n = 3$). Data analysis was carried out using GraphPad Prism v.8.0.2 (GraphPad, USA). Results were expressed as mean ±SD (standard deviation). To evaluate differences across multiple experimental cohorts, data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test pairwise multiple comparisons. For two-group comparisons between vehicle-control and T5-derived CFS-treated explants in the barrier gene expression analyses, statistical significance was determined using a two-tailed, unpaired Student's *t*-test. Differences were considered statistically significant at (*P < 0.05, **P < 0.01, ***P < 0.001). The FAA profiling was carried out as an exploratory single-run analysis using one pooled fermented sample (n=1), as described in Section 2.4.3. Accordingly, these data were reported as individual absolute concentrations without calculation of SD, inferential statistical testing or between-replicate comparisons.

3. RESULTS AND DISCUSSION

3.1. Growth Kinetics and Acidification Dynamics of *Lactiplantibacillus plantarum* T5 During Fermentation

As shown in Figure 1, viable cell counts demonstrated a typical bacterial growth curve for *L. plantarum* T5 during fermentation in 8% RSM. The viable cell counts increased from 7.4 ± 0.01 $\log_{10}$ CFU/ml at inoculation to a peak density of 8.4 ± 0.02 $\log_{10}$ CFU/ml at 20 h, indicating attainment of the stationary phase under the conditions used in this study. By 24 h, the count decreased slightly to 7.6 ± 0.04 $\log_{10}$ CFU/ml, suggesting the onset of an early decline phase. The culture media pH decreased from 6.5 ± 0.14 at the start to 4.7 ± 0.03 over the 24-h fermentation time. This acidification occurred concurrently with bacterial growth and was consistent with the expected production of organic acids during lactic fermentation. Although the culture was investigated for 24 h, the T5-derived CFS was collected at 20 h because the culture reached the stationary phase at that time point and the desired fermentation conditions were achieved. Previous studies have reported that the transition from the late-exponential to the stationary phase in lactic acid bacteria is commonly accompanied by the accumulation of organic

acids and other extracellular fermentation-associa-ted components [30,31]. The acidification pattern in the present study was broadly consistent with fermentation profiles reported for *L. plantarum* strains grown in dairy matrices [32]. However, the rate and extent of pH decrease may vary based on strain-specific metabolic activity, milk composition, inoculum size and fermentation conditions [32]. Overall, the present growth and acidification data supported the ability of strain T5 to grow and acidify the RSM media under the current conditions. The resulting T5-derived CFS represented an unfractionated, soluble fermentation-derived preparation. Therefore, the biological responses observed in the *ex vivo* experiments should be interpreted as effects of the complete CFS matrix and should not be attributed to single metabolite or component in isolation.

3.2. Composition of T5-derived CFS

3.2.1. Quantification of organic acids and exopolysaccharides in T5-derived CFS

The HPLC analysis verified lactic acid as the predominant organic acids in the T5-derived CFS, reaching a concentration of 2.44 mg/ml $\pm$ 0.02 , accompanied by acetic acid at 0.97 $\pm$ 0.04 mg/ml (Table 2).

Table 2. Bioactive component concentrations in *Lactiplantibacillus plantarum* T5 fermentation

Bioactive Component	concentration
Lactic acid (mg/mL)	2.44 ± 0.02
Acetic Acid (mg/mL)	0.97 ± 0.04
Exopolysaccharides (mg/L)	2705 ± 0.2

Data are presented as mean ± SD (n = 3).

These concentrations were in the range reported for dairy-adapted *L. plantarum* strains [16], reflecting the active primary metabolic capacity of strain T5 under the RSM fermentation conditions. The resulting organic acids composition, with the final supernatant pH of 4.70 $\pm$ 0.03, established an acidic physicochemical environment. In literature, lactic acid and acetic acid have been reported to affect skin acidification and contribute to antimicrobial regulation [13,33,34]. While skin surface pH under physiological conditions typically ranges 4.1-5.8 [35], the assessed organic acids content in the present preparation provided a descriptive biochemical rationale for its assessment in cutaneous models. In addition to organic acids, fermentation yielded 2705 ± 0.2 mg/l of crude EPS in skim milk matrix. This yield was higher than that documented for several other dairy-fermenting *L. plantarum* isolates such as strains HY7714 and RO30 [23,36]. However, direct quantitative comparisons in studies must be interpreted with caution, as assessed yields are affected by differences in milk composition, fermentation parameters, extraction protocols and analytical quantification methods. Previous literature has documented diverse, strain-specific cutaneous activities for purified *L. plantarum*-derived EPS fractions, including antioxidant, photoprotective and fibroblast-modulatory characteristics [20,23,36,37]. For example, EPS from *L. plantarum* HY7714 has been reported to downregulate matrix metalloproteinase-1 (*MMP*-1) expression and promote procollagen synthesis in ultraviolet (UV)-irradiated dermal fibroblasts [36,38], whereas other *L. plantarum* EPS preparations have been associated to enhanced fibroblast migration and *in vitro* re-epithelialization [15,23]. In the present study, these prior findings offered relevant biological background supporting the role of EPS as one of several functional components within the unfractionated T5-derived CFS matrix.

3.2.2. Exploratory free amino acid profiling of T5-derived CFS

As summarized in Table 3, exploratory FAA profiling of the T5-derived CFS demonstrated a total assessed FAA concentration of 13130 mg/l after 20 h of fermentation, compared to 2,340 mg/l in the unfermented RSM control. Because this preliminary screening was carried out on a pooled sample ($n = 1$), data were present descriptively as absolute concentrations without variance metrics (mean ± SD) or inferential statistical comparisons. Within the quantified individual FAAs in the fermented sample, glycine (1989.55 mg/l), alanine (1074.56 mg/l) and leucine (1067.90 mg/l) were present in the highest concentrations. Simultaneously, decrease in the concentration of free tryptophan was observed after fermentation.

The higher total amino acid concentration observed in the fermented preparation was descriptively consistent with the proteolytic activity typically associated with lactic acid bacteria during growth in dairy substrates [27,39,40]. As specific protease kinetics, degree of casein hydrolysis and biosynthetic amino acid fluxes were not directly quantified in this study, the precise relative contributions

of proteolysis against endogenous microbial metabolism could not be resolved from this single-point exploratory analysis. Furthermore, quantitative comparisons with other fermented matrices should be interpreted cautiously, as FAA yields depend heavily on strain-specific proteolytic machinery, starter inoculum, substrate composition, fermentation duration and derivatization protocols [27,39-41].

Table 3. Free amino acid profiles of reconstituted skim milk before and after 20 h of fermentation with *Lactiplantibacillus plantarum* T5.

Amino Acid (mg/L)		Reference stadrad Control (mg/L)	*L. plantarum* T5 (mg/L)	Net Change / Production (mg/L)
Aspartic acid	(Asp)	19.05	14.00	-5.05
Glutamic acid	(Glu)	51.47	14.79	-36.68
Serine	(Ser)	210.02	239.08	29.06
Glutamine	(Gln)	4.38	3.01	-1.37
Histidine	(His)	0	0	0
Glycine	(Gly)	287.71	1989.55	1701.83
Threonine	(Thr)	171.39	168.51	-2.88
Arginine	(Arg)	136.09	182.11	46.02
Alanine	(Ala)	425.46	1074.56	649.09
Tyrosine	(Tyr)	41.11	36.42	-4.69
Valine	(Val)	274.18	307.29	33.11
Methionine	(Met)	104.68	248.03	143.3
Tryptophan	(Trp)	24.79	20.62	-4.17
Phenylalanine	(Phe)	157.44	225.88	68.43
Isoleucine	(Ile)	157.79	210.05	52.26
Ornithine	(Orn)	0	0	0
Leucine	(Leu)	62.36	1067.9	1005.54
Lysine	(Lys)	87.54	408.61	321.07
Asparagine	(Asn)	78.39	158.7	80.31

In cutaneous biology, FAAs, particularly small hydrophilic species such as glycine and alanine, are recognized constituents of the stratum corneum natural moisturizing factor, which contributes to osmotic balance, water retention and epidermal hydration [42,43]. Branched-chain amino acids, including leucine, have been described in the literature as supportive substrates for cellular protein synthesis and tissue recovery processes. Additionally, the observed decrease in free tryptophan during fermentation might reflect its metabolic conversion into downstream aromatic derivatives. In literature, certain microbial tryptophan catabolites have been suggested as ligands for the aryl hydrocarbon receptor (AhR) pathway, which is involved in epidermal barrier differentiation and cutaneous homeostasis [44,45]. Because specific tryptophan derivatives and downstream AhR activation were not directly assessed in the present study, this pathway is a speculative hypothesis that warrants targeted metabolomic assessment in future investigations.

In summary, this exploratory profiling provides a descriptive baseline of the amino acid constituents present within the T5-derived CFS. With organic acids and EPSs, these FAAs contributed to the complex biochemical milieu of the supernatant. Because this profiling was exploratory (n=1) and individual components were not isolated or assessed independently, the tissue-level and gene-expression responses observed in the *ex vivo* experiments should be interpreted as the combined effects of the whole fermented matrix rather than the activity of single amino acid.

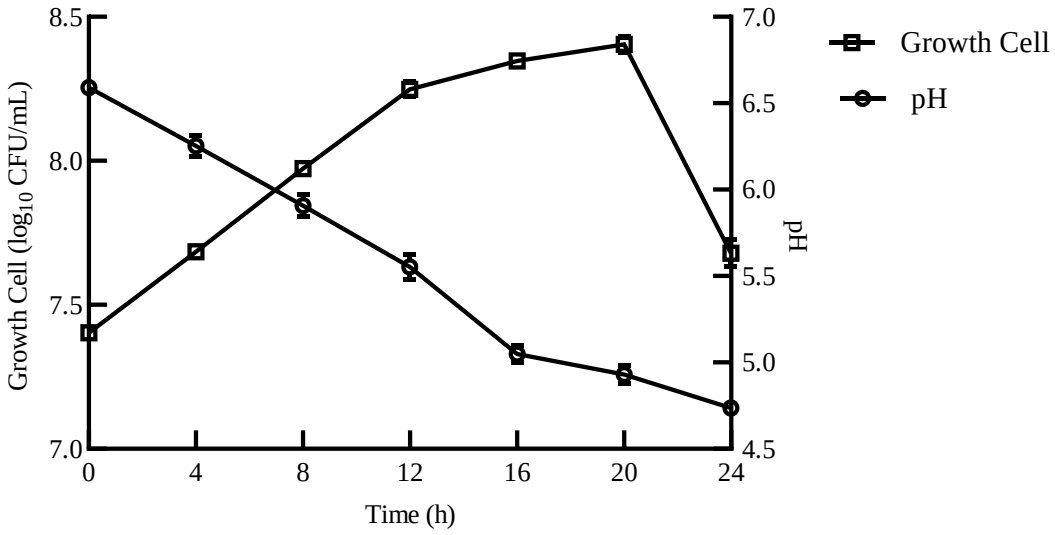

Figure 1. Growth kinetics and pH profiles of *Lactiplantibacillus plantarum* T5 during fermentation in 8% w/v reconstituted skim milk supplemented with 0.15% w/v L-cysteine hydrochloride. Viable cell counts ($\log_{10}$ CFU/ml) and pH assessments were assessed within 24 h at 4-h intervals. Data represent the mean ±SD (standard deviation) from three independent biological replicates ($n = 3$).

3.3. Effects of T5-derived Cell Free Supernatant on *Ex Vivo* Human Skin Tissue

3.3.1. *in vitro* cytotoxicity and biocompatibility

As shown in Figure 2, topical exposure of *ex vivo* human skin explants to the T5-derived CFS at concentrations of 25, 50, 75 and 100% (v/v) for 24 h did not induce significant decrease in tissue metabolic viability, compared to the PBS vehicle control. Quantitatively, relative tissue viability was greater than 100% in all concentrations, indicating that the native preparation was well tolerated by the skin explants under the assessed *ex vivo* conditions. Furthermore, treatment with 100% (v/v) T5-derived CFS resulted in a statistically significant increase in MTT reduction, compared with the PBS control and 25% (v/v) group ($P < 0.05$). For the MTT assay, this increased OD reflected an increase in mitochondrial dehydrogenase activity and cellular reducing capacity within the viable tissue layers rather than direct cellular proliferation or *de novo* tissue growth. This metabolic stimulation might be linked to the availability of readily usable low-molecular-weight fermentation components in the supernatant such as organic acids, EPSs and FAAs, which served as supportive substrates for cellular bio-energetics [46,47]. These findings were similar to those in prior studies reporting high cytocompatibility profiles for cell-free supernatants and non-viable preparations derived from *Lactiplantibacillus* species when used to epithelial and cutaneous models [48,46]. While the traditional use and Generally Recognized as Safe status of dairy-associated *L. plantarum* strains provide supporting history-ical context, the cytocompatibility is specific to the non-neutralized T5-derived CFS matrix and indicates its suitability for the *ex vivo* challenge assays.

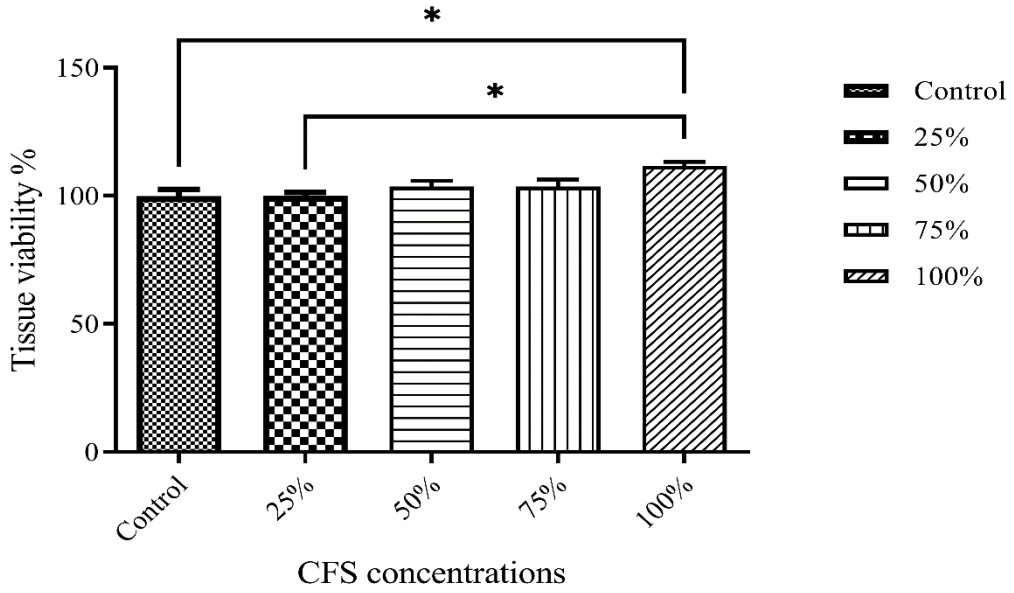

Figure 2. *Ex vivo* human skin tissue metabolic viability (%) after exposure to T5-derived CFS. Explants were treated topically for 24 h with increasing concentrations (25, 50, 75 and 100% v/v) of T5-derived CFS relative to the phosphate buffered saline vehicle control (100%). Tissue metabolic viability was assessed using the MTT assay. Data are expressed as mean ±SD (standard deviation) from three independent biological replicates ($n = 3$). Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$; ns = not significant).

3.3.2. Attenuation of sodium lauryl sulphate-induced tissue damages by t5-derived cell free supernatant pretreatment

The potential of the T5-derived CFS to attenuate surfactant-induced chemical stress was assessed in increasing concentrations of SLS (0, 0.1, 0.3, 0.5 and 1% w/v) after an 8-d topical pretreatment (Figure 3). In the absence of surfactant stress (0% w/v SLS, Figure 3A), pretreatment with 100% (v/v) T5-derived CFS resulted in statistically significant increase in baseline tissue metabolic activity, compared with the PBS vehicle control ($P < 0.05$). After acute exposure to 0.1% (w/v) SLS (Figure 3B), tissue viability in the PBS control group decreased to approximately 80%, whereas pretreatment with either 50 or 100% (v/v) T5-derived CFS significantly preserved tissue metabolic activity ($P < 0.001$). At 0.3% (w/v) SLS (Figure 3C), 50 and 100% (v/v) pretreatment cohorts preserved significantly higher viability levels relative to the PBS-treated challenged control ($P < 0.05$). Under higher surfactant stress (0.5% w/v SLS, Figure 3D), a statistically significant cytoprotective response was sustained exclusively in the 100% (v/v) T5-derived CFS group, compared to the control and the 50% (v/v) group ($P < 0.01$). At the highest challenge concentration of 1% (w/v) SLS (Figure 3E), both the 100% (v/v) preparation and the 50% (v/v) dilution significantly preserved tissue metabolic viability relative to the vehicle control ($P < 0.01$ and $P < 0.05$, respectively). Although the 100% (v/v) cohort demonstrated numerically higher metabolic preservation, the difference between the 50% and 100% treatment groups was not statistically significant ($P > 0.05$).

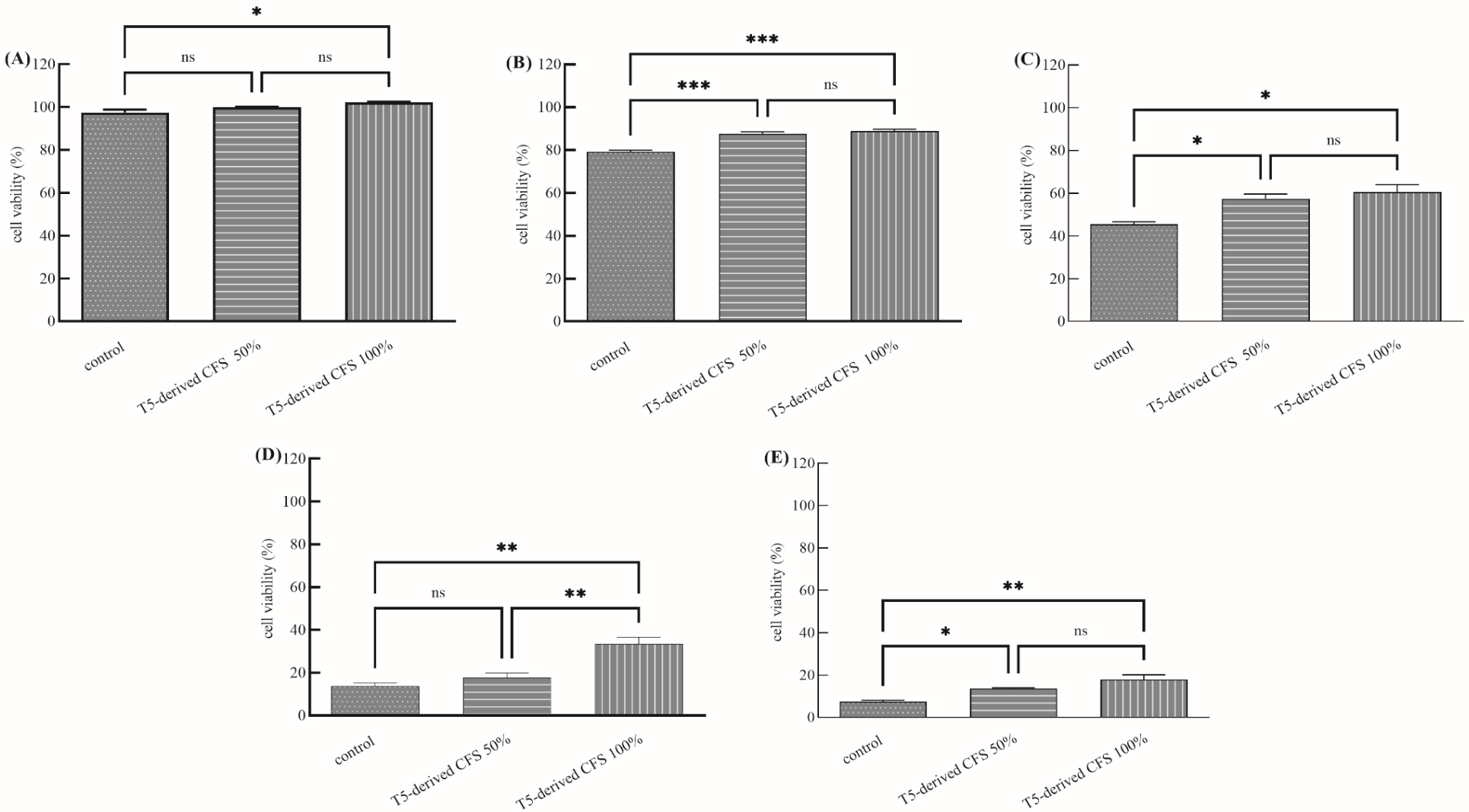

Figure 3. Modulation of *ex vivo* skin tissue viability using T5-derived CFS pretreatment under acute surfactant challenge. Skin explants were pretreated topically with phosphate buffered saline vehicle (control), 50% (v/v) and 100% (v/v) T5-derived CFS for 8 d, followed by acute 3-h exposure to increasing concentrations of SLS. (A) 0%, (B) 0.1%, (C) 0.3%, (D) 0.5% and (E) 1% (w/v). Relative tissue metabolic viability was assessed via MTT reduction. Data represent the mean ±SD (standard deviation) from three independent biological replicates ($n = 3$). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$, $P < 0.01$, $P < 0.001$; ns = not significant).

The assessed SLS concentration range (0.1–1.0% w/v) with a 3-h exposure window represented a well-established *ex vivo* model for inducing graded, acute disruption of the stratum corneum lipid matrix and plasma membrane (PM) integrity [49]. These dose-dependent observations were similar to those by Dinic et al. [13] and Jung et al. [50], which demonstrated that cell-free supernatants from *Lactobacillus* species could attenuate surfactant and stressor-induced epithelial cell damages in *ex vivo* models. Mechanistically, surfactant exposure disrupted inter-cellular lipid lamellae, altered protein conformation and compromised cellular metabolic competence [48]. The tissue preservation observed with T5-derived CFS might involve cooperative physical and metabolic contributions from its constituents. For example, crude EPSs and the viscous matrix might act as a physical buffer that attenuated direct surfactant-membrane partitioning, while fermentation-derived FAAs and organic acids might provide bioenergetic substrates that supported cellular stress-response pathways. Because bio-guided fraction-ation was not carried out in the present study, these mechanistic interactions are hypothetical. The observed *ex vivo* protective responses were therefore attributed to the collective biochemical profile of the intact T5-derived CFS matrix rather than individual isolated constituents [44,51].

3.3.3. Histological assessment of epider-mal architecture

To assess tissue architecture, *ex vivo* human skin explants were investigated using cross-sectional H&E staining after the 8-d topical pretreatment and, where usable, the SLS challenge. The unchallenged explants pretreated with T5-derived CFS for 8 d (Figure 4A) and those pretreated with PBS vehicle (Figure 4C) included preserved cutaneous architecture, including stratified nucleated epidermis and continuous stratum corneum. Minor loosening of the superficial stratum corneum in Figure 4C and localized contour irregularities in Figure 4A were considered compatible with routine sectioning and handling artifacts in explant specimens rather than clear evidence of tissue damage. In contrast, PBS-pretreated explants exposed to 0.3% (w/v) SLS for 3 h (Figure 4D) showed significant disruption of epidermal morphology, including desquamation, loss of stratum corneum continuity and pronounced intercellular edema consistent with spongiosis. Explants pretreated with 100% (v/v) T5-derived CFS before a similar 0.3% (w/v) SLS challenge (Figure 4B) included a more continuous and attached stratum corneum and less pronounced spongiosis than the PBS-pretreated challenged tissues.

These qualitative histological findings were consistent with the tissue metabolic-activity data in Figure 3 and supported an association between T5-derived CFS pretreatment and preservation of epidermal morphology under the assessed SLS challenge conditions. Previous studies have similarly reported that cell-free or fermented preparations derived from *Lactiplantibacillus* species can attenuate structural alterations caused by external chemical or biological stressors [44,51]. In the present study, however, the observed morphological preservation reflected response to the unfractionated T5-derived CFS matrix and could not be attributed to individual metabolite.

3.3.4. Expression profile of skin barrier-associated genes

To investigate transcriptional responses associated with the histological observations, the transcript levels of the selected epidermal differentiation and cell-junction-associated genes were quantified using qRT-PCR in *ex vivo* human skin explants (Figure 5). Relative to the PBS vehicle control, topical treatment with the T5-derived CFS significantly increased the transcript levels of filaggrin (*FLG*; Figure 5A), claudin-1 (*CLDN*1, Figure 5B) and zonula occludens-1 (*ZO*1, Figure 5C). The *FLG* transcript levels increased approximately threefold ($P < 0.05$), whereas *ZO*1 transcripts increased approximately twofold ($P < 0.01$). The *CLDN*1 expression significantly increased after T5-derived CFS treatment ($P < 0.05$). In contrast, loricrin (*LOR*, Figure 5D) showed an upward numerical trend that did not reach statistical significance ($P > 0.05$).

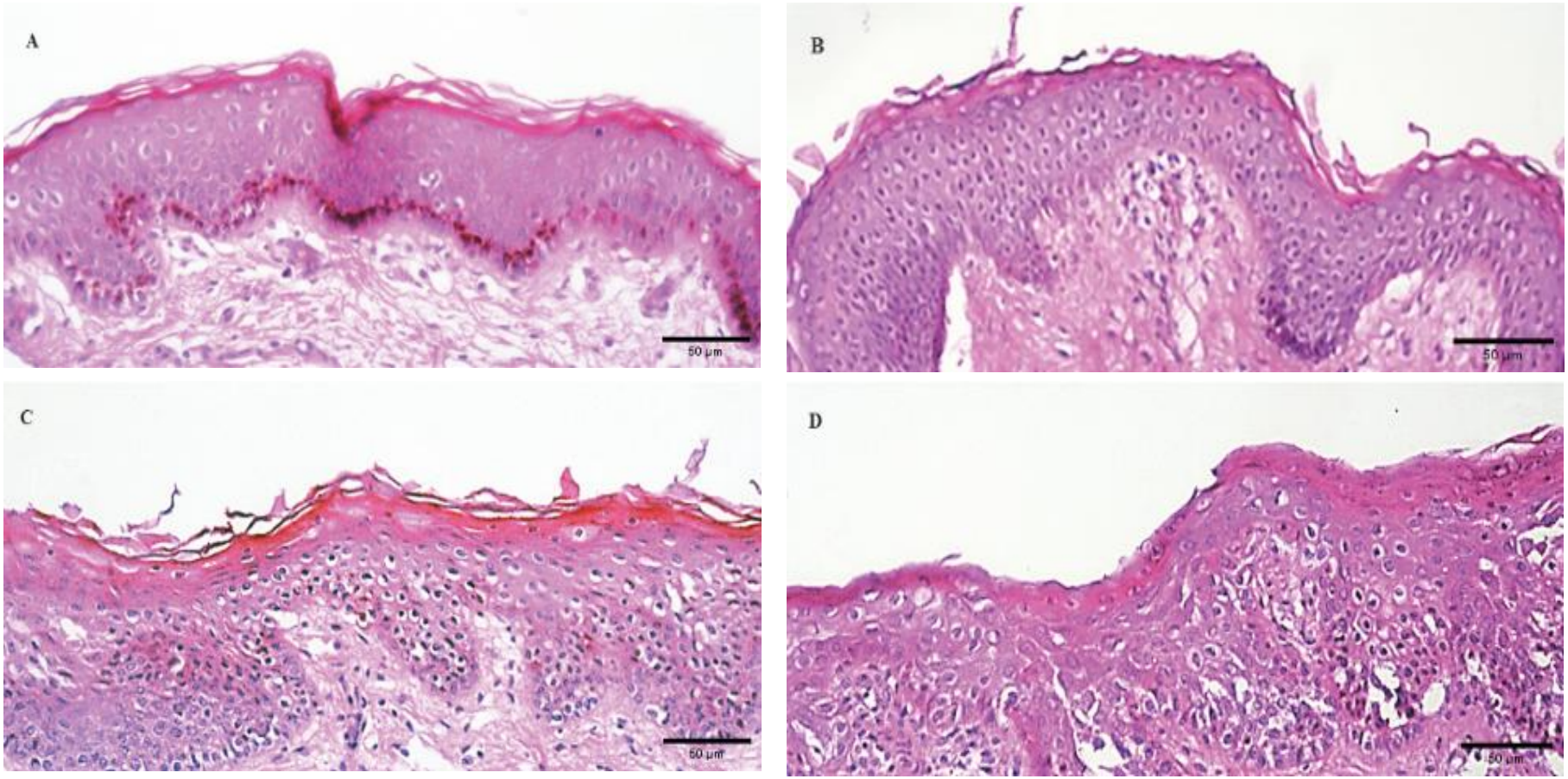

Figure 4. Representative histological morphology of *ex vivo* human skin explants under baseline and surfactant-challenge conditions. Cross-sections were stained with hematoxylin and eosin (scale bars = 50 μm). (A) Baseline explant pretreated with 100%(v/v) T5-derived CFS without surfactant challenge, showing preserved epidermal stratification; (B) explant pretreated with 100% (v/v) T5-derived CFS followed by acute 3-h exposure to 0.3% w/w sodium lauryl sulphate showing attenuation of structural disruption; (C) baseline explant pretreated with phosphate buffered saline vehicle, showing typical cutaneous morphology; and (D) challenged explant pretreated with PBS vehicle followed by 3-h exposure to 0.3% w/w sodium lauryl sulphate, showing pronounced epidermal degradation, loss of stratum corneum continuity and intercellular spongiosis.

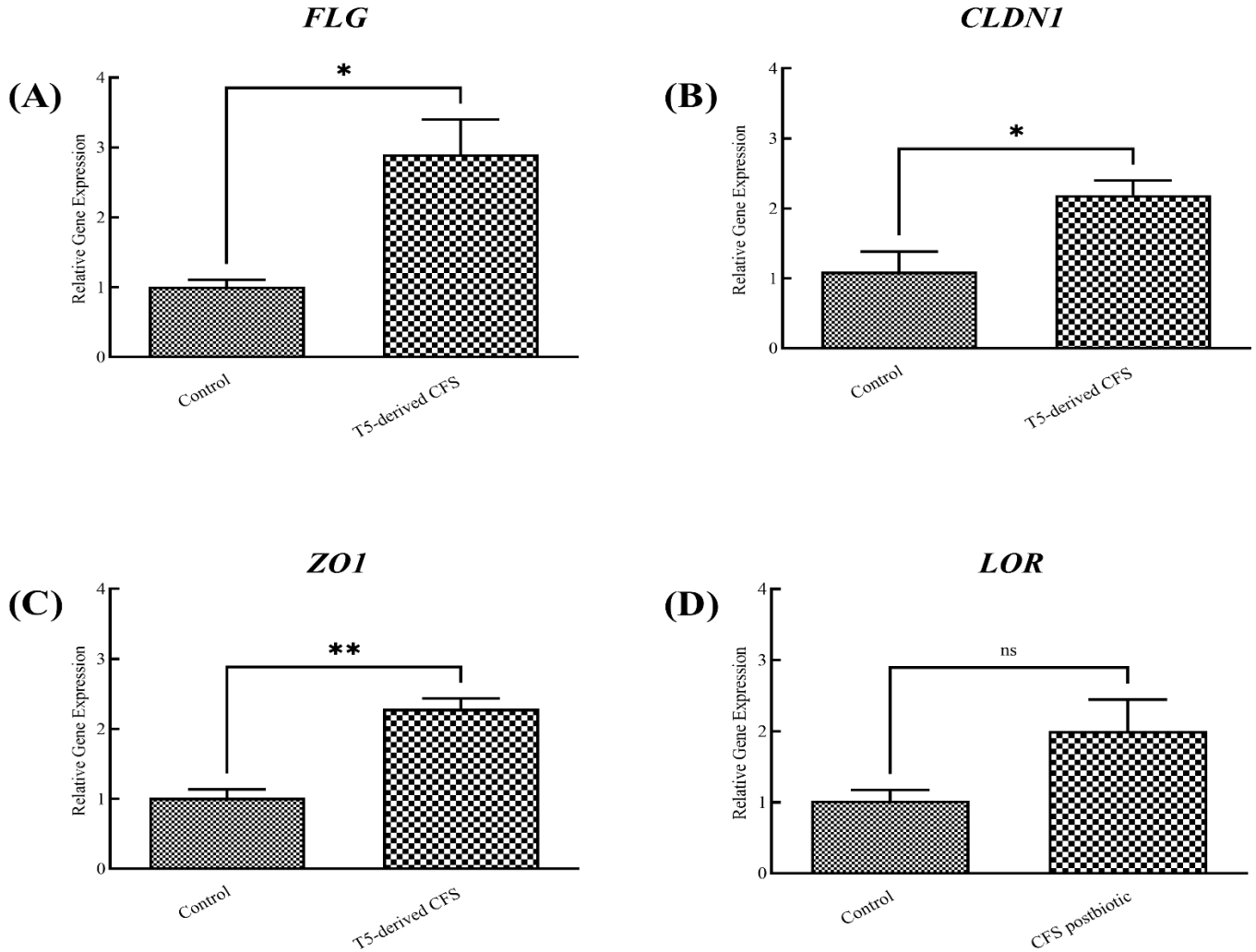

Figure 5. Relative transcript levels of epidermal differentiation- and junction-associated genes in *ex vivo* human skin explants. Explants were treated topically with T5-derived CFS or phosphate buffered saline vehicle control for 48 h. Transcript levels were quantified by qRT-PCR and normalized to *GAPDH* ($n = 3$ independent biological replicates). (A) Filaggrin (*FLG*), (B) claudin-1 (*CLDN*1), (C) zonula occludens-1 (*ZO*1) and (D) Loricrin (*LOR*). Data are present as mean ±SD (standard deviation). Statistical analysis by unpaired t-test (*$P < 0.05$, **$P < 0.01$; ns = not significant).

The increased *FLG* transcript level was similar to the established role of filaggrin in epidermal differentiation and cornified-envelope formation. Similarly, the concomitant increases in *CLDN*1 and *ZO*1 transcripts indicated a transcriptional response involving genes associated with epithelial cell-cell junctions. These interpretations were consistent with those in previous reports of barrier-associated responses to *L. rhamnosus* lysates in reconstructed human epidermis models [50] and to *L. plantarum* JBMI F5-derived extracts in photoaged tissue models [51]. Other studies have associated metabolites or cell-free preparations derived from *Lactiplantibacillus* species with the maintenance of epithelial homeostasis under external stress conditions [34,51-53]. The coordinated changes in *FLG*, *CLDN*1 and *ZO*1 transcripts might be linked to regulatory processes involved in epidermal differentiation and junctional organization. The aryl hydrocarbon receptor (AhR) axis and MAPK-linked signalling have been suggested in the literature as potential mediators of barrier-associated responses to cell-free microbial preparations [37,54,55]. However, these pathways were not directly investigated in the present study and should therefore be regarded as literature-based mechanistic hypotheses rather than mechanisms demonstrated by the current data. Overall, the present results directly demonstrate increased transcript levels of *FLG*, *CLDN*1 and *ZO*1 after exposure to the unfractionated T5-derived CFS matrix. These transcriptional changes should not be interpreted as direct evidence of increased protein abundance or restored barrier function.

3.3.5. Study limitations

Several methodological considerations should be addressed when interpreting these findings. First, the biological assessments were carried out using intact, unfractionated T5-derived CFS matrix in its native acidic state, precluding the identification of specific active components or direct causal links between individual metabolites and the observed tissue responses. Second, the initial amino acid profiling was carried out as an exploratory single-run screening ($n = 1$) on a pooled sample, serving a descriptive purpose without biological variance estimates. Third, the molecular assessment was restricted to mRNA expression of the selected barrier markers (*FLG*, *CLDN*1, *ZO*1 and *LOR*), which provided directional transcriptional insight but did not verify protein abundance, localization or intercellular junction assembly. Fourth, although epidermal architecture was preserved under surfactant challenge, functional barrier performance was not directly verified using biophysical endpoints such as transepidermal water loss or electrical resistance. While the *ex vivo* human skin model preserves native tissue architecture, it lacks microvascular perfusion, systemic immune recruitment and long-term host-microbiome dynamics, limiting the direct extrapolation of these acute *ex vivo* findings to clinical efficacy or commercial formulation stability.

4. CONCLUSION

This study characterized the cell-free supernatant derived from *L. plantarum* T5 cultured in RSM and assessed its cutaneous effects using *ex vivo* human skin explant model. The T5-derived CFS comprised a complex mixture containing lactic acid, acetic acid, EPSs and FAAs. Under baseline *ex vivo* conditions, topical use of the native T5-derived CFS did not compromise tissue metabolic viability. Furthermore, pretreatment with the unfractionated supernatant was associated with preserved tissue metabolic activity and epidermal morphology after acute SLS challenge, alongside upregulated transcript levels of the barrier-associated genes *FLG*, *CLDN*1 and *ZO*1. While these findings suggest the barrier-supportive potential of T5-derived CFS, they reflect the collective activity of the intact matrix rather than isolated metabolites and do not establish functional barrier restoration, protein-level alterations, or specific intracellular signalling mechanisms. Overall, this work highlights the potential of indigenous food-derived bacterial fermentates as functional preparations for topical assessment, providing a foundation for future bio-guided fractionation, functional barrier quantification and *in vivo* investigations.

5. DECLARATION

5.1. Acknowledgements

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Central Branch, for its institutional support and the resources provided throughout the course of this study.

5.2. Declaration of competing interest

The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The lead author, Razieh Rezaee, is the developer of the novel investigational formulation utilized in this study. This formulation was independently designed, optimized, and validated within the scope of her PhD dissertation research. The authors confirm that the development of this formulation for academic investigation does not involve any commercial conflict of interest or external financial influence."

5.3. Authors' Contributions

R.R.: Conceptualization, Methodology, Investigation (Formulation Development & Experimental Execution), Formal Analysis, Writing – Original Draft, Visualization
M.T.E.: Supervision, Methodology, Validation, Formal Analysis, Writing – Review & Editing, Final Approval.
M.N.K.: Methodology, Investigation, Formal Analysis, Writing – Review & Editing.
A.H.: Conceptualization, Formal Analysis, Writing – Review & Editing.

5.4. Using Artificial Intelligent Chatbots

During the preparation of this work, the authors utilized [AI Tool] exclusively for language refinement, grammar correction, and enhancing the overall clarity and readability of the manuscript. The AI tool was not used to generate, interpret, or analyze any scientific data, experimental results, or conclusions presented in this study.

5.5. Ethical Consideration

This study did not involve any human participants or live animal subjects. All experiments were conducted using discarded human foreskin explants obtained from routine elective paediatric circumcisions in accordance with the Declaration of Helsinki, as described in Section 2.5.

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آنالیز ترکیبات زیست‌فعال و اثرات پوستی مایع رویی فاقد سلول *لاکتی‌پلانتی‌با سیلوس پلانتاروم* T5 جدا شده از ترخینه ایرانی

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واژگان کلیدی:

مایع رویی بدون سلول، فیلاگرین، پوست جدا شده انسانی، *لاکتی‌پلانتی باسیلوس پلانتاروم*، موانع پوستی، سدیم لاریل سولفات

چکیده

مقدمه و هدف: میکروبیوم پوست نقش کلیدی در حفظ سلامت پوست و عملکرد سد دفاعی آن ایفا می‌کند و عدم تعادل (دیس‌بیوز) آن زمینه‌ساز بروز اختلالات پوستی گوناگون است .اگرچه پروبیوتیک‌ها به‌طور گسترده مورد پژوهش قرار گرفته‌اند، اما ترکیب زیستی و اثرات پوستی فرآورده‌های فاقد سلولِ ویژه هر سویه، که از غذاهای تخمیری سنتی مشتق شده باشند، کمتر شناخته شده‌اند .این مطالعه با هدف شناسایی و تعیین کمیت اجزای حاصل از تخمیر در مایع رویی فاقد سلول مشتق از باکتری *لاکتی‌پلانتی‌باسیلوس پلانتاروم* (T5-CFS) T5جداسازی‌شده از غذای سنتی ایرانی و ارزیابی اثرات محافظتی آن در یک مدل پوست انسانی به‌صورت برون‌تن (*ex vivo*) انجام شد.

مواد و روش‌ها: باکتری *لاکتی‌پلانتی‌باسیلوس پلانتاروم* T5 در شیر بدون چربی بازسازی‌شده کشت داده شد . مایع رویی فاقد سلول حاصل با استفاده از روش کروماتوگرافی مایع با کارایی بالا (HPLC) جهت تعیین کمیت اسیدهای آلی و اسیدهای آمینه آزاد مورد آنالیز قرار گرفت و میزان اگزوپلی‌ساکاریدها نیز به روش فنل-سولفوریک اسید سنجش شد .ریزنمونه‌های پوست ختنه‌گاه انسانی (*ex vivo*) تیمارشده با غلظت‌های مختلف T5-CFS، از نظر میزان زیست‌پذیری و آسیب‌های بافتی ناشی از سدیم لوریل سولفات با آزمون MTT ارزیابی شدند .مورفولوژی بافت با رنگ‌آمیزی هماتوکسیلین-ائوزین (H&E) بررسی شد و بیان ژن‌های مرتبط با سد دفاعی پوست با استفاده از واکنش زنجیره‌ای پلیمراز کمی و بی‌درنگ (qRT-PCR) سنجیده شد .

یافته ها و نتیجه گیری: مایع رویی فاقد سلول سویه T5 حاوی اسید لاکتیک (۲/۴۴ میلی‌گرم بر میلی‌لیتر)، اسید استیک (۰/۹۷ میلی‌گرم بر میلی‌لیتر)، اگزوپلی‌ساکارید (۲۷۰۵ میلی‌گرم بر لیتر) بود. بررسی میزان آمینواسیدها نشان دهنده افزایش اسیدهای آمینه آزاد کل از ۲۳۴۰ به ۱۳۱۳۰ میلی‌گرم بر لیتر بود. در شرایط برون‌تن(*ex vivo*) ، تیمار با T5-CFS بدون ایجاد سمیت، زیست‌پذیری بافت و مورفولوژی اپیدرم را حفظ کرد،آسیب‌های ناشی از سدیم لوریل سولفات را کاهش داد و بیان رونوشت ژن‌های کلیدی سد دفاعی *ZO1* ،*CLDN1* و *FLG*) را به‌طور معناداری افزایش داد. مایع رویی پلانتاروم حاوی یک ترکیب پیچیده مرتبط با تخمیر بود و با مورفولوژی اپیدرمی حفظ‌شده و افزایش بیان رونوشت‌های مرتبط با سد دفاعی در پوست انسان در شرایط برون تن مرتبط بود. در کل، یافته‌ها نشان داد این فرآورده حاوی مجموعه‌ای متنوع از اجزای مشتق‌شده از تخمیر است که می توانند در پاسخ‌های مشاهده‌شده مرتبط با سد دفاعی نقش داشته باشند. با این حال، رابطه علیت مختص به هر جزء و بازیابی عملکردی سد دفاعی اثبات نشده است. این یافته‌ها پتانسیل T5-CFS را به‌عنوان یک کاندید زیستی برای توسعه فرمولاسیون‌های موضعی تأیید می‌کند.