UNLOCKING THE THERAPEUTIC POTENTIAL: A DEEP DIVE INTO THE BIOLOGICAL ACTIVITIES OF COMMERCIAL LACTOFERRIN

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1. INTRODUCTION

Lactoferrin (Lf) is an 80 kDa iron-binding glycoprotein primarily found in milk and other exocrine secretions of the human body, such as saliva, tears, and gastrointestinal fluids. It exists in two forms: apo-Lf, the iron-unsaturated form, and holo-Lf, the iron-saturated form. Studies have demonstrated Lf's biological activities, including cell proliferation, immune function stimulation, and antimicrobial effects (ASHRAF et al., 2023). Human lactoferrin (hLf) and bovine lactoferrin (bLf) share 70% amino acid sequence homology and exhibit similar biological activities, with bLf being commercially available for therapeutic use (ASHRAF et al., 2023).

Lf has been shown to influence wound healing by promoting collagen gel contraction and stimulating the proliferation of fibroblasts and keratinocytes. Its antimicrobial, antioxidant, and anti-inflammatory properties make it a potential therapeutic agent for wound care and the treatment of skin conditions such as psoriasis and atopic dermatitis. In human and animal studies, topical application of Lf has demonstrated anti-inflammatory effects, reducing cytokine levels and accelerating wound closure (MORENO-EXPÓSITO et al., 2018). Additionally, Lf-loaded hydrogel membranes show antibacterial properties that enhance wound healing (JANARTHANAN et al., 2020).

Despite the similarities between hLf and bLf, differences in glycosylation and receptor-binding regions suggest distinct biological functions. For example, bLf inhibits the growth of a broad range of pathogens, though it remains unclear if hLf offers comparable protection. Variations in immunogenicity have also been observed depending on the source and extraction method of Lf, highlighting the importance of understanding these differences for therapeutic use (ALMOND et al., 2012).

Lf's versatility extends beyond infection control, as it enhances phagocytic activity in the presence of viruses and can bind to various compounds, such as lipopolysaccharides and specific receptors (BELVEDERE et al., 2021). This gives Lf potential as a therapeutic alternative; it can be used alone or in combination with other bioactives in various applications, including regenerative medicine, where it plays a role in both bone and epithelial tissue regeneration (ICRIVERZI et al., 2020). Lf is also utilized in nutraceutical formulas, dietary supplements or functional foods designed to provide health benefits beyond basic nutrition (LI et al., 2022). Considering these capabilities, this study aims to investigate the bioactivities of commercially available Lf derived from cow's milk, with the goal of enhancing our understanding of the functionality and applications of different forms of Lf.

2. METHODOLOGY

2.1 Materials. The lactoferrin (Lf) used in the experiments was commercially sourced from Lactoferrin Company (Byron Bay, AUS), extracted from cow's milk, spray-dried, and had a purity level of over 95%, with a pH range of 5.2 to 7.2.

2.2 Chemical analysis. Attenuated total reflectance - Fourier transform infrared spectroscopy (ATR-FTIR) was performed on the bLf powder to assess its composition. The FTIR spectra were baseline-corrected and normalized for analysis.

2.3 Cell-based assays

2.3.1 Cell culture. Biological assays were performed using the human skin keratinocyte lineage (HaCaT; Cell Lines Service, 300493). The cells were cultured in complete DMEM medium (Dulbecco's Modified Eagle's Medium; Gibco, Carlsbad, CA, USA) containing 1% antibiotic solution (Penicillin-Streptomycin 10,000 U/mL; Gibco) and 10% fetal bovine serum (FBS; Gibco) in a humidified incubator (37°C, 5% CO2; Thermo Fisher Scientific). Cells were treated with various concentrations of bLf (5, 10, and 50 µg/mL).

2.3.2 Cell viability. Cell viability was assessed using alamarBlue™ over 1, 3, and 7 days. Fluorescence was measured at 560 nm and 590 nm using a microplate reader.

2.3.3 Cell proliferation. CyQUANT™ assay was performed to quantify cell proliferation by evaluating total DNA content. Cells were exposed to either complete or FBS-free media with/without 5 µg/mL bLf, and fluorescence intensity was measured after 1, 3, and 7 days.

2.3.4 Morphological analysis. Morphological changes were assessed by culturing cells in 24-well plates and treating them according to the experimental groups for 12, 24, and 48 hours. Cells were fixed and stained with ActinRed for actin filaments and DAPI for nuclei. Images were captured via fluorescence microscopy.

2.3.5 Wound healing assay. A scratch assay was performed on confluent cell monolayers treated with 5 µg/mL bLf. The rate of scratch closure was monitored over 48 hours using automated imaging, and migration rates were quantified via ImageJ software.

2.4 Antimicrobial Activity. The antimicrobial activity of bLf was evaluated by determining the minimum inhibitory concentration (MIC) using the microdilution assay technique against *S. aureus* and *P. aeruginosa*. Bacterial growth inhibition was assessed by measuring turbidity at 625 nm using a spectrophotometer.

3. RESULTS AND DISCUSSION

FTIR analysis of commercially available bLf revealed an O–H bond peak at 3278 cm^{-1} (indicating water presence), amide I and II peaks at 1637 cm^{-1} and 1529 cm^{-1} (related to peptide bonds), and a probable amide III peak at 1451 cm^{-1}, with potential interference from the sample's origin and extraction method.

The cytotoxicity of bLf was evaluated in HaCaT cells, and it was found that concentrations of 5 and 10 µg/mL were non-toxic and even promoted cell proliferation, especially at 10 µg/mL. At 50 µg/mL, however, a decrease in cell viability was observed. These findings are consistent with the study published by UCHIDA et al. (2017), which demonstrated bLf's ability to promote proliferation and differentiation, although at high concentrations it can reduce cell viability. This reduction contrasts with studies on human lactoferrin (hLf), which reported no cytotoxic effects at higher concentrations. (ABDALLA et al., 2020; BELVEDERE et al., 2021). The difference may be attributed to variations in Lf origin, its method of

extraction, or its combination with other molecules, such as silver nanoparticles, which were not used in the current study (FATHIL et al., 2023).

The CyQUANT™ assay demonstrated an increase in cell proliferation in all groups from days 1 to 3, except for the 5 µg/mL (-) group, where a reduction was observed by day 7, likely due to the cells reaching confluence. This suggests that Lf, particularly at lower concentrations and combined with serum, supports cell proliferation. The results align with established data that Lf does not induce apoptosis but can enhance proliferation under certain conditions. However, the influence of fetal bovine serum (FBS) on these outcomes, along with the concentration of Lf, must be considered for accurate interpretation of its proliferative capacity (TANG et al., 2010).

In the scratch assay, the group treated with 5 µg/mL of bLf showed the fastest wound closure within the first 24 hours, while higher concentrations (50 µg/mL) exhibited delayed closure, aligning with the cytotoxicity data. By 48 hours, all groups achieved near-complete closure except for the highest concentration group (Fig. 1).

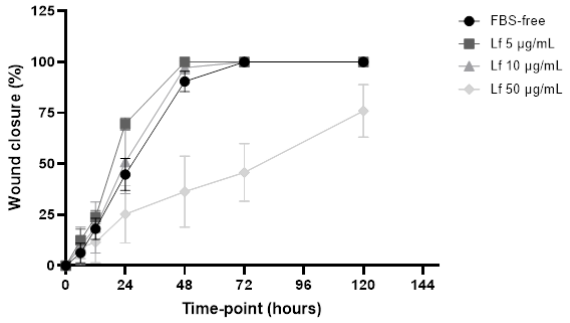

Figure 1. Graphic representation of mean and standard deviation of wound closure (%) of HaCaT cells after treatment with Lf at different concentrations: 5 µg/mL, 10 µg/mL, and 50 µg/mL.

These results are consistent with ABDALLA et al. (2021), which demonstrated that bLf can stimulate keratinocyte migration and wound closure. However, the association with other molecules such as silver nanoparticles can influence the effectiveness of bLf in migration assays, as shown by ABDALLA et al. (2021).

The bLf did not demonstrate antimicrobial activity against *S. aureus* or *P. aeruginosa* in this study, even at higher concentrations. This contrasts with other reports of Lf's antimicrobial efficacy, particularly when conjugated with other compounds like glycosaminoglycans or nanoparticles (BELVEDERE et al., 2021). The lack of activity in this study may be due to the use of isolated bLf without additional antimicrobial agents. This highlights the importance of Lf's combination with other molecules in enhancing its antimicrobial effects, which should be considered when interpreting the results.

4. CONCLUSION

In conclusion, the discrepancies observed between this study's results and existing literature emphasize the impact of lactoferrin's purity on its bioactivity. The varying glycoprotein profiles and their unclear mechanisms necessitate further research, particularly regarding extraction methods, to better understand and optimize lactoferrin's applications

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