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(Research Paper)

Evaluation of Functionality of Bacterial Lipopeptides in Homogenized Milk and Properties of Milk Powder Fortified with the Lipopeptide

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Abstract
Fortified foods are developed for public health and as carriers of functional bioactive compounds. This study investigated the activity of anticancer lipopeptides (LP) in homogenized milk and evaluated the properties of LP-fortified milk powder. Our results showed that the LP activity decreased with increasing milk fat content. The addition of LP to homogenized milk resulted in only minor or non-significant changes in the protein content, moisture content, bulk density, tapped density, Carr index (CI), Hausner ratio (HR) of milk powders, as well as the pH and particle size of the rehydrated milk powders. However, a significant color difference and reduced solubility were observed in the fortified samples compared to the non-fortified controls. Despite the reduced LP activity in milk, particularly in fat-containing milk, measurable bioactivity was retained. Importantly, since the only negative effect of LP was on solubility, it can be added to milk powder without a major loss of quality. Although the activity of bacterial LP has been studied in non-homogenized milk, this is the first report evaluating LP activity in homogenized milk.

Key words: *Bacillus*, Dairy, Solubility, Bioactivity, Functional food.

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Introduction

Dairy products are rich in proteins, fatty acids, calcium, potassium, and B vitamins and are widely consumed. Therefore, they are often suggested as matrices for food enrichment and fortification (1). Recently, dairy-based functional foods have attracted the attention of international organizations that are working to increase public awareness of their use (2). One strategy for producing functional foods is fortification, which involves adding beneficial components. The fortification process should avoid causing major changes to the texture and organoleptic properties of the base food product. Ideally, any resulting changes would serve to improve these properties (1). Generally, any changes must remain within the range of consumer acceptability. Dairy products have a relatively stable matrix and can effectively release added components in the body; therefore, they are suitable vehicles for fortifying. However, the properties and dosage of the added material, as well as the effect of processing on the texture of the final product and the stability of its nutritional value, are important considerations in this regard.

Recently, researchers have focused on dairy product fortification. For example, skim milk powder (SMP) fortification with soya extract has been investigated. The results showed that the levels of minerals and polyphenolic compounds, as well as the antioxidant activity of the final product, increased (3). In another study, ice cream was prepared using sheep milk and fortified with probiotic bacteria, whey protein, oil extracted from *Schizochytrium* spp. (as a source of omega-3), inulin (as a prebiotic), and locust bean extract (as a stabilizer). The results demonstrated that the percentages of protein, fiber, and omega-3 increased in the final product (4).

In 1989, the term "nutraceutical" was introduced. Functional foods are beneficial for health and are considered a form of co-therapy (5). When patients consume these foods, they do not need to change their dietary habits, making them suitable for patients or children who dislike taking medications (6). Nutraceutical foods are useful for preventing diseases. Previous studies have shown that surfactin lipopeptides extracted from *Bacillus* sp. exhibit anticancer activity and are candidates for cancer therapy. Ghafouri et al. (7) reported that skim milk powder (SMP) prepared from non-homogenized milk could be a suitable matrix for fortification with lipopeptides. They showed that although the activity of the lipopeptides decreased upon addition to milk powder, their stability increased over time. They also reported that in milk containing non-homogenized fat, the decrease in lipopeptide activity was greater than that in SMP. Given that milk powders containing fat have a higher nutritional value, they may be more suitable for patients. In the present study, the activity of lipopeptides was investigated in homogenized milk powders containing fat.

In this study, a mixture of three lipopeptides, surfactin, fengycin, and kurstakin, was used to fortify milk powder. Surfactin, produced by various strains of *Bacillus subtilis*, is a potent biosurfactant belonging to the cyclic lipopeptide family and is synthesized via nonribosomal peptide synthetases (NRPS) (8). Its structure comprises a cyclic peptide ring consisting of seven amino acids linked to a β-hydroxy fatty acid chain containing 13–16 carbon atoms, forming a cyclic lactone ring (9). Beyond its biosurfactant properties, surfactin exhibits a broad spectrum of biological activities, including anticancer, antimicrobial, antiviral, antimycoplasma, and antiprotozoal effects (10). Fengycin, another major lipopeptide produced by *Bacillus* species, is also a non-ribosomal peptide consisting of a β-hydroxy fatty acid chain linked to ten amino acid residues (11). It has been reported to possess anticancer properties and antifungal activity against filamentous fungi (12). Kurstakin, which has been isolated from various bacteria, including *Bacillus* species outside the *B. cereus* group as well as *Enterobacter cloacae* and *Citrobacter* species, shares a conserved peptide core among its analogs but varies in its lipid tail composition (13). In this study, the lipopeptide mixture was added to homogenized milk, and its biosurfactant and antibacterial activities were evaluated. Additionally, the physicochemical properties of the resulting fortified skim milk powder were investigated.

Materials and methods

Bacterial culture and LP extraction

In the present study, a bacterium capable of producing anticancer lipopeptides (*Bacillus mojavensis* HF) was used (7). Previous studies have shown that this strain can produce surfactin, fengycin, and kurstakin lipopeptides (14), and a mixture of these lipopeptides exhibits anticancer activity (7).

The bacterium was cultured in a medium containing yeast extract (1.5%), glycerol (1.5%), peptone (0.5%), K□HPO□ (0.1%), and MgSO□ (0.05%), adjusted to pH 6.8. At the stationary phase of growth, the culture was centrifuged, and the supernatant was collected. The pH of the supernatant was adjusted to 2.5 using HCl, followed by

refrigeration for 1 hour (14). LP was extracted from the precipitate using a chloroform–methanol mixture (2:1, v/v). The obtained LP extract was stored at –80 °C for further analysis.

Fortification of homogenized milk with different fat contents with LP

Three types of homogenized milk samples, including skim milk (SM, containing ≤ 0.5% fat), low-fat milk (LM, containing 1.2% fat), and whole-fat milk (WM, containing 3% fat), were purchased from Pegah Company (Iran). The composition of these milk samples is presented in Table 1.

Table 1. Components of homogenized milk samples used in the present study

Milk sample	Solid phase (%)	Fat (%)	Protein (%)	Lactose (%)
SMP	9	≤0.5%	3.4	4.8
LMP	10.2	1.2	3.2	4.5
WMP	12	3	3.2	4.5

To prepare LP-fortified milk, LP was dissolved in the milk samples to a final concentration of 0.13%. This concentration was used for all analyses, except for the antibacterial test. Previous studies have shown that this LP concentration is sufficient to detect biosurfactant activity in milk, as confirmed by surface tension analysis (7). The samples were kept at room temperature with gentle manual stirring. Control samples without LP addition were prepared simultaneously using the same procedure.

Investigation of LP functionality in milk with different fat content

To investigate the activity of LP in milk samples with different fat contents, two analyses were performed: antibacterial activity and biosurfactant activity tests. These analyses are described in the following sections.

Antibacterial activity test

The antibacterial activity of milk samples with different fat contents was investigated against *S. aureus* (ATCC 6538). For this purpose, LP was added to the milk samples under sterile conditions at a final concentration of 4% (within the detectable range) and kept at room temperature for 30 min. During this time, the samples were gently stirred manually. Then, 100 µL of each sample was pipetted onto a blank disk. The disks were placed on Mueller-Hinton agar plates that had been swabbed with *S. aureus* at 0.5 McFarland concentration. The samples were incubated at 37 °C for 24 h, and the inhibition zones around the disks were measured. The control sample was prepared by adding LP to deionized water (dH□O), following the same procedure used for the other samples.

Biosurfactant activity of milk samples fortified with LP

Since biosurfactants can decrease the surface tension of liquids, the activity of LP in milk samples was investigated by measuring their surface tension and comparing it with milk without LP. For this purpose, a tensiometer was used, and the analyses were performed at 25 °C (15).

Milk Powder Preparation from Homogenized Milk Sample with Different Fat Contents, Fortified with LP

Milk samples were dried using a spray dryer equipped with a vertical cylindrical chamber (500 mm in length and 150 mm in diameter). The feed solution (pump) rate was set at 30%. The inlet/outlet temperatures were 150 °C/30 °C and the spray gas flow rate was 90% (33.75 m³/h). The resulting milk powders were collected and allowed to cool to ambient temperature. All samples were stored at 4 °C and protected from light (16, 17).

Investigation of physicochemical properties of milk powders (protein, moisture and pH)

The protein content of the samples was measured using the Kjeldahl method (18). To determine the moisture content, the samples were heated at 103 °C until a constant weight was achieved. The moisture percentage was calculated using the following formula:

$$\text{Moisture}(\%) = \frac{W_1 - W_2}{W_1} \times 100$$

Where:

- W□ = initial sample weight (before heating)
- W□ = sample weight after heating

The pH of the reconstituted milk samples was measured using a pH meter.

Colorimetric properties of milk powders

Color parameters were analyzed using a HunterLab colorimeter. The color was determined based on L*a*b* parameters, where L* represents lightness, a* indicates the green–red axis, and b* indicates the blue–yellow axis

Bulk/ tapped density, flowability and cohesiveness measurements

To determine the bulk density, 1 g of milk powder was placed into a 10 mL graduated cylinder, and the initial volume was recorded. Tapped density was measured using the same procedure; however, after adding the powder, the cylinder was tapped for 10 min, and the final volume was recorded. The densities were calculated using the following formula:

$$\text{Density} = \frac{W}{V}$$

Where:

- W = sample weight (g)
- V = sample volume (mL)

Note: This formula was applied to calculate both bulk density (using the initial volume) and tapped density (using the final volume after tapping).

Flowability and cohesiveness were evaluated based on the CI and HR, respectively, which were calculated using the following equation (16, 19, 20):

$$CI = \frac{\rho_T - \rho_B}{\rho_T} \times 100$$

$$HR = \frac{\rho_T}{\rho_B}$$

Where ρ_T is the tapped density, ρ_B is the bulk density, CI is the Carr Index, and HR is the Hausner ratio.

Solubility of milk powders

Milk powders were dissolved in water, and the amounts were adjusted so that all samples had the same solid content. To this end, the moisture content of each powder was measured, and the appropriate amount of powder was added to ensure uniform solids content in all samples. The samples were gently stirred for 5 minutes and then incubated at room temperature for 15 min. After incubation, the samples were gently mixed and centrifuged at 5070 × g for 5 min. The supernatant was discarded, and the pellets were washed with distilled water, followed by centrifugation under the same conditions. The pellets were then dried at 70 °C until a constant weight was reached.

The percentage of insoluble material of milk powders was calculated using the following formula:

$$\text{Insolubility of milk powders} = \left(\frac{W_s}{W}\right) \times 100$$

Where:

- Ws: weight of the precipitate
- W: initial sample weight

Particle size distribution analysis (PSD)

The particle size distribution of the reconstituted MPs was determined by laser diffraction using a Malvern Mastersizer FZ-100 instrument (Horiba, Japan) (21).

Results

Investigation of antibacterial activity of LP-fortified milk

Since surfactin, which is present in LP, has been reported to possess antibacterial activity against *S. aureus* (22), we evaluated the antibacterial activity of LP against this bacterium. The results of the antibacterial activity assay for fortified milk (Fig 1 and Table 2) indicated that the activity of LP decreased in all milk samples compared to LP dissolved in dH_2O (control). Furthermore, the antibacterial activity decreased as the fat content of the milk increased.

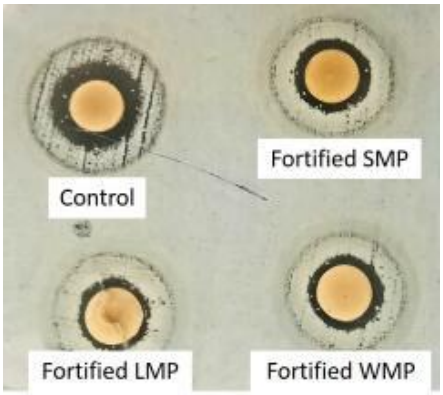

Fig 1. Antibacterial activity of LP-fortified milk samples with varying fat contents. The control represents LP dissolved in dH_2O.

Table 2. Antibacterial activity of LP-fortified milk samples against *S. aureus*. Samples represent homogenized milk with 0% (SMP), 1.2% (LMP), and 3% (WMP) fat contents.

Sample	Inhibition zone (mm)	Antibacterial activity
dH_2O, SMP, LMP and WMP	0.0 ± 0.0^{e}	-
dH_2O+ LP	0.76 ± 19.16^{a}	Very strong
SMP+ LP	0.57 ± 17.33^{b}	strong
LMP+LP	0.57 ± 15.66^{c}	strong
WMP+P	0.57 ± 14.66^{d}	strong

Results are the mean ± standard deviation of 3 replicate. Values with different letters indicate significant differences at a 95% confidence level. Very strong antibacterial activity: > 19 mm, strong: 10-19 mm, moderate: 5-9 mm, and weak: < 5 mm (inhibition zone diameter) (23).

Investigation of the biosurfactant activity of LP in fresh milk and milk powders

Lipopeptides are amphiphilic molecules capable of interacting with both polar and non-polar molecules in a sample. This property leads to a decrease in the free energy at the interface between these phases, thereby reducing the surface tension of the sample (24, 25). Previous studies have demonstrated that effective biosurfactants can reduce the surface tension of water from approximately 72 mN/m to below 35 mN/m (26). In the present study, the reduction in the surface tension of dH_2O by LP fell within this range, indicating that LP possesses significant biosurfactant activity.

The results of the surface tension analysis for milk and milk powder samples fortified with LP (Table 3) indicated that this parameter decreased in all samples upon the addition of LP. This finding demonstrates that the biosurfactant activity of LP was retained within the milk matrix. LP exhibited its strongest biosurfactant activity in the skimmed milk (SM) and skimmed milk powder (SMP) samples.

Table 3. Surface tension of milk and milk powder with varying fat contents, with and without LP fortification

Sample		Surface tension (mN/m)	
		Samples without LP	**Samples with LP**
dH_2O		0.14±62.20 ^{aA}	0.07±28.30^{hE}
milk	SM	0.08±43.02 ^{b}	0.08±29.42^{g}
	LM	0.07±40.80 ^{c}	0.14±34.3^{e}
	WM	0.05±35.76 ^{d}	0.08±32.74^{f}
Milk powder	SMP	0.05±43.66^{B}	39.730.08± ^{BC}
	LMP	0.10±34.60^{C}	38.600.07± ^{BC}
	WMP	0.10±35.46 ^{C}	34.760.07± ^{C}

Results are the mean ± standard deviation of 3 replicates. Values with different letters indicate significant differences at a 95% confidence level. Lowercase and uppercase letters were used for within-group comparisons of milk and milk powder samples, respectively.

Investigation of physicochemical properties of LP-fortified milk powders (protein, moisture and pH)

As shown in Table 4, the protein content of LP-fortified SMP and WMP was significantly higher compared to unfortified samples. This increase could be attributed to the addition of the peptide moiety of the lipopeptides (LP) to the milk powders. However, the difference was small.

The moisture content of SMP and LMP did not significantly differ from that of their respective unfortified controls (unfortified samples). However, a slight increase in moisture content was observed in WMP.

Although the mean pH of the samples ranged within a narrow range (6.60–6.66), this parameter was significantly lower in the reconstituted milk samples containing LP.

Table 4. Protein, moisture, and pH of rehydrated LP-fortified milk powders with varying fat content

Sample	Protein (%)	Moisture (%)	pH
SMP	0.01±31.59 ^{b}	0.19±5.21 ^{a}	0.005±6.62 ^{b}
SMP+LP	0.3±32.017 ^{a}	0.36±5.42^{a}	0.01±6.56 ^{c}
LMP	0.05±26.93 ^{c}	0.30±5.05 ^{a}	0.01±6.65 ^{a}
LMP+LP	0.93±27.45 ^{c}	0.10±5.15 ^{a}	0.01±6.61 ^{b}
WMP	0.04±24.45^{e}	0.02±2.17^{c}	0.02±6.66 ^{a}
WMP+LP	0.39±24.97^{d}	0.003±3.38^{b}	0.01±6.60 ^{b}

Results are the mean ± standard deviation of 3 replicates. Numbers with different letters indicate significant differences at a 95% confidence level.

Color properties of milk powders

As shown in Table 5, upon the addition of LP to all milk powder types, the L* parameter (lightness) decreased, while the a* parameter (redness) increased. The b* parameter (yellowness) also increased in SMP and WMP samples. Collectively, these changes indicate that the addition of LP resulted in milk powders that were darker, redder, and more yellow. This color alteration is attributed to the inherent color of the LP, which influenced the overall color profile of the milk powders. Previous studies have shown that a ΔE value greater than 2 indicates a significant color difference between samples (27). In this study, the addition of LP to all milk samples resulted in a significant color difference.

Bulk and tapped density, flowability, and cohesiveness of MPs with different fat contents, fortified with LP

The results for bulk density, tapped density, CI, and HR are presented in Table 6 (CI reflects flowability, whereas HR indicates powder cohesiveness). The results showed that these parameters remained largely unchanged in the LP-fortified MPs compared to the control samples.

Table 5. Color properties of MPs with varying fat content, with and without LP fortification

Sample	L*	a*	b*	ΔE
SMP	0.78±97.44 [a]	-0.30±0.19 [c]	0.30±0.21 [d]	
SMP+LP	0.17±94.91 [b]	0.13±1.50 [a]	0.32±3.08 [c]	0.39±4.22
LMP	0.36±93.61 [c]	-0.25±1.35 [d]	0.83±11.06 [a]	
LMP+LP	0.93±87.03 [d]	-0.14±0.37 [c]	0.32±10.68 [a]	0.92±6.72
WMP	0.29±97.49 [a]	0.22±0.56 [b]	0.19±3.24 [c]	
WMP+LP	0.070±94.64 [b]	0.31±1.32 [a]	0.18±4.58 [b]	0.43±3.24

Results are the mean ± standard deviation of 3 replicates. Numbers with different letters indicate significant differences at a 95% confidence level. ΔE results show the color difference between each sample and its control.

Table 6. Bulk/tapped density, CI, and HR of MPs with different fat contents, fortified with LP.

Sample	**Bulk density (g/mL)**	**Tapped density (g/mL)**	**CI (%)**	**HR**
SMP	0.00±0.26 [bc]	0.00±0.40 [b]	0.71±35.68 [a]	0.017±1.55 [a]
SMP+LP	0.01±0.27 [abc]	0.01±0.40 [b]	6.81±31.72 [a]	0.15±1.47 [a]
LMP	0.01±0.29 [a]	0.01±0.46 [a]	4.30±36.01 [a]	0.10±1.56 [a]
LMP+LP	0.00±0.28 [ab]	0.01±0.46 [a]	1.870±39.59 [a]	0.05±1.65 [a]
WMP	0.00±0.26 [bc]	0.01±0.39 [b]	3.55±33.93 [a]	0.08±1.51 [a]
WMP+LP	0.01±0.25 [c]	0.02±0.41 [b]	6.75±37.73 [a]	0.18±1.62 [a]

Results are the mean ± standard deviation of 3 replicates. Numbers with different letters indicate significant differences at a 95% confidence level.

Insolubility of milk powders

As shown in Fig 2, insolubility significantly increased in LP-fortified milk powders. This indicates that the addition of LP decreased the solubility of the milk samples.

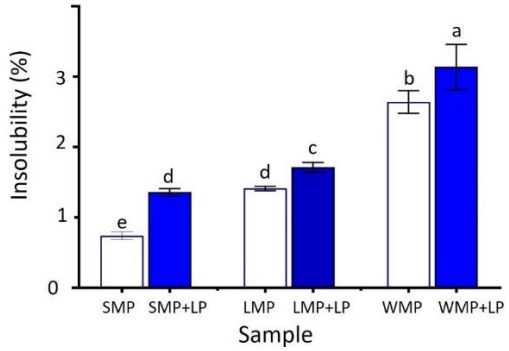

Fig 2. Insolubility of LP-fortified MPs compared to their control samples (MPs without LP). All data are reported as the mean ± standard deviation (n=3).

Particle size analysis

As illustrated in Fig 3a, particle size analysis of solubilized milk powders showed no significant difference between SMP and SMP+LP. In the LMP samples, fortification with LP resulted in an increase in the average particle size. However, the PSD span for LMP+LP was greater than 1 (Fig 3b), indicating a broad particle size distribution (a mixture of fines and coarse particles). A PSD span greater than 1 reflects greater heterogeneity among particles, which likely contributed to the lack of a statistically significant difference in mean particle size. Conversely, WMP+LP samples displayed a significantly smaller particle size than the WMP controls.

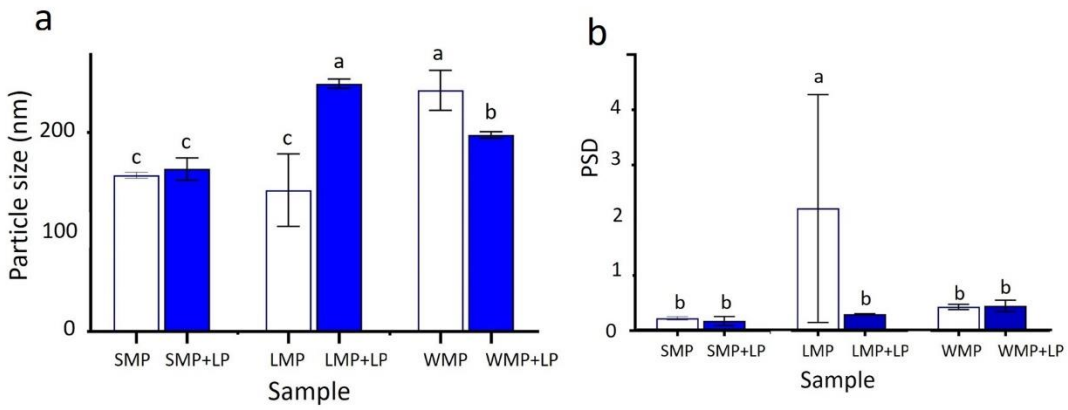

Fig 3: Particle size and PSD of MPs prepared from milk with varying fat percentages, with and without LP fortification. All data are reported as mean ± standard deviation (n=3).

Discussion

In the present study, the activity of anticancer LP derived from *Bacillus mojavensis* HF was investigated in homogenized milk with varying fat contents. Previous studies have shown that the activity of the LP decreased in fat-containing non-homogenized milk, which the authors attributed to interactions with fat (7). Given the smaller fat particle size and greater dispersion in homogenized milk relative to non-homogenized milk (28), the present study evaluated LP activity in homogenized milk. The results showed that in homogenized milk, the activity (antibacterial and surface tension) of LP also decreased as the amount of fat increased. Herein, it is hypothesized that the lipid moiety of LP may interact with milk fat, leading to a reduction in its activity (7). This result suggests that although fat globules in homogenized milk are involved in a uniform emulsion (29), they may still interact with LP. This may be because LP has biosurfactant activity, and biosurfactants are known to partition at interfaces. Therefore, the LP molecules likely adsorb onto the surface of the fat globules (which are now coated with casein proteins), reducing their availability in the aqueous phase and consequently diminishing their antibacterial activity and surface-tension-lowering effects.

Addition of LP to homogenized milk caused a slight increase in moisture content in WMP. This increase could be attributed to the ability of LP to interact with or form structures (e.g., micelles) involving fat molecules (30). As amphiphilic molecules, lipopeptides can interact with both fat and water molecules, potentially leading to an increased capacity to retain moisture. In general, moisture content tended to decrease with increasing fat content in the samples. This may be because the non-polar nature of fat globules could reduce the water-binding capacity of the milk matrix, facilitating water loss.

Powder density is an important quality attribute in the dairy industry, and decreased density is unfavorable due to increased storage, transportation, and packaging costs (31). In dairy processing, flowability is essential for pneumatic conveying, tank filling and emptying, silo storage, bagging, packaging, and mixing operations. Greater flowability and lower cohesiveness simplify process control (32). Bulk density, tapped density, CI, HR, and particle size of fortified samples remained largely unchanged. Maintaining the physical quality of the original product is a key challenge in food fortification. These findings demonstrate that the fortified milk powders retained similar bulk and tapped densities as the control, suggesting that packaging and transport logistics would likely be unaffected. Furthermore, the consistent flowability and cohesiveness imply that the powder would exhibit good handling properties and resist caking and clumping during storage and use.

A significant color difference was observed between fortified and non-fortified samples. The insolubility of milk powders increased with increasing fat content and with the addition of LP to the milk samples. Previous studies suggest that insolubility in fortified milk may result from the interaction of added molecules with milk proteins (33, 34). Alteration or denaturation of these proteins, potentially followed by aggregation or precipitation, can lead to decreased solubility (33, 19).

The particle size of the SMP and LMP samples did not change significantly with the addition of LP. Conversely, WMP+LP samples displayed a

significantly reduced particle size compared to WMP controls. This suggests a complex role for LP: in SMP and LMP, potential structural changes in milk proteins affecting precipitation rates may underlie their lower solubility, whereas in WMP, LP appears to facilitate finer particle dispersion. This could be attributed to LP's potential involvement in the formation of fat-associated interfacial structures (in the WMP sample), thereby limiting its interaction with proteins.

Most dairy powders require reconstitution before use, making it essential to evaluate their reconstitution properties, including wettability, solubility, and dispersibility. In a study on curcumin-enriched spray-dried milk powder, curcumin-loaded milk powder exhibited no significant differences in most physicochemical properties compared to raw milk powder, except for a reduction in wettability (34). Similarly, soy extract-enriched milk powder showed negative effects on all reconstitution properties due to the high fat content of the extract (3). Bensel et al. (2017) prepared three types of milk powder (buffalo, cow, and a buffalo-cow mixture) enriched with varying honey concentrations. Increasing honey levels resulted in higher powder adhesion, cohesion, bulk and tapped density, caking tendency, particle size, and porosity, while flowability decreased. These effects were attributed to honey's hygroscopic nature, which increased stickiness and moisture content, leading to particle aggregation and reduced flowability. The authors recommended using low-permeability packaging and storing the powders in cool, dry conditions to prevent moisture absorption and spoilage during storage (35). In line with the studies mentioned above, fortification generally did not improve powder reconstitution properties. Similarly, our results showed that the addition of 0.13% LP to skim milk decreased solubility without negatively affecting the other powder properties, representing the first report on LP activity in homogenized milk.

Conclusion

Bacteria have great potential for application in the dairy industry (36, 37). This study investigated the antibacterial and biosurfactant activities of LP—a bacterial lipopeptide with known anticancer properties—in homogenized milk samples of varying fat contents. In addition, the characteristics of milk powder fortified with LP were assessed. The results showed that although LP activity decreased in milk and decreased even further in higher-fat milk samples, it still retained measurable activity. Since the only negative effect of LP was on solubility, LP can be added to milk powder without major loss of quality. The findings of this study indicate that LP can be incorporated into milk, a commonly consumed dairy product, to produce a functional food. Nevertheless, if the bacterial strain is to be incorporated into the product, its safety must first be evaluated and confirmed.

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