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Research Article

Natural preservation of butter using whey protein hydrolysates: Antioxidant, antimicrobial, and sensory evaluation

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Abstract

Whey protein hydrolysates (WPHs) are recognized as a valuable source of bioactive peptides with multifunctional properties, especially their potent antioxidant and antimicrobial activities. These characteristics make them attractive natural alternatives to synthetic preservatives and align with the growing consumer demand for clean-label and functional foods. In this study, lyophilized whey protein hydrolysates (LWPH) were incorporated into a butter-based model system to assess their potential as natural preservatives. Four formulations were prepared: (1) control (without additives), (2) butter with 1% (w/w) LWPH, (3) butter with 0.02% (w/w) butylated hydroxytoluene (BHT), and (4) butter with a combination of 1% (w/w) LWPH + 0.02% (w/w) BHT. All samples were stored at 4 ± 1 °C for 60 days, and quality parameters were periodically monitored. Lipid oxidation was determined by thiobarbituric acid (TBA) assay, and microbial quality was evaluated through total viable count method. The results showed that the LWPH + BHT formulation significantly retarded lipid oxidation, but had weak antimicrobial activity. Moreover, the treated samples maintained better overall sensory acceptability throughout storage. These findings indicate that LWPH can serve as effective natural preservatives for extending the shelf life and maintaining the quality of lipid-rich foods such as butter.

Keywords: Butter, Hydrolysates, Quality attributes, Whey protein.

Introduction

In recent years, there has been an increasing interest in the application of bioactive peptides derived from whey proteins as natural antioxidants and preservatives in food systems, aiming to reduce or replace synthetic additives (Sumny & Kempka, 2025). Whey proteins such as α-lactalbumin, β-lactoglobulin, and minor fractions like lactoferrin are rich sources of peptides that can be released through enzymatic hydrolysis, fermentation, or processing. The amino acid composition and structural conformation of these proteins largely determine the type and activity of the peptides produced, influencing their antioxidant, antimicrobial, and other bio-functional properties (Dalaka et al., 2023).

One of the key challenges in food preservation is lipid oxidation, which adversely affects flavor, color, nutritional quality, and shelf life. Several studies have demonstrated that whey protein hydrolysates (WPHs) effectively inhibit lipid oxidation in food models. For instance, Liu et al. (2022) reported that

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the incorporation of 15% WPH into pork patties improved oxidative stability and water-holding capacity while reducing peroxide value and lipid oxidation during storage. Similarly, Arshad et al. (2022) found that milk protein hydrolysates derived from whey and casein suppressed lipid peroxidation in meat products and improved overall product quality during refrigerated storage. Beyond meat systems, the antioxidant potential of whey-derived peptides has also been explored in dairy products. Auestad & Layman, (2021) demonstrated that whey protein hydrolysates could delay oxidative deterioration and improve sensory acceptability in ghee and butter models. Likewise, Rodzik et al. (2020) reported that whey-derived peptides enhanced oxidative stability in cream-based emulsions by scavenging reactive oxygen species and chelating pro-oxidant metal ions. These findings highlight the applicability of WPHs as natural antioxidants in lipid-rich dairy matrices such as butter.

Whey-derived peptides also exhibit multiple biological activities, including immunomodulatory, antihypertensive, and antimicrobial effects (Saubenova et al., 2024). Their multifunctionality makes them promising candidates for both food preservation and functional food development. Dalaka et al. (2023) showed that peptides released from sweet whey after *in vitro* digestion demonstrated enhanced antioxidant capacity in oxygen radical absorbance capacity (ORAC), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays, indicating that gastrointestinal processes can generate more potent antioxidant fragments. Growing consumer awareness about the risks associated with synthetic antioxidants has accelerated the demand for natural and clean-label ingredients (Zaky et al., 2022). Despite promising evidence, variations in enzyme type, hydrolysis conditions, and peptide structure can significantly affect antioxidant potency and stability in real food systems. Moreover, long-term effects on storage stability, sensory attributes, and potential allergenicity remain underexplored (Quintieri et al., 2025; Sumny & Kempka, 2025).

Therefore, the present study aimed to evaluate the antioxidant and antimicrobial effects of lyophilized whey protein hydrolysates (LWPH) in a traditional butter model system, both individually and in combination with the synthetic antioxidant butylated hydroxytoluene (BHT). This investigation contributes to the growing evidence supporting the use of whey-derived bioactive peptides as natural functional agents for improving the shelf life and quality of lipid-rich dairy products such as butter.

Materials and Methods

Materials

Whey protein concentrate (WPC; 81% protein) was obtained from Hilmar Ingredients, USA. Pepsin enzyme and analytical-grade reagents, including butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), sodium hydroxide (NaOH), hydrochloric acid (HCl), chloroform, diethyl ether, ethylenediaminetetraacetic acid (EDTA), and methanol, were purchased from Sigma-Aldrich, USA and Merck, Germany. Plate count Agar (PCA) and peptone water were purchased from Merk (Germany). Commercial unsalted butter was purchased from a local market in Urmia, Iran, produced by a local dairy manufacturer. Deionized water was used in all preparations.

Preparation of whey protein hydrolysate

Whey protein concentrate was dissolved in deionized water to a final concentration of 5% (w/v) and stirred for 30 min at room temperature. The pH was adjusted to 2.0 using a pH meter (Metrohm827, Switzerland) and 0.1 N HCl to provide optimal conditions for pepsin activity. Enzymatic hydrolysis was performed with 2% (w/w) pepsin based on protein content at 37 °C for 3 h under constant stirring (Sarmadi & Ismail, 2010; Udenigwe & Aluko, 2012). The enzymatic reaction was terminated by adjusting the pH to 7.0 with 0.1 N NaOH, followed by centrifugation at 7000 × *g* for 10 min at 25 °C using an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany). The supernatant was filtered through Whatman No. 1 filter paper (Whatman, Maidstone, UK) and freeze dried in a freeze-drier (Christ Alpha 1–2 LD, Martin Christ, Osterode am Harz, Germany). The resulting LWPH was stored at –21 °C until use (Korhonen & Pihlanto, 2006; Borges et al., 2025).

Preparation of butter samples

Four butter formulations were prepared as follows: 1) Control (C): Butter without additives; 2) BHT: Butter with 0.02% (w/w) BHT; 3) LWPH: Butter with 1% (w/w) LWPH; and 4) LWPH + BHT: Butter with 1% (w/w) LWPH + 0.02% (w/w) BHT. Each formulation (100 g) was thoroughly mixed using a magnetic stirrer (Heidolph MR Hei-Standard, Heidolph, Schwabach, Germany) under sterile conditions, packed in sterile airtight containers, and stored at 4 ± 1 °C for 60 days. Analyses were performed on days 0, 30, and 60 (Park & Nam, 2015).

Lipid extraction

For each sampling time (0, 30, and 60 days), 10 g of butter was homogenized (Heidolph DIAX 900, Heidolph, Schwabach, Germany) with 20 mL of chloroform and 40 mL of methanol in a 2:1 (v/v) ratio. Additional 20 mL chloroform and 20 mL methanol were added sequentially to ensure complete lipid extraction. The solvent was removed using a rotary evaporator (Heidolph Laborota 4003, Germany), and the lipid extract was transferred into opaque bottles and stored at –80 °C until analysis (Bligh & Dyer, 1959; Zaky et al., 2024).

Determination of free fatty acids (FFA)

A 0.2 g portion of the extracted fat was dissolved in 50 mL of a 1:1 (v/v) ethanol–diethyl ether mixture. The solution was titrated with 0.10 N NaOH in the presence of phenolphthalein indicator until a stable pink color persisted for at least 30 seconds. The FFA content was expressed as percentage of oleic acid (% oleic acid) and calculated using the following equation:

$$\text{Free Fatty Acid (FFA) (\% oleic acid)} = \frac{28.2 \times N \times V}{W}$$

where V = volume of NaOH (mL), N = normality of NaOH, and W = weight of lipid sample (g) (Egan et al., 1981; Demir et al., 2024).

Determination of lipid oxidation

Lipid oxidation was determined using thiobarbituric acid reactive substances (TBARS) method. Approximately 5 g of butter was homogenized with 15 mL of 7.5% (w/v) trichloroacetic acid (TCA) containing 0.1% (w/v) propyl gallate and 0.1% (w/v) EDTA. The homogenate was filtered, and 5 mL of the filtrate was mixed with 5 mL of 0.02 M thiobarbituric acid (TBA). The mixture was incubated in a boiling water bath at 100 °C for 30 min, cooled to room temperature, and the absorbance was measured at 532 nm using a spectrophotometer (Novaspec II; Pharmacia LKB, Uppsala, Sweden). TBARS values were expressed as mg malondialdehyde (MDA)/kg butter (Shantha & Decker, 1994; Shahidi & Zhong, 2010).

Microbiological analysis

Microbiological quality was evaluated by determining the total viable count (TVC) using PCA. Butter samples (10 g) were homogenized with 90 mL of sterile 0.1% (w/v) peptone water using a Stomacher 400 (Seward, Worthing, UK). Serial dilutions were prepared, and 0.1 mL of each dilution was spread on PCA plates, which were incubated at 37 °C for 48 h. The microbial counts were expressed as $\log_{10}$ CFU/g butter (Hussein et al., 2020).

Sensory evaluation

A semi-trained panel of 10 members (5 male and 5 females; aged 22–35 years) conducted sensory evaluation on days 0, 30, and 60. Coded samples were presented in random order at room temperature. Sensory attributes (color, texture, odor, and overall acceptability) were rated using a 9-point hedonic scale (1 = dislike extremely, 9 = like extremely). Panelists rinsed their mouths with water between samples to minimize carry-over effects (Stone et al., 2012).

Statistical analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (SD). Data were analyzed using SPSS software (Version 20; IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to compare means. Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Free fatty acids

In the control group, FFA levels increased from 0.38 ± 0.03% at day 0 to 1.30 ± 0.08% at day 60, reflecting progressive lipid hydrolysis (**Table 1**). In contrast, butter samples treated with BHT, LWPH, and LWPH+BHT exhibited significantly ($P<0.001$) lower FFA contents (0.60 ± 0.07%, 1.13 ± 0.01%, and 0.86 ± 0.07%, respectively). These results are consistent with the results of other researches (Li et al., 2023; Uçar et al., 2024). According to international standards (Codex Alimentarius, 2023), FFA values above 1.5% are considered indicative of lipid spoilage. The reduced FFA accumulation in LWPH-treated samples indicates that whey-derived peptides can partially inhibit enzymatic hydrolysis of triglycerides. This inhibition is likely attributed to metal-chelating and radical-scavenging actions, which preserve lipid integrity throughout storage (Cais-Sokolińska et al., 2024; D'Souza et al., 2020). Previous studies have also shown that whey peptides enhance emulsion stability and mitigate both lipid and protein oxidation in dairy systems (Shokery et al., 2025).

Lipid oxidation

Lipid oxidation is a key determinant of butter shelf life. TBARS values increased progressively across all treatments, indicating ongoing oxidative reactions during storage, consistent with previous findings (Hussein et al., 2020). However, LWPH and LWPH+BHT samples exhibited significantly lower TBARS values, demonstrating that WPHs exert measurable antioxidant activity in real food systems (**Table 1**). Based on Codex Alimentarius (2023), TBARS values lower than 0.5 mg MDA/kg indicate acceptable oxidative quality. On day 60, the LWPH + BHT treatment showed significantly lower lipid oxidation compared with the control.

These findings are consistent with those of Alizadeh & Aliakbarlu (2020), who reported enhanced antioxidant activity of whey protein fractions following ultrasound and ohmic pretreatments. Similarly, Liu et al. (2022) showed that WPHs reduced protein aggregation and lipid peroxidation in pork patties during freeze–thaw cycles. The antioxidant effect of WPHs is largely due to peptides containing hydrophobic and histidine residues capable of proton donation and metal chelation (Saubenova et al., 2024). Interestingly, our data suggest an additive effect when LWPH was combined with BHT, aligning with Park & Nam, (2015), who noted that natural and synthetic antioxidants can complement each other through distinct mechanisms. This synergy enables lower synthetic additive concentrations while maintaining oxidative stability, addressing consumer concerns about chemical preservatives.

Microbial stability

Microbiological analyses revealed reduced TVC in all LWPH-containing samples compared with the control. The LWPH+BHT treatment exhibited the lowest microbial load by day 60, confirming that WPHs possess antimicrobial properties that reinforce their antioxidant activity (**Table 1**). All treatments showed a weak antimicrobial activity. In accordance with microbiological standards (Codex Alimentarius, 2023), TVC values higher than 7 $\log_{10}$ CFU/g indicate spoilage.

This antimicrobial effect agrees with previous reports showing that whey derived peptides disrupt bacterial cell membranes, increase permeability, and induce leakage of cytoplasmic contents (Nagpal et al., 2011). They may also chelate essential trace metals such as Fe^{2+} and Zn^{2+}, disrupting microbial metabolism (Nagpal et al., 2022). Likewise, Takeda et al. (2023) found that WPHs inhibited microbial proliferation in meat systems while preventing color degradation. Furthermore, the ability of LWPH to retard microbial growth aligns with the clean-label trend in food preservation. Quintieri et al. (2025) emphasized that whey peptides extend shelf life while enhancing safety by inhibiting pathogens such as *Listeria monocytogenes* and *Escherichia coli*. These dual activities (antioxidant and antimicrobial) underline the multifunctionality of WPHs as sustainable natural preservatives.

Sensory attributes and consumer acceptability

The sensory evaluation showed that LWPH and LWPH+BHT formulations retained superior scores for flavor, odor, and overall acceptability after 60 days (**Table 2**). This improvement is attributed to the suppression of oxidation, derived volatiles, such as

aldehydes and ketones, which cause rancid flavors (Clark et al., 2009).

Notably, LWPH addition did not produce off-flavors or undesirable sensory characteristics, a key advantage over plant-based antioxidants like rosemary or oregano extracts that often impart strong herbal aroma (Shahidi & Zhong, 2010). From an industrial viewpoint, this sensory neutrality makes LWPH a promising additive for a wide range of dairy and lipid-based foods. Similar trends have been reported other researchers. Arshad et al. (2022) observed that milk protein hydrolysates improved the sensory profile of meat products during storage, while Saubenova et al. (2024) highlighted their positive effects on texture and mouthfeel. Thus, LWPH contributes not only to preservation but also to the sensory quality of butter.

Table 1. Chemical analysis of butter samples for free fatty acids (FFA) and thiobarbituric acid reactive substances (TBARS), and total viable count (TVC) during 60 days of refrigerated storage**.**

FFA (% as oleic acid)	**Storage time (day)**			**P-value**		
Group	0	30	60	Treatment	time	Interaction
Control	0.38±0.03^{Aa}	0.71±0.05^{Ba}	1.30±0.08^{Ca}	<0.001	<0.001	<0.001
BHT	0.31±0.02^{Aa}	0.50±0.03^{Bc}	0.60±0.07^{Cd}	<0.001	<0.001	<0.001
LWPH	0.38±0.03$^{\mathbf{Aa}}$	0.63±0.03^{Bb}	1.13±0.01^{Cb}	<0.001	<0.001	<0.001
LWPH + BHT	0.34±0.02$^{\mathbf{Aa}}$	0.55±0.04^{Bc}	0.86±0.07^{Cc}	<0.001	<0.001	<0.001
TBARS (mg MDA/kg)						
Group	0	30	60	Treatment	Time	Interaction
Control	0.14±0.01^{Aa}	0.30±0.02^{Ba}	0.40±0.02^{Ca}	<0.001	<0.001	<0.001
BHT	0.13±0.01^{Aa}	0.17±0.01^{Bd}	0.25±0.01^{Cc}	<0.001	<0.001	<0.001
LWPH	0.13±0.02^{Aa}	0.27±0.01^{Bb}	0.37±0.03^{Cb}	<0.001	<0.001	<0.001
LWPH + BHT	0.14±0.01^{Aa}	0.25±0.02^{Bc}	0.33±0.03^{Cb}	<0.001	<0.001	<0.001
TVC (log_{10} CFU/g)						
Control	4.10±0.12^{Aa}	6.07±0.13^{Bb}	7.54±0.11^{Ca}	<0.002	<0.001	<0.323
BHT	3.99±0.15^{Aa}	5.88±0.15^{Bc}	7.13±0.14^{Cc}	<0.002	<0.001	<0.323
LWPH	4.10±0.13^{Aa}	6.19±0.10^{Ba}	7.36±0.14^{Cb}	<0.002	<0.001	<0.323
LWPH + BHT	4.00±0.13^{Aa}	6.02±0.09^{Bc}	7.33±0.10^{Cb}	<0.002	<0.001	<0.323

Different lowercase letters (a–d) within each column indicate significant differences between treatments at the same storage time ($p < 0.05$). Different uppercase letters (A–C) within each row indicate significant differences across storage times for each treatment ($p < 0.05$). LWPH = Lyophilized whey protein hydrolysate; BHT = Butylated hydroxytoluene.

Comparison with other natural antioxidants

While plant-derived antioxidants such as polyphenols are widely utilized, WPHs offer distinct advantages. They are flavor-neutral, highly compatible with dairy matrices, and derived from whey, a valuable by-product of cheese manufacturing. Hence, their use promotes sustainable utilization of dairy by-products, reducing environmental waste (Shahidi & Ambigaipalan, 2015). Emerging studies suggest the interaction effects between peptides and plant-based antioxidants. For example, Zaky et al. (2022) reported that combining bioactive peptides with polyphenols enhances both radical scavenging and microbial inhibition. Future research should explore such hybrid systems in butter and plant-based analogues for improved preservation and functionality.

Table 2. Sensory attributes of butter samples during 60 days of storage.

Parameter	Treatment	Storage time (days)		
		0	30	60
Color	Control	9.00 ± 0.00^{Aa}	8.00 ± 1.00^{Ba}	6.67 ± 0.58^{Ca}
	LWPH	8.67 ± 0.58^{Aa}	8.33 ± 0.58 ^{Ba}	7.00± 0.00^{Ca}
	BHT	9.00 ± 0.00^{Aa}	8.00± 0.00 ^{Ba}	6.67 ± 0.58^{Ca}
	LWPH + BHT	9.00 ± 0.00^{Aa}	7.67± 0.58^{Bb}	7.33 ± 0.58^{Ca}
Odor	Control	8.67 ± 0.58^{Aa}	5.33 ± 0.58^{Bb}	2.67 ±0.58^{Cc}
	LWPH	9.00 ± 0.00^{Aa}	5.67 ± 0.58^{Bb}	3.00 ±1.00^{Cbc}
	BHT	9.00 ± 0.00^{Aa}	7.67± 0.58^{Ba}	4.33 ± 0.58^{Ca}
	LWPH + BHT	9.00 ± 0.00^{Aa}	6.67± 0.58^{Ba}	3.67 ± 0.58^{Cb}
Texture	Control	9.00 ± 0.00^{Aa}	7.33 ± 0.58^{Ba}	4.67 ± 0.58^{Cc}
	LWPH	8.67 ± 0.58^{Aa}	7.00 ± 0.00^{Ba}	5.00 ± 0.00^{Cb}
	BHT	9.00 ± 0.00^{Aa}	7.67 ± 0.58^{Ba}	5.67 ± 0.58^{Ca}
	LWPH + BHT	8.67 ± 0.58^{Aa}	7.33 ± 0.58^{Ba}	5.67 ± 0.58^{Ca}
Overall acceptability	Control	9.00 ± 0.00^{Aa}	5.67 ± 0.58^{Bb}	2.66 ± 0.58^{Cc}
	LWPH	8.67 ± 0.58^{Aa}	7.00 ± 0.00^{Ba}	3.67 ± 0.58^{Cb}
	BHT	8.33 ± 0.58^{Aa}	6.67 ± 0.58^{Ba}	4.67 ± 0.58^{Ca}
	LWPH + BHT	9.00 ± 0.00^{Aa}	7.33 ± 0.58^{Ba}	4.33 ± 0.58^{Ca}

Means with different lowercase letters (a–c) within columns differ significantly ($p < 0.05$). Means with different uppercase letters (A–C) within rows differ significantly across storage times ($p < 0.05$). LWPH = Lyophilized whey protein hydrolysate; BHT = Butylated hydroxytoluene.

The dual antioxidant and antimicrobial properties of LWPH make it an excellent candidate for industrial applications in butter and other high-fat dairy systems. Its incorporation can extend shelf life, reduce the need for synthetic additives, and meet consumer demand for natural, clean-label foods. Beyond dairy applications, WPHs have shown potential in meat (Liu et al., 2022), plant-based (Saubenova et al., 2024), and nutraceutical products (Quintieri et al., 2025). Nutritionally, whey-derived peptides provide additional health benefits, including antihypertensive, immunomodulatory, and anti-inflammatory effects (Guha et al., 2021; Sumny & Kempka, 2025). Therefore, their inclusion in food systems may simultaneously enhance product stability and deliver functional health-promoting attributes.

Conclusion

The findings of this study demonstrate that LWPH exhibit significant antioxidant and antimicrobial properties when applied to a butter-based food model. The incorporation of LWPH, alone or in combination with the synthetic antioxidant BHT, led to a marked reduction in lipid oxidation during a 60-day refrigerated storage period. The LWPH+BHT treatment showed the most effective performance, suggesting an additive interaction between the natural peptides and synthetic antioxidant components. Additionally, sensory evaluation results indicated that LWPH helped preserve the organoleptic characteristics of butter, including texture, odor, and overall acceptability. This is particularly important in fat-rich dairy products where oxidative degradation and off-flavor development are major concerns during shelf life. Overall, these results support the use of LWPH as a natural food preservative with functional benefits. Its dual role in oxidative stability and microbial control offers a promising alternative or complement to conventional synthetic additives. Further research is recommended to explore LWPH applications in other dairy and lipid-based systems, and to optimize concentration and formulation strategies for commercial scale-up.

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Conflicts of interest

The authors declare no conflict of interest.

Disclaimer

None.

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