

Influence of trichloroacetic acid concentration on protein removal and structural preservation of exopolysaccharides obtained from milk fermentations

Influencia de la concentración de ácido tricloroacético en la remoción de proteínas y preservación estructural de exopolisacáridos obtenidos de fermentaciones lácteas

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ABSTRACT

This study evaluated the effect of two concentrations of trichloroacetic acid (TCA) on protein removal during the extraction of exopolysaccharides (EPS) produced by milk fermentation using kefir grains (KGs) or *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 (Lc2772). The results showed that treatment with 10% TCA solution yielded more lyophilized solids (LS) in both fermentations compared to 80% TCA. Moreover, the solids obtained with 10% TCA exhibited higher sugar concentrations— 34.95 ± 0.78 and 23.22 ± 0.25 mg_{eq} glucose mL⁻¹ for LS-Lc2772 and LS-KGs, respectively. In contrast, 80% TCA resulted in up to a 60% reduction in sugar content, suggesting potential polysaccharide hydrolysis. Although 80% TCA was more effective at removing proteins, it adversely affected EPS integrity, potentially compromising their functional properties. These findings highlight the need to develop alternative protein removal methods that preserve the structural integrity of EPS.

Keywords: kefir; lactic acid bacteria; protein removal

RESUMEN

Este estudio evaluó el efecto de dos concentraciones de ácido tricloroacético (TCA) en la eliminación de proteínas durante la extracción de exopolisacáridos (EPS) producidos por fermentación de leche utilizando gránulos de kéfir (GK) o *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 (Lc2772). Los resultados mostraron que el tratamiento con 10 % de TCA produjo una mayor cantidad de sólidos liofilizados (SL) en ambas fermentaciones en comparación con el TCA al 80 %. Además, los sólidos obtenidos con TCA al 10 % presentaron mayores concentraciones de azúcares: 34.95 ± 0.78 y 23.22 ± 0.25 mg_{eq} de glucosa mL⁻¹ para SL-Lc2772 y SL-GK, respectivamente. En contraste, el uso de TCA al 80 % resultó en una reducción de hasta un 60 % en el contenido de azúcares, lo que sugiere una posible hidrólisis del polisacárido. Aunque el TCA al 80 % fue más eficaz en la eliminación de proteínas, afectó negativamente la integridad de los EPS, comprometiendo potencialmente sus propiedades funcionales. Estos hallazgos resaltan la necesidad de desarrollar métodos alternativos de eliminación de proteínas que preserven la integridad estructural de los EPS.

Palabras clave: kefir; bacterias ácido-lácticas; remoción de proteínas

INTRODUCTION

Several studies have explored the functional properties of exopolysaccharides (EPS) produced during the fermentation processes of various microorganisms. These metabolites, known as postbiotics, are compounds generated during fermentation that offer health benefits (Cuevas-González *et al.*, 2020; Wegh *et al.*, 2019). Reported biological activities of EPS include immunomodulatory, antioxidant, antihypertensive, and hypoglycemic effects (Jiang *et al.*, 2023; Niu *et al.*, 2023). Additionally, EPS are utilized in the food industry to enhance important characteristics such as rheological and sensory properties (Ayivi *et al.*, 2020; Korcz and Varga, 2021; Tang *et al.*, 2017). Fermented dairy products, such as yogurt and kefir, are significant sources of EPS.

Kefir is produced by fermenting milk with kefir grains (KGs), a consortium of microorganisms comprising lactic acid bacteria (LAB), acetic acid bacteria, and yeasts embedded in a matrix composed of proteins, lipids, and EPS. LAB constitutes approximately 60% of the microbial composition (Guzel-Seydim *et al.*, 2021). The EPS produced by KGs are known as kefiran (Figure 1), a heteropolysaccharide composed of glucose and galactose subunits, with a molecular weight ranging from 55 to 10,000 kDa, depending on the producing microorganisms, fermentation conditions, and extraction process (Gentry *et al.*, 2023; Yadav *et al.*, 2024). Due to its structural characteristics, kefiran has shown potential for use in the food industry as an emulsifying or gelling agent (Simionelli *et al.*, 2022), and even in forming biofilms (Montoille *et al.*, 2021).

LAB is the leading group of microorganisms present in KGs. These bacteria are widely used in the food industry due to their GRAS (Generally Recognized As Safe) status and the beneficial properties they confer on foods. The structural composition of EPS also depends on the producing strain, as each possesses specific genes involved in its production (Soumya and Nampoothiri, 2021). Generally, fermenting milk produces heteropolysaccharides composed of the monosaccharides glucose, galactose, and occasionally rhamnose, which can be linked in different positions: 1→3, 1→4, and 1→6, both in α- or β-configuration. For example, Table 1 presents oligomer fragments that make up EPS produced by different strains of *Lactobacillus delbrueckii* subsp. *bulgaricus*.

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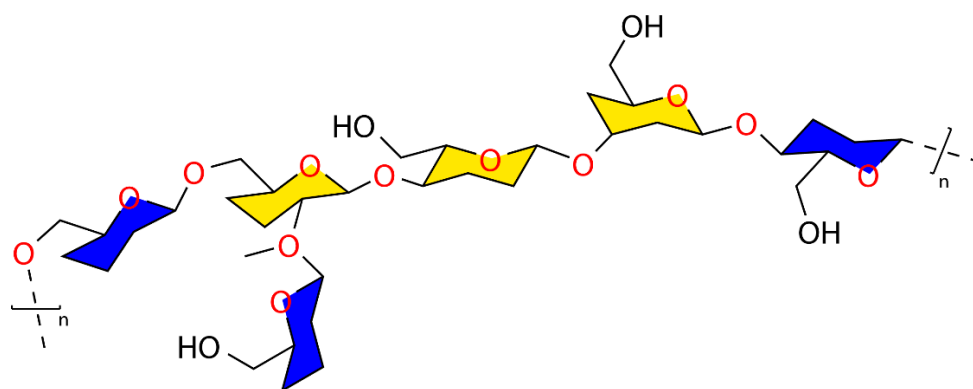


Figure 1. Micheli *et al.* (1999) reported the chemical structure of a kefiran subunit. D-glucopyranose residues are shown in blue, and D-galactopyranose residues in yellow. The polymerization degree ($n/2$) ranges from 55 to 10,000.

Figura 1. Micheli *et al.* (1999) reportaron la estructura química de una subunidad de kefirán. Los residuos de D-glucopiranososa se muestran en azul y los de D-galactopiranososa en amarillo. El grado de polimerización ($n/2$) varía de 55 a 10,000.

Table 1. Structures of the EPS produced by different strains of *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Tabla 1. Estructuras de los EPS producidos por diferentes cepas de *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Structure	Strain	Reference
	OLL1073R-1	Van Calsteren <i>et al.</i> (2015)
	LBB.B26	Sánchez-Medina <i>et al.</i> (2007)
	NCC556	Lamothe <i>et al.</i> (2002)

The most common process for EPS extraction involves protein precipitation using trichloroacetic acid (TCA), followed by neutralization and solvation with aqueous sodium hydroxide and absolute ethanol, respectively. Finally, dialysis removes small molecules like monosaccharides and ions (Yadav *et al.*, 2024). However, the amount of TCA used is a critical step, as there are differences among various authors that use concentrations ranging from 4% to 80% (w/v) (Ayala-Hernandez *et al.*, 2008; Ayyash *et al.*, 2020; Rimada and Abraham, 2003; Tang *et al.*, 2020; Tiwari *et al.*, 2021). It is important to note that all authors report EPS as the total solid obtained in the extraction process, measured as total sugars; however, this solid may also contain proteins and other components such as minerals, as no additional purification process is usually reported. Therefore, we hypothesized that the concentration of TCA could play a critical role affecting the integrity of the EPS: higher concentrations would effectively removed a greater proportion of protein it might also cause hydrolysis of the polysaccharide, while lower concentrations

would help to better preserved both EPS integrity and overall yield. To test this hypothesis, as part of a project aimed at obtaining kefiran for use as an adsorbent agent, this study evaluated the effect of two TCA concentrations on the removal of proteins potentially associated with EPS produced during milk fermentation with KGs and *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 (Lc2772).

MATERIAL AND METHODS

Kefir grains conditioning

In Mexico, KGs, traditionally known as “búlgaros,” are commonly shared from person to person. The KGs used in this study were obtained from a household in Mexico City. Before their use in EPS production, the grains were conditioned by continuous fermentation for one week as follows: sterilized glass jars were filled with 1 L of ultra-pasteurized whole milk (Alpura®, Mexico) and 100 g of KGs (wet weight), and maintained at room temperature.

Activation of lactic acid bacteria

A volume of 1 mL of an active culture of Lc2772 was inoculated into 9 mL of skim milk previously sterilized at 121 °C for 15 min. The culture was incubated at 37 °C for 24 h or until a bacterial concentration of approximately 10⁸ CFU/mL was achieved.

Fermentation using kefir grains and *Lactobacillus delbrueckii* subsp. *bulgaricus*

Conditioned KGs were filtered, rinsed with distilled water, and gently blotted dry with a paper towel. For fermentation, 200 mL of ultra-pasteurized whole milk (Alpura®, Mexico) and 20 g (wet weight) of kefir grains (KGs) were added to a 250 mL Erlenmeyer flask previously sterilized at 121 °C for 15 min. The mixture was incubated at 30 °C for 24 h without agitation.

For fermentation with Lc2772, 12 g of whole milk powder (Alpura®, Mexico) were dissolved in 90 mL of sterile water to obtain 100 mL of reconstituted milk. Then, 90 mL of this milk were transferred to a 150 mL Erlenmeyer flask, pasteurized at 121 °C for 5 min, and cooled to 4 °C in an ice bath. Subsequently, the milk was inoculated with 10 mL of a previously activated Lc2772 culture and incubated at 37 °C for 24 h without agitation.

Extraction of exopolysaccharides

EPS were extracted from both fermented milk samples (KGs and Lc2772). For the KGs sample, the grains were first removed using a strainer. Next, 16 mL of trichloroacetic acid (TCA; JT-Baker, USA), at either 10% or 80% (w/v), were added to each sample. The mixtures were gently stirred and allowed to stand for 5 minutes to facilitate protein denaturation. Subsequently, the samples were centrifuged at 10,000 rpm for 30 minutes at 4 °C using an Avanti J-E centrifuge (Beckman Coulter, USA). The supernatants were collected, and the pH was adjusted to 7.0 using 4 N sodium hydroxide (NaOH; JT-Baker, USA).

EPS precipitation was performed by adding three volumes of absolute ethanol (JT-Baker, USA) to the neutralized solutions and resting at 4 °C for 12 h. Precipitated samples were centrifuged at 4,000 rpm for 20 min at 4 °C. The resulting supernatants were discarded, and the precipitates were resuspended in 10 mL of deionized water.

The resuspended solids were dialyzed against deionized water at a 1:100 (v/v) ratio for 48 h at 4 °C, with water changes at 3, 6, 12, and 24 h. Dialysis was carried out using cellulose membranes with a molecular weight cutoff of 10 kDa (Sigma-Aldrich, USA). Finally, the dialyzed samples were freeze-dried at –65 °C using a Scientz-10N lyophilizer (Ningbo Scientz Biotechnology, China) and labeled as SL-GK and SL-Lc2772, corresponding to EPS extracted from the kefir grains and Lc2772 fermentations, respectively.

Each lyophilized sample (1 mg) was dissolved in 1 mL of deionized water for subsequent determination of total sugar and protein contents.

Determination of total sugar and protein content

Total sugar content was quantified using the phenol-sulfuric acid method described by Dubois *et al.* (1956). Briefly, 1 mL of EPS solution was transferred into a test tube, and 1 mL of 5% (w/v) phenol was added. The mixture was gently homogenized, and 5 mL of concentrated sulfuric acid (98% H₂SO₄) were slowly added. After thorough mixing, the reaction mixture was allowed to cool to room temperature. Absorbance was measured at 490 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). A glucose calibration curve (Sigma-Aldrich, USA) ranging from 20 to 100 µg/mL was used for quantification.

Protein content was determined using the Bradford assay (Bradford, 1976). A 1:10 dilution of the EPS solution was prepared, and 100 µL of this dilution were transferred to a 1.5 mL microtube (Eppendorf, Germany). Then, 1 mL of Bradford reagent (Sigma-Aldrich, USA) was added, and the mixture was gently homogenized and incubated in the dark for 5 min. Absorbance was recorded at 595 nm using a UV-Vis spectrophotometer. A bovine serum albumin (BSA) standard curve (Sigma-Aldrich, USA) in the 20–100 µg/mL range was used for protein quantification.

Statistical analysis

Since only two extraction treatments (10% and 80% TCA) were compared for each fermentation type, data were analyzed using a Student's t-test for independent samples at a 95% confidence level to determine significant differences between means. Analyses were carried out using NCSS software, version 23.0.3.

RESULTS AND DISCUSSION

Yield of extracted solids

EPS extraction using 10% TCA yielded significantly more lyophilized solids than 80% TCA for both fermentations (Table 2). Specifically, solids recovered from KGs fermentation reached 166.6 ± 2.14 mg with 10% TCA, 26% higher than 80% TCA (131.3 ± 8.14 mg). A similar trend was observed for Lc2772, where 10% TCA produced 128.9 ± 3.04 mg of solids, representing a 13% increase compared to that obtained with 80% TCA.

Table 2: Total solids obtained from the fermented samples using the two TCA concentrations for extraction.

Tabla 2: Sólidos totales obtenidos de las muestras fermentadas utilizando las dos concentraciones de TCA para la extracción.

	Total solids (mg)	
	TCA 10%	TCA 80%
KGs	166.6 ± 2.14	131.3 ± 8.14
Lc2772	128.9 ± 3.04	113.7 ± 3.40

KG: kefir grains; **Lc2772:** *Lactobacillus delbrueckii* spp. *bulgaricus* NCFB 2772.

These results agree with Hernández-Martínez *et al.* (2024), who reported that mild extraction and fermentation conditions favored the recovery of EPS from secondary whey fermented with KGs. This experiment yielded $632.6 \pm 30.8 \text{ mg L}^{-1}$ of freeze-dried solids with characteristic glycosidic peaks at $1000\text{--}1200 \text{ cm}^{-1}$ in Fourier Transform Infrared Spectroscopy (FTIR) spectra, confirming the presence of polysaccharide-type bonds. The higher yield obtained at 10% TCA in the present study can be attributed to limited acid hydrolysis and reduced loss of soluble polysaccharide fragments during dialysis. Conversely, high TCA concentrations likely promoted partial depolymerization and precipitation of smaller sugar chains, as observed in acid-induced degradation studies of polysaccharides, where protonation of glycosidic oxygen destabilizes the linkages and accelerates hydrolysis (Chen *et al.*, 2023).

Although the recovered solids include proteins and minerals, the 10% TCA treatment appears to better preserve the polysaccharide fraction relevant for functional applications.

Total sugar content in EPS

As shown in Figure 2, both fermentations demonstrated a marked decrease in sugar content when extracted with 80% TCA. For kefir grains, the EPS extract contained $23.22 \pm 0.25 \text{ mg}_{\text{eq}} \text{ glucose mL}^{-1}$ with 10% TCA, but dropped by approximately 60% when a higher concentration of TCA was used. Similarly, in the Lc2772 fermentation, the sugar concentration was $34.95 \pm 0.78 \text{ mg}_{\text{eq}} \text{ glucose mL}^{-1}$ with 10% TCA and decreased by nearly 50% using 80% TCA.

The pronounced reduction in total sugar content suggests that strong acid conditions promote cleavage of glycosidic bonds, leading to chain scission and the formation of low-molecular-weight fragments that are lost during dialysis. This behavior aligns with the acid-mediated degradation

mechanism previously described for polysaccharides, where the extent of depolymerization depends on acid concentration, temperature, and exposure time (Chen *et al.*, 2023). Under severe hydrolytic conditions, the polymer backbone undergoes extensive cleavage, whereas milder treatments primarily affect side chains, allowing the polysaccharide to retain its conformational stability.

Consistent with this interpretation, Hernández-Martínez *et al.* (2024) reported characteristic glycosidic peaks in the FTIR spectra of EPS obtained from kefir grain fermentations, highlighting the sensitivity of these linkages to acid treatment. Together, these observations reinforce the idea that acid strength is a decisive factor controlling EPS recovery and structural preservation, explaining the higher yield and molecular integrity obtained under 10% TCA extraction in the present study.

Although structural degradation in the present study is inferred from decline in total sugar quantification, future analyses using gel permeation chromatography (GPC) and FTIR spectroscopy are recommended to directly assess molecular-weight distribution and functional-group composition. Such complementary characterization would confirm the extent of hydrolysis and further clarify the relationship between extraction conditions and EPS structural preservation.

Comparative analysis with previous studies

These findings are consistent with previous reports showing that acid strength directly influences the structural integrity of exopolysaccharides. Several studies have demonstrated that both, the microorganism and the extraction method, strongly affect EPS yield and composition. For instance, Ayala-Hernández *et al.* (2008) reported that strong acid treatment disrupts EPS–protein interactions, whereas milder conditions preserve complex integrity. Similarly, Tang *et*

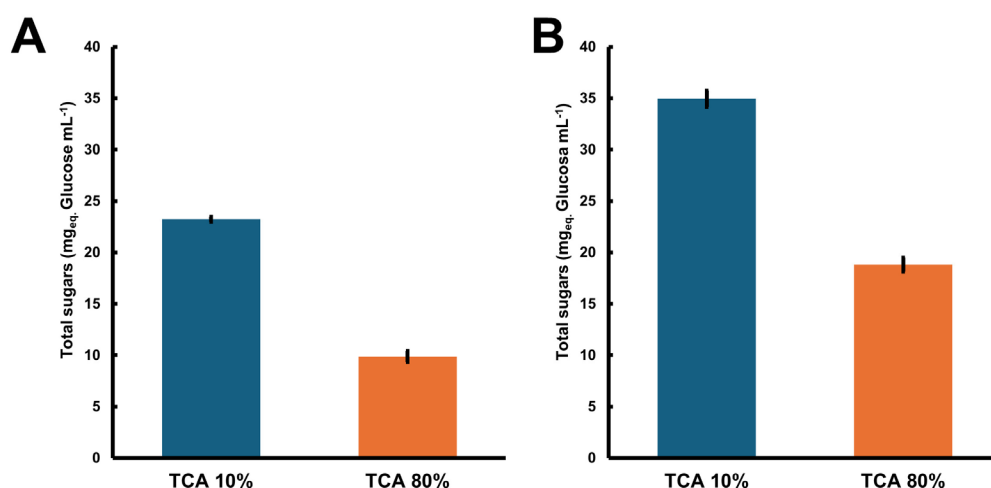


Figure 2. Comparison of the total sugar content in EPS obtained from milk fermented with (A) kefir grains and (B) *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772, using two different trichloroacetic acid (TCA) concentrations during EPS extraction.

Figura 2. Comparación del contenido total de azúcares en los EPS obtenidos de leche fermentada con (A) gránulos de kéfir y (B) *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772, utilizando dos diferentes concentraciones de ácido tricloroacético (TCA) durante la extracción de EPS.

al. (2020) observed that excessive acid exposure promoted cleavage of glycosidic bonds in *Lactobacillus delbrueckii* EPS, leading to a loss of viscosity and molecular weight.

In agreement, Ayyash *et al.* (2020) found that optimized mild acid extraction conditions maintain functional groups associated with bioactivity. Likewise, Zaghloul and Ibrahim (2022) reported a high carbohydrate content (~91.5%) for EPS extracted from *Lactiplantibacillus plantarum* El6 using 10% TCA, highlighting the effectiveness of moderate conditions in preserving EPS structure. Furthermore, Pintado *et al.* (2024) emphasized the importance of sequential purification steps to improve EPS purity and reduce protein contamination in *Lactobacillus acidophilus* strains. Altogether, these studies reinforce that 10% TCA provides an optimal balance between protein removal efficiency and preservation of EPS structural and functional integrity.

Protein content in extracted EPS

Figure 3 shows that 80% TCA significantly reduced protein levels in both EPS extracts. In the KGs fermentation, protein content dropped to $7.16 \pm 0.25 \text{ mg}_{\text{eq}} \text{BSA mL}^{-1}$, while in the Lc2772 fermentation, it decreased to $19.74 \pm 0.12 \text{ mg}_{\text{eq}} \text{BSA mL}^{-1}$. This confirms the efficiency of higher TCA concentrations for protein precipitation.

However, this improved protein removal comes at the expense of polysaccharide yield and possibly bioactivity. The microbial consortium utilizing proteins, polysaccharides, and lipids in kefir grain formation may explain the lower protein content observed in KGs samples.

The findings from this study clearly demonstrate that the concentration of trichloroacetic acid (TCA) used during EPS extraction significantly affects both the yield and quality of the recovered material. The higher amount of lyophilized

solids obtained with 10% TCA suggests that milder conditions better preserve extractable components, likely due to reduced denaturation and loss of labile macromolecules such as polysaccharides.

Protein removal was more efficient with 80% TCA, as expected from the enhanced precipitation under high acidity. However, this increased efficiency in protein elimination comes at the cost of polysaccharide yield and possibly compromises bioactivity or techno-functional properties. The balance between protein removal and polysaccharide preservation must, therefore, be considered based on the intended application of the EPS.

For applications requiring functional EPS, such as food texture modification or biofilm formation, 10% TCA offers a better compromise for the extraction process. Moreover, its combination with proteolytic enzymes (e.g., trypsin, papain) may allow for selective protein removal while minimizing damage to the EPS structure.

Future research should investigate the functional properties of the recovered EPS—such as viscosity, antioxidant capacity, and gelling behavior—to assess how extraction conditions influence their potential applications. These insights would support the development of optimized extraction protocols tailored to specific functional or technological objectives.

TCA concentration plays a critical role in both protein removal and the preservation of EPS structural integrity.

Milder acidic conditions (e.g., 10% TCA) help preserve the structural integrity of the EPS and could be combined with selective enzymatic treatments, such as protease application, to enhance protein removal without compromising polysaccharide quality.

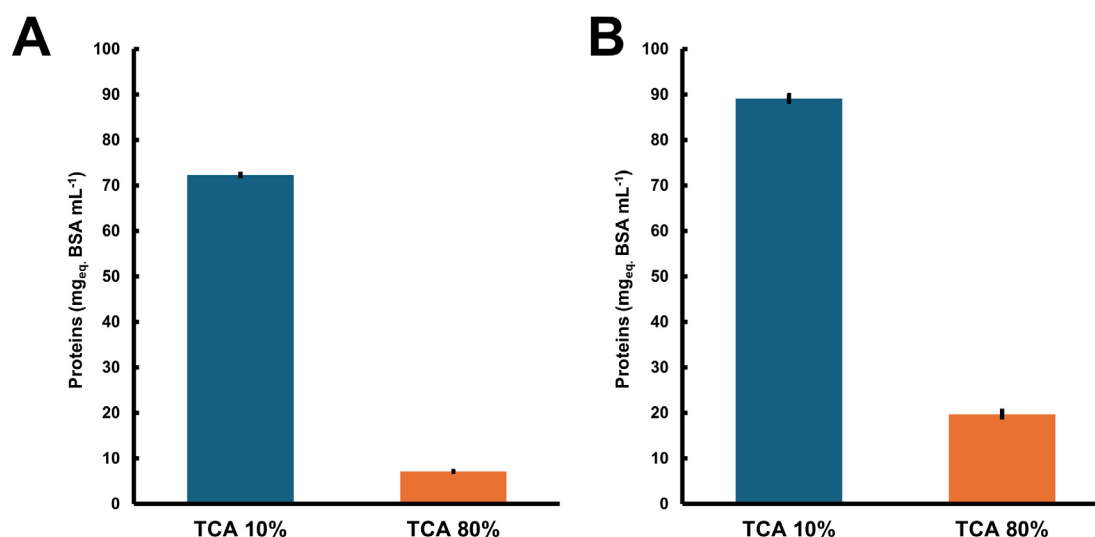


Figure 3. Comparison of the total protein content in EPS obtained from milk fermented with (A) kefir grains and (B) *Lactobacillus delbrueckii* susp. *bulgaricus* NCFB 2772, using two different trichloroacetic acid (TCA) concentrations during EPS extraction.

Figura 3. Comparación del contenido total de proteínas en los EPS obtenidos a partir de leche fermentada con (A) gránulos de kéfir y (B) *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772, utilizando dos diferentes concentraciones de ácido tricloroacético (TCA) durante la extracción de EPS.

CONCLUSIONS

This study shows that the concentration of trichloroacetic acid (TCA) used during EPS extraction plays a critical role in determining both the yield and structural integrity of the recovered material. Treatment with 10% TCA produced a higher yield of lyophilized solids with greater sugar content, indicating better preservation of polysaccharide structure. In contrast, 80% TCA achieved more efficient protein removal but caused a marked reduction in sugar content, likely due to acid-induced depolymerization of glycosidic linkages.

Overall, these results highlight the fact that mild acidic conditions favor the recovery of structurally intact EPS, while strong acid treatment compromises polymer stability and functional properties. Balancing protein removal efficiency with the preservation of polysaccharide integrity is therefore essential for optimizing EPS extraction protocols.

From a biotechnological standpoint, the use of 10% TCA represents a practical compromise between purification and preservation, offering a reliable strategy for obtaining functional EPS suitable for applications in food formulation, texture modification, or biofilm development. Future studies should focus on integrating selective enzymatic treatments (e.g., protease-assisted extraction) and physicochemical analyses (FTIR, GPC, rheological properties) to further refine EPS purification methods, and evaluate their techno-functional and bioactive properties.

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CONFLICTS OF INTEREST

The manuscript was prepared and reviewed with the participation of all authors, who declare that no conflicts of interest could compromise the validity of the results presented.

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