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Evaluation of Isolation Method of Exopolysaccharide Produced by *Lactobacillus Acidophilus*

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ABSTRACT

Exopolysaccharides (EPS) are produced by various microorganisms, including lactic acid bacteria (LAB), which are recognized as food-grade organisms. Furthermore, these compounds have been strongly suggested to possess health-promoting properties. This study examines the potential of two strains of Lactobacillus acidophilus, namely L. acidophilus LAC-1 and L. acidophilus ATCC 4356 which was used as a positive control strain for EPS production, to produce EPS when cultivated on MRS medium supplemented with 2% (w/v) lactose. The study also evaluates the performance of the EPS isolation method, particularly with regard to potential interference from proteinaceous materials in EPS quantification. Both strains exhibited comparable growth and EPS production patterns. Analysis of various EPS and supernatant fractions revealed the presence of interferents, including proteinaceous materials from the fermentation medium. These types of interferents likely interact with the carbohydrates in the medium, leading to an overestimation of the levels of crude EPS produced. Nonetheless, employing more efficient EPS isolation methods can mitigate this issue. In the current study, it was observed that by incorporating at least two purification/precipitation steps in the isolation process, the presence of sugars and proteins in the medium is significantly reduced, reaching in the case of protein a maximum reduction of 89% for L. acidophilus LAC-1 and 91% for L. acidophilus ATCC 4356 strains.

Keywords
Exopolysaccharides, Carbohydrates, Interferents, Extraction, Analytical chemistry.

Introduction

Lactic acid bacteria (LAB) are used in the food industry for the manufacture of several plant- and animal-fermented products. In the dairy industry, LAB are extensively employed owing to the capacity of certain strains to improve the flavour and texture of milk products [1,2]. LAB may produce exopolysaccharides (EPS) – which are either excreted in the growth medium as slime or remain attached to the bacterial cell wall, thus forming capsular EPS [3,4]. The interest in EPS has significantly increased in the last few years, because of technological advantages when used as additives in a wide variety of food products in which they serve as thickening, stabilizing, emulsifying or gelling agents [5]. In addition to those technological benefits, certain EPS produced by LAB are also claimed to have beneficial physiological effects on the consumer [6-8]. It has been speculated that the increased viscosity of EPS-containing foods may lead to larger residence time for the ingested fermented milk in the gastrointestinal tract, and therefore be beneficial to colonization by probiotic bacteria [9-12]. *L. acidophilus*, is a prominent species among LAB, inhabits both human and animal gastrointestinal tracts. Its metabolic activity contributes to the development of flavor and aroma, enhancing the sensory qualities of fermented foods while also

inhibiting spoilage [13-15]. Notably utilized in the production of yogurt, liquid yogurt, cheese, fermented cream, and dairy desserts, *L. acidophilus* also generates EPS, which substantially influences the texture, mouthfeel, and rheological properties of fermented dairy beverages and products [16-18].

L. acidophilus ATCC 4356 is a probiotic strain that has been studied for its therapeutic effects on various diseases and is known to produce EPS, which offer numerous health benefits [16,19-21]. *L. acidophilus* LAC-1 is another probiotic strain that has demonstrated good viability when incorporated into various whey cheese matrices and fermented foods and good resistance to simulated gastric [22-24]. It has also shown potential as an EPS-producing strain [25], making it promising for use in the food industry. The type of strain, the culture condition and the medium composition may influence the quantity of microbial EPS produced by a certain species [26,27]. In particular, the type of carbon source plays a major role upon EPS productivity and may also affect the composition of EPS itself [12,28]. EPS is typically produced at 10-1000 mgL^{-1} levels and are characterized by high molecular mass (above 10^6 Da) [29,30]. For many lactobacilli strains, such complex media as de Man Rogosa and Sharpe (MRS), All Purpose Tween (APT) and a synthetic medium (made with enriched milk ultrafiltrate or cheese whey) are currently used in studies focused on EPS production. The medium composition is a very important factor influencing EPS production, and some complex nutrients of the medium (e.g. beef extract, peptone and yeast extract) may interfere in the analysis of constituent monomers and associated structure [30-32].

Selection of an adequate EPS production medium is important and can have a major influence on effective EPS recovery. Not only does it impact production yield, but it can also introduce certain medium components that might not be effectively removed during the isolation process. These components could potentially interfere with detection and quantification methods, further emphasizing the importance of choosing an appropriate EPS production medium [4,33,34]. In the MRS broth medium, some of these interfering substances may include proteins and polymeric components containing carbohydrates. During the alcohol precipitation step in the EPS isolation method, these substances can be recovered alongside EPS, potentially leading to an overestimation of EPS quantification [34,35].

In EPS separation, isolating EPS from the culture medium supernatant is typically achieved through centrifugation, and EPS precipitation commonly involves one or more steps utilizing cold ethanol [4,36,37]. The degree of EPS purification attained will vary based on the study's objectives. It may be less critical when solely quantifying EPS production but becomes crucial when attempting to characterize the recovered EPS [38]. A critical step involves the selection of the appropriate methodology for isolating and purifying the EPS to ensure optimal yields along with a high degree of purity [37,39]. In cases where more complex media are used, additional pre-treatment steps become necessary to minimize the presence of proteins in the final EPS-rich extracts [40-42]. Most of the reported protocols involve removing cells through centrifugation, followed by EPS precipitation using a polar organic solvent that is miscible with water, such as chilled ethanol which is widely used for this purpose [38,43,44]. One of the frequently employed procedures for protein elimination involves precipitation with trichloroacetic acid (TCA) at final concentrations typically ranging between 4% and 20%. Alternatively, protein elimination can be achieved through digestion with proteases such as pronase E, proteinase K, or flavourzyme. In some cases, a sequential combination of both TCA precipitation and protease digestion is used. Following protein removal, centrifugation is performed to separate the precipitated proteins, and the EPS is subsequently concentrated through precipitation with ethanol [45-48].

In this study, *L. acidophilus* LAC-1 was assessed for its potential to produce EPS and *L. acidophilus* ATCC 4356 was used as an EPS production positive control strain. The effectiveness of the method for isolating the EPS obtained was evaluated based on its ability to eliminate potential interfering compounds from the culture medium. These compounds could otherwise interfere with the crude EPS quantification method.

Materials and Methods

Bacterial Strains and Culture Conditions

L. acidophilus LAC-1 was obtained as DELVO-PRO® freeze-dried concentrated starter culture from DSM Food Specialties (Moorebank, Australia); *L. acidophilus* ATCC 4356 which was used as a positive control strain for EPS production was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The strains were maintained frozen (-80 ºC) and were sub-cultured in 20 mL of MRS broth (Merck, Darmstadt, Germany) - (peptone from casein 10.0 g/L; meat extract 8.0 g/L); yeast extract 4.0 g/L; D(+)-glucose 20.0 g/L; dipotassium hydrogen phosphate 2.0 g/L; Tween® 80 1.0 g/L; di-ammonium hydrogen citrate 2.0 g/L; sodium acetate 5.0 g/L; magnesium sulfate 0.2 g/L; manganese sulphate 0.04 g/L) (1% (v/v)) inoculum, and incubated under anaerobic conditions (Gas-Pak Plus system, from Becton Dickinson, Maryland MA, USA) at 37 °C for 48 h. Before experimental use, the cultures were propagated twice in the same medium and environmental conditions.

Fermentation Performance

Batch fermentations took place in flasks containing 500 mL of De-Man Rogosa and Sharpe (MRS) broth media [49] with 2% (w/v) lactose. The experiments were carried out at 37 ºC, without pH control. A 5% (v/v) standard inoculum was prepared from each sub-culture of *L. acidophilus*, previously grown in the corresponding medium for 20 h at 37 ºC and was used to start up the fermentation batch. Fermentations lasted up to 24 h.

Sampling, Growth Determination and Chemical Composition

Samples were aseptically withdrawn from the fermentation vessel in duplicate at (0, 6 and 24 h), to determine viable cell numbers expressed in log CFU mL^{-1} (after plating on MRS agar), optical density was measured spectrophotometrically at 650 nm (OD_{650}).

The polymer dry mass was gravimetrically determined and then

used for analysis of total carbohydrate that was estimated by the phenol-sulfuric acid colorimetric test [50] that was determined based on a standard calibration curve prepared using glucose as standard (1.5 – 400 mg L^{-1}) and the samples were analyzed using a spectrophotometer at a wavelength of 490 nm. The protein concentrations were measured using the micro-protein determination kit (Micro Lowry, Onishi & Barr - TP0200, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), which employs the Biuret and Folin and Ciocalteau's Phenol as reagents, and bovine serum albumin (BSA) as the protein standard. The total residual sugars (viz. lactose and glucose) and organic acids were determined by high pressure liquid chromatography - HPLC system (Lachrom, Merck Hitachi, Germany) that was composed of an ion exchange Aminex HPX - 87H Column (300 x 7.8 mm), which was maintained at 65°C; and two detectors in series, refractive index and UV spectrophotometry (220 nm). The mobile phase used was 13 mM L^{-1} sulfuric acid, and the flow rate was 0.6 mL min^{-1}; the running time was 30 min, and the injection volume was 50 µL. Prior to analysis, all samples were pre-treated in order to eliminate protein interference; 1 mL of sample was thus precipitated with 200 µL of 35% (v/v) perchloric acid (PCA), allowed to stand for 10 min in ice, added with 55 µL of 7.0 M KOH (to neutralize PCA), centrifuged for 10 min at 4000 r.p.m, and the supernatant filtered through a 0.2 µm membrane filter [51]. All determinations were done in triplicate and expressed as mean values and associated standard deviations.

Isolation and Screening for EPS Production

The EPS isolation based on the method of Degeest and De Vuyst [42,52] was optimized. A flowchart of the protocol developed to isolated EPS in our study is shown in Figure 1. Aliquots of the cultures (5 mL) were aseptically withdrawn and heated at 100ºC for 15 min to inactivate enzymes potentially capable of polymer degradation. To precipitate proteins, one volume of 20% (v/v) trichloroacetic acid (TCA) was added to the culture, which was heated and stirred for 1 h. Cells and precipitated proteins were removed by centrifugation (4000 r.p.m, 20 min, 4ºC). The clear supernatant was collected, and the EPS was precipitated overnight with an iso-volume of chilled ethanol, followed by centrifugation of the precipitate (4000 r.p.m, 20 min, 4ºC). The resulting supernatant was recovered – SP1 whereas the pellet – EPS1 was redissolved in ultra-pure water; and the residual proteinaceous material was once again precipitated using one volume of 20% (v/v) TCA, removed by centrifugation, and EPS was once again precipitated as described above. The second fraction of supernatant – SP2 and pellet – EPS2 were duly collected. The different fractions collected in each step of the isolation method (EPS1, EPS2, SP1 and SP2) fractions were recovered by freeze-drying. The amounts were gravimetrically determined and then used for analysis of total carbohydrate, and protein by the methods previously described in 2.3.

Statistical Analyses

The study employed a series of statistical tests to evaluate different aspects of the data were conducted to assess the effect of the isolation method on the total yield of crude EPS and fractions collected from the method (EPS1, EPS2, SP1, SP2) and protein interferent from both strains under study over the fermentation time. Initially, the normality of the distribution was assessed using the Shapiro–Wilk test, with a significance threshold set at $p<0.05$. Subsequently, for variables that showed statistical significance, post hoc analysis was conducted using the Bonferroni method. Additionally, significant differences were explored through a two-way ANOVA, assuming homogeneity of variance at the $p<0.05$ significance level. The statistical software program utilized for these analyses was STATISTICA, version 14.0.0.15.

Results

Bacterial Growth and Exopolysaccharide Production

The MRS broth medium, supplemented with 20 g L^{-1} of lactose, was analyzed for various fractions of EPS and supernatant (usually discarded) to evaluate potential interferences.

The fermentation characteristics of both *L. acidophilus* strains at 24 h are summarized in Table 1. Regarding optical density measurements at 650 nm (OD_{650}) and viable cell numbers (log CFU mL^{-1}), both strains exhibited similar growth behavior in this medium and showed no statistically significant differences ($p>0.05$). The only exception was at time 0 h, where a slightly lower value was observed for *L. acidophilus* LAC-1. By 24 hours, *L. acidophilus* LAC-1 showed higher OD650 but lower viable cell numbers compared to *L. acidophilus* ATCC 4356.

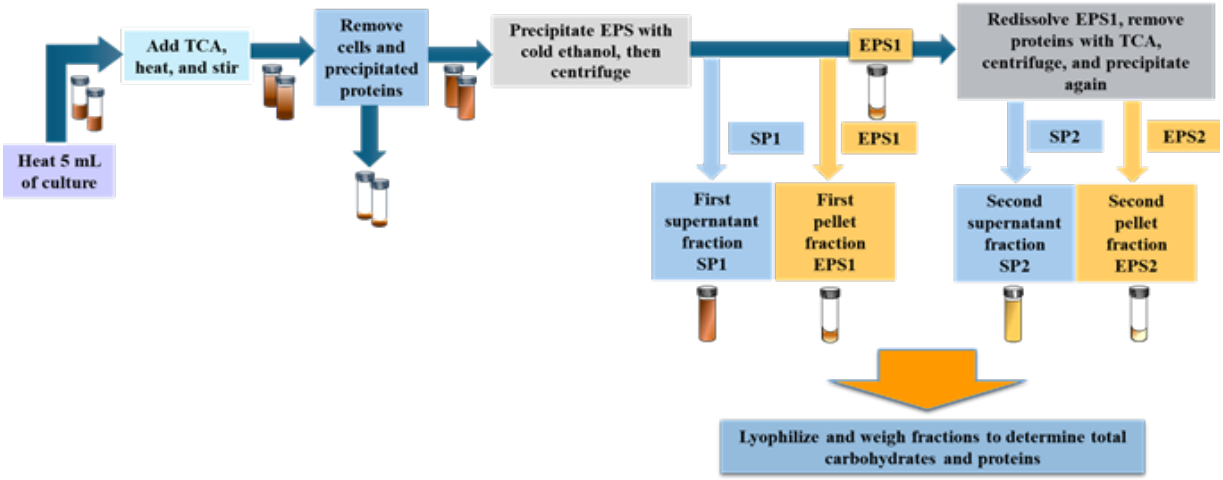

Figure 1: Flowchart of EPS isolation method optimized.

Regarding the EPS production, by 24 hours, *L. acidophilus* LAC-1 and *L. acidophilus* ATCC 4356 produced different concentrations of EPS1 and EPS2 with *L. acidophilus* ATCC 4356 showing slightly higher levels for both fractions but, no statistically significant differences (p>0.05) was found between the two strains (Table 1).

The EPS maximum concentration was observed after 6 h of fermentation, whereas a reduction in EPS concentration was observed by the end of the 24 h incubation period, this trend was confirmed for both strains. It was observed for both strains and for all fermentation times statistically significant differences (p<0.05) between the EPS1 and EPS2 fractions were observed. The EPS concentration values reported decreased with the degree of purification, that is, comparison between EPS1 with one precipitation step versus EPS2 with two precipitation steps involved. At the start of fermentation (0 h), EPS levels were already high for both *L. acidophilus* LAC-1 and ATCC 4356. This suggests that the values, measured using the phenol sulfuric acid method, might be influenced by contaminants in the culture medium that alone or complexed with medium carbohydrates are not eliminated during the isolation process, as similar EPS levels were observed in the uninoculated medium (results not shown).

The EPS dry mass values for both strains in the EPS1 and EPS2 fractions, obtained after freeze-drying and weighing, were higher compared to those quantified by the phenol sulfuric acid method. The amount of EPS measured as dry mass decreased with the degree of purification, showing statistically significant differences (p<0.05) between the EPS1 and EPS2 fractions for both strains. At 0 h of fermentation, significant protein concentrations were detected in EPS1 for both *L. acidophilus* LAC-1 and *L. acidophilus* ATCC 4356. However, a notable decrease in protein concentration was observed in EPS2 as the degree of purification increased for both strains. These values are likely associated with contaminants in the culture medium derived from nitrogen sources such as yeast extract, peptone, tryptone, cassitone, or beef extract present in the MRS medium. In the EPS1 fraction, protein levels at 6 hours were significantly higher (p<0.05) compared to other fermentation times for both strains. After 24 hours of fermentation, the analysis revealed contaminating protein concentrations in EPS1 and EPS2 for both *L. acidophilus* LAC-1 and *L. acidophilus* ATCC 4356. Evaluation of protein concentration at each precipitation step with TCA and ethanol showed a significant (p<0.05) reduction from the first EPS1 fraction to the second EPS2, with a maximum reduction of 89% for *L. acidophilus* LAC-1 and 91% for *L. acidophilus* ATCC 4356 strains.

Sugar and Lactic Acid Analysis

The monitoring of residual sugars and lactic acid (see Figure 2) revealed that both strains exhibited consistent behaviour throughout the fermentation process.

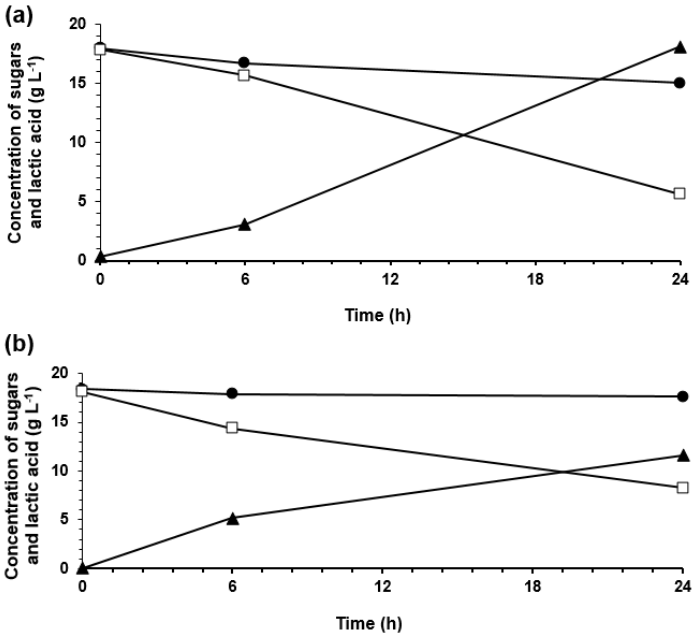

Figure 2: Consumption/production of lactose (●), glucose (☐) and lactic acid (▲) in a culture of (a) *L. acidophilus* LAC-1 and (b) *L. acidophilus* ATCC 4356.

The primary metabolic conversion observed involved the conversion of glucose into lactic acid through the homofermentative glycolytic degradation pathway. The initial concentration of glucose decreased from 18 g L^{-1} at the start of fermentation to 6 g L^{-1} after 24 h for *L. acidophilus* LAC-1 and from 18 to 8 g L^{-1} for *L. acidophilus* ATCC 4356.

Table 1: Bacterial growth, total EPS production, EPS dry mass and total protein concentration obtained from cultures of *L. acidophilus* LAC-1 and *L. acidophilus* ATCC 4356, using MRS broth supplemented with lactose. EPS1: Exopolysaccharide after first precipitation with ethanol; EPS2: Exopolysaccharide after second precipitation with ethanol; SD: Standard deviation using three replicate determinations.

Culture	Time (h)	OD_{650}	Cell number log CFU ml^{-1} ± SD	EPS1 mg L^{-1} ± SD	EPS2 mg L^{-1} ± SD	EPS1 Dry mass mg g^{-1} ± SD	EPS2 Dry mass mg g^{-1} ± SD	Protein in EPS1 mg L^{-1} ± SD	Protein in EPS2 mg L^{-1} ± SD
LAC-1	0	0.3	7.09 ± 0.10^{c}	297 ± 7^{a}	118 ± 12^{b}	3.5 ± 0.1^{ab}	2.5 ± 0.1^{b}	173 ± 5^{c}	19 ± 1^{d}
	6	0.86	8.36 ± 0.01^{a}	343 ± 35^{a}	153 ± 26^{b}	4.0 ± 0.2^{a}	2.3 ± 0.1^{bc}	252 ± 8^{a}	32 ± 1^{d}
	24	5.41	8.43 ± 0.02^{a}	265 ± 18^{a}	116 ± 20^{b}	3.6 ± 0.2^{a}	0.7 ± 0.0^{d}	$160 \pm 2c$	23 ± 2^{d}
ATCC 4356	0	0.22	7.57 ± 0.00^{b}	292 ± 2^{a}	114 ± 8^{b}	3.6 ± 0.5^{a}	2.8 ± 0.3^{b}	188 ± 5^{c}	18 ± 1^{d}
	6	1.29	8.59 ± 0.04^{a}	336 ± 12^{a}	141 ± 7^{b}	3.9 ± 0.5^{a}	2.5 ± 0.1^{b}	225 ± 8^{b}	22 ± 1^{d}
	24	3.64	8.67 ± 0.04^{a}	316 ± 7^{a}	118 ± 16^{b}	3.8 ± 0.5^{a}	0.4 ± 0.9^{d}	212 ± 5^{c}	19 ± 2^{d}

[a-d] Superscript different letters in the same row represent statistically different values (p< 0.05) between the two strains (*L. acidophilus* LAC-1 and *L. acidophilus* ATCC 4356) and across the evaluated parameters: cell number, as well as the pairwise analysis of EPS1 and EPS2, EPS1 and EPS2 dry mass, and protein content in EPS1 and EPS2.

In the case of the initial lactose concentration (18 g L^{-1}), *L. acidophilus* LAC-1 consumed only 3 g L^{-1}, while *L. acidophilus* ATCC 4356 did not utilize any of it. This may be due to lactose consumption occurring only when the levels of glucose were extremely low in the culture medium. The lactic acid content obtained after 24 h of fermentation was notably high, with 18 g L^{-1} for *L. acidophilus* LAC-1 and 12 g L^{-1} for *L. acidophilus* ATCC 4356. However, despite a thorough analysis, citric, formic, succinic, and acetic acids were not detected in the fermentation medium. Analysis of the total carbohydrates in supernatant fractions SP1 and SP2 (Table 2) indicates that in SP2, the levels were significantly lower (p<0.05) when compared to SP1 for both strains. For the SP1 supernatant, significant differences (p<0.05) in total carbohydrate values between the two strains were evident at 0 h and 24 h of fermentation. Conversely, in the case of the SP2 supernatant, no significant differences (p>0.05) were observed between the two *L. acidophilus* strains at any of the fermentation times.

Table 2: Total carbohydrates in SP1 and SP2 in cultures of *L. acidophilus* LAC-1 and *L. acidophilus* ATCC 4356. SP1 = Supernatant after first precipitation with ethanol; SP2 = Supernatant after second precipitation with ethanol; SD = Standard deviation using three replicate determinations.

	LAC-1		ATCC 4356	
Time (h)	SP1 g L^{-1} ± SD	SP2 g L^{-1} ± SD	SP1 g L^{-1} ± SD	SP2 g L^{-1} ± SD
0	36.80 ± 2.19^{b}	0.27 ± 0.04^{c}	39.71 ± 1.62^{a}	0.37 ± 0.02^{c}
6	29.54 ± 3.28^{b}	0.48 ± 0.01^{c}	33.63 ± 3.13^{b}	0.59 ± 0.02^{c}
24	26.66 ± 4.85^{b}	0.35 ± 0.01^{c}	8.27 ± 2.02^{d}	0.40 ± 0.03^{c}

$^{a-d}$ Superscript different letters in the same row represent statistically different values (p< 0.05) between the two strains (*L. acidophilus* LAC-1 and *L. acidophilus* ATCC 4356) for the evaluated parameters by pairwise analysis of SP1 and SP2.

A comparison of both supernatant fractions reveals that, in the first precipitation method step, the collected supernatant, SP1, as expected, contains the majority of the carbohydrates present in the culture medium. In the second precipitation method step, the resulting supernatant, SP2, showed a considerably lower carbohydrate content, which may be attributed to those carbohydrates that were not completely removed during the initial purification step.

Discussion

The growth patterns of *L. acidophilus* LAC-1 and *L. acidophilus* ATCC 4356 strains were similar (p>0.05). However, the *L. acidophilus* ATCC 4356 strain exhibited slightly higher growth capacity. Some authors have reported identical cell level results for *L. acidophilus* ssp. during fermentation in MRS medium.

Avonts et al. [53] in their study, observed that maximum viable cell numbers of 7.9 log CFU mL^{-1} were achieved for *L. acidophilus* IBB 801 after 24 h of fermentation and for *L. acidophilus* ATCC 4356 after 5 h of fermentation whereas 8.8 log CFU mL^{-1} were obtained for *L. johnsonii* La1 (formerly *L. acidophilus* La1) strain in MRS medium; this improved growth behaviour is in agreement with the results achieved in our study. Regarding the EPS production, both strains exhibited a similar production trend, reaching their maximum values by 6 h of fermentation subsequently experiencing a decline in production rates up to 24 h. In some studies, various LAB strains have reported EPS production ranging from 45 to 350 mg L^{-1} under non-optimized fermentative culture growth conditions, while values of 150 to 600 mg L^{-1} have been reported under ideal culture growth conditions [54-56].

Abedfar et al. [57] reported that *L. acidophilus* (LC_155899.1), isolated from rice bran sourdough, produced 268 mg L^{-1} of purified EPS in MRS broth under facultative anaerobic conditions at 37 °C for 24 h. Additionally, Deepak et al. [58] observed that *L. acidophilus* (10307) produced 180 mg L^{-1} of EPS when cultivated in MRS medium. In the aforementioned studies, the reported EPS production values were found to be within the same order of magnitude as those observed in our study for both strains.

The decrease in EPS concentration at 24 h observed for both *L. acidophilus* strains may be associated with polymer degradation, potentially due to the presence of glycohydrolase activity, which could contribute to the gradual breakdown of the polymer. Several authors [59-61] have suggested that an increase in incubation time, particularly during extended fermentation, leads to a decrease in the total EPS amount. This phenomenon could be attributed to either enzymatic degradation, which may vary by strain, or alterations in the culture conditions (temperature, pH value, carbohydrate source, etc.). Another explanation for the decrease in EPS at 24 h could be attributed to the higher acidification of the culture medium resulting from lactic acid production, which peaks at 24 h for both strains. The pH of the fermentation medium is one of the critical factors for the biosynthesis of bacterial exopolysaccharides [62-64]. This increase in acidity may have contributed to the decline in EPS production between 6 h and 24 h, especially considering that the batch fermentations were conducted without pH control. Notably, the decline in EPS concentration from 6 h to 24 h seems more pronounced in the case of the *L. acidophilus* LAC-1 strain compared to *L. acidophilus* ATCC 4356 which correlates well with the 1,5-fold higher lactic acid production by *L. acidophilus* LAC-1 (18 g L^{-1}) than by *L. acidophilus* ATCC 4356 (12 g L^{-1}).

Most of the reported procedures for isolating EPS from complex media involve TCA precipitation to separate cells and remove proteins through centrifugation. Subsequently, EPS is precipitated using a water-miscible polar organic solvent and chilled ethanol precipitation, a method commonly employed to concentrate the EPS [54,65,66]. The selection of an appropriate method for EPS isolation is a crucial prerequisite for EPS quantification, and it depends on the culture medium used. This is because complex media used for EPS production may introduce interference from their components in the EPS analysis. Thus, pre-treatment and additional purification steps may be necessary [4,37].

In this study, besides evaluating the EPS production capacity of *L. acidophilus* strains, it was crucial to assess the isolation method and understand how its purification steps could impact the final quantification of the EPS produced by the strains. We observed

that when an additional purification step was included in the EPS isolation process, the values significantly decreased from EPS1 to EPS2 by the end of fermentation, reaching 58% for *L. acidophilus* LAC and 37% for *L. acidophilus* ATCC 4356 positive control strain for EPS production at 24 h.

This reduction was expected, as, regardless of the isolation method used, an increase in the degree of EPS purification tends to result in a decrease in the amount of precipitated EPS. This reduction may be associated with the loss of EPS1 since EPS pellets can be easily lost during the purification process, leading to underestimated results due to the potential loss of polysaccharides that might occur during the isolation steps [38].

Another explanation for the decrease in EPS values from the first purification stage to the second stage may be attributed to the enhanced purity of the EPS. As the degree of purification increases, potential interferences, such as proteinaceous materials present in the fermentation medium, are progressively eliminated. The use of TCA precipitation in various EPS isolation methods allows for obtaining more purified EPS by facilitating the precipitation and removal of contaminating materials like proteins or polypeptides from complex culture media. Rimada et al. [38] demonstrated that the methods involving TCA precipitation reduced contamination by approximately 50%. Therefore, while TCA precipitation enables the isolation of purer EPS fractions, it can also result in the loss of EPS due to the coprecipitation of the biopolymer with proteins. This reduction in the recovered EPS amount aligns with our study, where a similar level of reduction for both strains was observed in EPS2 when a second purification step with TCA was employed. Furthermore, TCA itself can potentially introduce contamination because it is not entirely removed by the isolation method due to its hygroscopic properties, which may contribute to an increase in the mass of the EPS. Likewise, other authors have reported that various components of the culture medium, as well as complex synthetic media (with or without specific carbohydrates), whether originating from the media itself or frequently added to stimulate bacterial growth, can potentially introduce interference when chemical methods are employed for total carbohydrate determination in the quantification of EPS [34,67].

In this study, we observed that, with the selected isolation method, a substantial portion of the carbohydrates in the culture medium remains in the supernatants (SP1) for both strains during the first purification step using 20% (v/v) TCA and ethanol precipitation (Table 2). However, when we introduced a second purification/precipitation step to the isolation process, although carbohydrates were not completely eliminated, a substantial reduction in carbohydrates was observed in the SP2 supernatants for both *L. acidophilus* strains in a range of 0.27 – 0.59 g L^{-1}.

Considering the low concentrations of sugars observed in SP2, our results suggest that when a second purification step is incorporated into the EPS isolation method, the likelihood of carbohydrate interferences in the quantification of EPS in EPS2 is diminished. A single purification/precipitation step alone was insufficient to eliminate all carbohydrates from the medium.

The EPS values obtained at the beginning of fermentation for both EPS1 and EPS2, regardless of the strain, primarily correspond to the quantification of the culture medium components. This is because at 0 h of fermentation, it is not expected that EPS has been produced yet. These findings highlight that the commonly used MRS medium for growing LAB and EPS production introduces contaminants into EPS-rich extracts. The complex medium ingredients present in MRS medium, including yeast extract (a source of mannoproteins and other glycans), beef extract (with high glycogen content), and proteose peptone, are likely responsible for introducing proteinaceous material that can interfere with EPS quantification. Kimmel et al. [31] found that these interfering substances in MRS broth accounted for 94% of the total background EPS-equivalents and even after 48 h of dialysis, some of these interfering substances could not be removed. To overcome the issue of culture medium interference in studies involving EPS quantification, it is common practice to subtract the total carbohydrate values of the non-inoculated culture medium from the carbohydrate values of the inoculated medium at all fermentation time points to obtain a more accurate EPS value. However, Torino et al. [34] emphasized the need for caution when employing this approach, as it may not always yield the most accurate results. The EPS value obtained through this method can potentially be either inflated or underestimated. On one hand, certain compounds present in the culture medium, such as yeast extract, beef extract, and peptone, can interact with the carbohydrates in the medium, forming carbohydrate-nitrogen complexes. These complexes may precipitate along with EPS during the ethanol precipitation step, leading to an inflation of the EPS quantity. On the other hand, some of these interfering compounds in the culture medium are metabolized by microorganisms during their growth, often through metabolic pathways different from those used for EPS synthesis. As a result, subtracting these values from their respective EPS values can lead to results that fall below the estimated values, especially when dealing with strains that produce reduced quantities of EPS. Consequently, the interference level (EPS-equivalent) in fermented media may be lower than that in non-inoculated media.

Vaningelgem et al. [68] also concluded that the presence of glucomannans derived from yeast extract and peptone in the culture medium represents the carbohydrate polymeric material that causes interference in the quantification of EPS produced in complex culture media. Consequently, if the strain being studied utilizes materials that react with carbohydrates during growth, the level of interference in the fermentation medium will be reduced, particularly as fermentation progresses towards its conclusion, as these compounds are likely to have been consumed by the strains.

Higher protein values were observed at 6 h compared to 24 h for both *L. acidophilus* strains in both EPS1 and EPS2 fractions, exhibiting a similar pattern to that observed for EPS production. This phenomenon may be attributed to interfering substances that could raise the quantified protein concentrations. Specifically, certain substances have the potential to interfere with the protein

quantification method used, such as the Micro Protein Assay Kit, which relies on colorimetric detection and can be influenced by various reducing agents [69].

Considering that the maximum EPS production was observed at 6 h for both strains, it is conceivable that there is a higher concentration of reducing sugars present in the medium at this fermentation time, potentially intensifying interference with the protein quantification method. Additionally, since the EPS obtained in our study is crude EPS and has not undergone further purification steps as a subsequent process of dialysis, it may further intensify these interfering factors, thereby justifying the observed increase in protein concentration at 6 h of fermentation.

Upon evaluating the protein concentration in both EPS fractions (EPS1 and EPS2), we observed that, while the protein was not entirely eliminated, its concentration was significantly reduced from the first to the second purification step. This leads to the conclusion that two purification steps involving 20% (v/v) TCA followed by ethanol precipitation effectively decrease the amount of protein material contaminating the EPS. Our results align with the findings of Rimada et al. [38], who determined that a two-step ethanol precipitation method was the most effective for isolating kefir polysaccharides from milk and whey. Hence, it is evident that while TCA effectively removes contaminants from the culture medium, such as proteins or polypeptides, the choice of precipitation methods involving TCA must be assessed in line with the study's objectives. This is because it can also lead to the loss of EPS due to the co-precipitation of the biopolymer with proteins. Therefore, when the study's goal is to characterize EPS, preference should be given to EPS precipitation methods utilizing TCA, as they yield EPS with a higher degree of purity.

Conclusion

According to the results reported the ability of *L. acidophilus* LAC-1 and *L. acidophilus* ATTC 4356 (positive control strain for EPS production) strains to produce EPS was confirmed, and EPS production reached a maximum concentration at 6 h followed by a decrease at 24 h of fermentation for both tested strains. Regarding residual sugars, it was found that both strains consumed glucose preferentially, over the 24 h fermentation period, which was converted via glycolytic degradation homofermentatively to lactic acid, this conversion being more pronounced for the *L. acidophilus* LAC-1 strain, which showed higher lactic acid concentrations after 24 h. The isolation method tested in the present study, which includes two purification steps with 20% (v/v) TCA and ethanol precipitation, proved to be effective in eliminating carbohydrates and interfering proteins from the culture medium. The results revealed that a single purification/precipitation step was not enough to eliminate interference from the MRS medium with the addition of 2% (w/v) lactose and that the method used with two purification steps was more efficient in eliminating carbohydrates and proteins from the medium. If on the one hand the two purification/precipitation steps can lead to some loss of EPS throughout the isolation process, on the other hand our results have shown that it allows to eliminate a large part of the interferents that can interfere with the analytical quantification methods, causing the EPS results to be overestimated. Based on our findings, we can conclude that both strains demonstrated the capacity to produce EPS, making them promising candidates for industrial applications. The EPS isolation method assessed in our study proved to be effective in removing interferences from the medium, specifically proteins from the culture medium. This method may be considered suitable for isolating EPS produced by the two *L. acidophilus* strains from a complex culture medium.

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