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# Use of Legume Wastewater Extracts on the Storage Stability of Freeze-Dried *Lactiplantibacillus plantarum* WCFS1

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## Abstract

Chickpeas and lentils contain prebiotic carbohydrates, including galacto-oligosaccharides (GOS), that confer health benefits but can also lead to undesirable effects like bloating and flatulence. Legume processing reduces these disadvantages and usually consists of the soaking and cooking of the legume seeds. In their industrial production, the processing water is considered waste. As different carbohydrates have recognized stabilization properties on lactic acid bacteria during dehydration processes, this study aimed to investigate the protective effect of GOS-containing wastewater extracts from cooking chickpeas and lentils, as well as fructo-oligosaccharides, and sucrose (used for comparative purposes), on the stability of freeze-dried *Lactiplantibacillus plantarum* WCFS1, by measuring biological (culturability, storage stability, acidifying activity), chemical (pH), and physical (water content) properties. The best storage stability was observed for mixtures with GOS-containing wastewater legume extracts. Protection of lactic acid bacteria strains with GOS-containing wastewater legume extracts would limit their spoilage in food production, positively impacting the environment.

**Keywords** Freeze-drying · Galacto-oligosaccharides · Lactic acid bacteria · Pulses · Viability · Wastewaters

## Abbreviations

BF	Before freezing
C-BW	Chickpea “boiling” water
C-CW	Chickpea cooking water
CFU	Colony-forming unit(s)
DSC	Differential scanning calorimetry

DP	Degree of polymerization
F	Freezing
FD	Freeze-drying/dried
FOS	Fructo-oligosaccharide(s)
GOS	Galacto-oligosaccharide(s)
L-BW	Lentils’ “boiling” water
L-CW	Lentil cooking water
MRS	De Man, Rogosa, and Sharpe
PCA	Principal component analysis
RH	Relative humidity
SUC	Sucrose
Tg	Glass transition temperature

Gonçalo Nuno Martins and María Guerrero Sánchez contributed equally to this work.

## Highlights

- Legume wastewaters were used for protecting *Lactiplantibacillus plantarum* WCFS1.
- Wastewaters containing GOS were better than FOS and sucrose.
- *Lactiplantibacillus plantarum* WCFS1 survived in storage at 37 °C for 3 weeks.
- Boiling legumes without soaking provided the best protective compounds.

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## Introduction

Chickpeas (*Cicer arietinum* L.) and lentils (*Lens culinaris Medikus* var. *variabilis*) are two of the most important legume crops worldwide (Jukanti et al., 2012; Ninou et al., 2019). Traditionally, they have been consumed in developing countries, where protein-energy malnutrition is prevalent because they are a staple food and a low-cost source of protein. However, in recent years, the inclusion of these legumes in daily diet has grown significantly in North America, Australia, and many European countries, mainly due as a result of consumers increasingly healthy and balanced diet choices (Madurapperumage et al., 2021; Ninou et al., 2019; Siddiq et al., 2022; Sidhu et al., 2022).

Chickpeas and lentils have a high nutritional quality and are a good source of carbohydrates and proteins, together constituting about 80% of the total dry seed mass (Siddiq et al., 2022). Legumes' carbohydrates contain a wide range of prebiotic substances including galacto-oligosaccharides (GOS), particularly from the raffinose family of oligosaccharides, and fructo-oligosaccharides (FOS) (Johnson et al., 2020; Martins et al., 2019). These prebiotic carbohydrates are selectively utilized by the host microorganisms and fermented into active metabolites (short-chain fatty acids, branched-chain fatty acids, vitamins, bile acid derivatives) conferring significant human health profits (Gibson et al., 2017; Johnson et al., 2020). Benefits of legumes include treating and preventing constipation, controlling cholesterol levels, and reducing the risk of obesity, diabetes, and certain types of cancer (Maphosa & Jideani, 2017; Martins et al., 2019; Siddiq et al., 2022). Despite these known benefits, chickpeas and lentils contain several compounds that have "anti-nutritional" activity (phytic acid, tannic acid), inhibit mineral absorption, have a bitter taste, increase cooking time, or are non-digestible ( $\alpha$ -GOS) and can cause bloating and flatulence (Munthali et al., 2022; Ninou et al., 2019; Njoumi et al., 2019). In order to reduce the undesirable attributes of chickpeas and lentils, as well as to increase the nutrients' availability and improve their organoleptic characteristics, legume processing methods are applied (Njoumi et al., 2019; Sharma et al., 2022).

Legume processing frequently consists of two main steps: soaking and cooking. Soaking increases hydration, softening the seed coat, and making it easier for water to penetrate during cooking, allowing for faster cooking. The soaked seeds can then be cooked in different ways, but boiling is the easiest and most common method. Cooking improves palatability causing textural changes and chemical reactions that produce volatile compounds important for flavor development (Chigwedere et al., 2019; Serventi, 2020). In the industrial production of legumes, the water resulting from their processing is considered waste and known as *aquafaba* or *liluva*. While *aquafaba*

refers to chickpea cooking water, *liluva* consists of water from different legumes and different processing methods (Hippolite et al., 2023). The global legume market (chickpeas, lentils, and other grains) represented a CAGR 11.5 Billion US dollars in 2022 (Market Research Future, 2024). Also in 2022, chickpeas and lentil production was around 18.1 (Shahbandeh, 2024) and 6.7 (FAOSTAT, 2023) million metric tons, respectively. Given a joint chickpea and lentil production of 24.7 million metric tons, assuming 1/10 of these legumes are sold pre-cooked or canned (2.47 million metric tons), and a standard 1:4 grain to water ratio is used in their cooking, a rough estimate of the amount of cooking wastewater produced would be 9.88 million metric tons, or around 10,000 million liters. Recycling legume wastewater would limit their wastage, positively impacting the environment (Hippolite et al., 2023; Serventi, 2020). Several studies have shown the potential of legume wastewater in food applications as texture improvers (foaming agents, gel lingers, thickeners) and as a source of prebiotics. In addition, these waters have a high content of proteins, soluble and insoluble carbohydrates, saponins, and minerals (Huang et al. 2017; Mustafa et al., 2018; Serventi et al., 2018; Stantiall et al., 2018).

*Lactiplantibacillus plantarum* is a lactic acid bacterium with a large and flexible genome that helps it adapt to diverse habitats including dairy products, fruits, vegetables, sausages, the digestive tract/feces of animals and humans, and soil. For decades, *L. plantarum* strains have been used as starter cultures in the production of fermented products because of their organoleptic properties and as probiotic cultures because of their beneficial properties such as antimicrobial activity, immunological effects, and modulation of the intestinal microbiota (Behera et al., 2018; Carboni et al., 2023; Echegaray et al., 2023; Garcia-Gonzalez et al., 2021).

Freeze-drying is a standard method for preserving probiotic strains facilitating their transportation and storage. However, it exposes the strains to environmental changes that can lead to structural cell damages, or even their death (Broeckx et al., 2016; Fonseca et al., 2015). A recent review by Penha Rodrigues Pereira et al. (2024) describes the main challenges faced in increasing probiotic survival and how to tackle them. To limit the encountered stresses' potential negative effects during the freeze-drying process and to improve the survival of bacteria after storage, the addition of protective molecules to the cell concentrates is an effective strategy (Chen et al., 2023; Guerrero Sanchez et al., 2022). Sugars such as sucrose and prebiotics such as FOS and GOS have been demonstrated to be efficient protective compounds of lactic acid bacteria after freeze-drying and storage stages (Gagneten et al., 2024; Romano et al., 2016; Tymczynszyn et al., 2011). They are commonly used to preserve probiotic strains because they can replace water molecules removed during drying, thus maintaining membrane integrity. Additionally, they have the ability

to form glassy states with high viscosity and low molecular mobility, which limits diffusive damaging reactions (Gagnetenet et al., 2024; Golowczyk et al., 2013; Romano et al., 2016; Sosa et al., 2016; Tymczynszyn et al., 2011).

It was recently reported that *L. plantarum* CIDCA 83114 was able to grow in a culture medium prepared with GOS-containing wastewater legume extracts (Martins et al., 2023). In addition, the wastewater from cooking dry chickpeas was capable of protecting *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 during freeze-drying and storage by forming a protective glassy matrix surrounding the bacterium (Passot et al., 2024). In this work, we intend to continue studying the applicability of these waste products, namely the cooking wastewaters obtained from lentil seeds. Four GOS-containing wastewater extracts were obtained by applying different procedures to chickpeas and lentils and were repurposed for the protection of lactic acid bacteria during freeze-drying and storage. The GOS mixtures' protective capability over *L. plantarum* WCFS1 during the freeze-drying and storage was studied by determining the biological activity (culturability, storage stability, acidifying activity) and chemical (pH) and physical (water content) properties of the cell concentrates.

## Materials and Methods

The experimental approach used in this study and the activities measured are shown in Fig. 1.

## Chickpeas and Lentil Seeds' Wastewaters

### Production

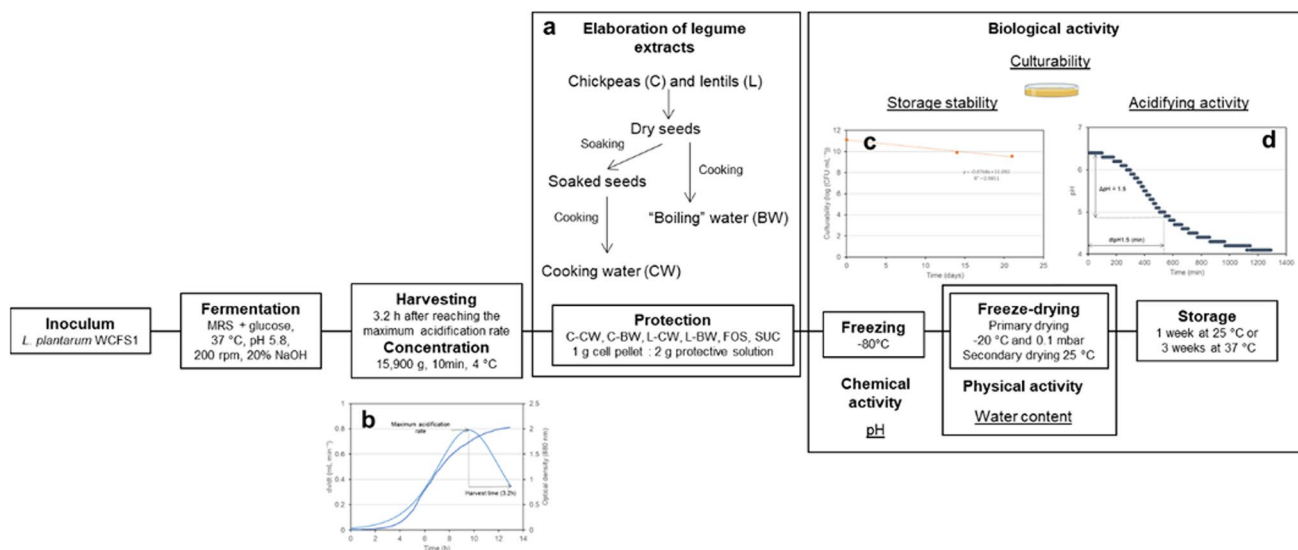
Chickpea and lentil seeds were provided by Continente (Matosinhos, Portugal) and Don Elío (INTA Arroyo Seco, Argentina), respectively. The seeds were kept at 25 °C in dry conditions throughout the making of this work. GOS-containing wastewater extracts were prepared as described previously by Martins et al. (2023). Briefly, cooking waters were produced from raw and soaked chickpeas and lentil seeds in a 1:5 seeds-to-water mass ratio and cooked for 30 min (Fig. 1a). The supernatants were separated and freeze-dried. In total, four extracts were produced: chickpea cooking water (C-CW), chickpea "boiling" water (C-BW), lentil cooking water (L-CW), and lentil "boiling" water (L-BW).

### High-Performance Liquid Chromatography

The carbohydrate content of the produced legume extracts was determined by high-performance liquid chromatography with refractive index detection as described previously by Martins et al. (2023) and may be found in Table 1.

### Glass Transition Temperature

The glass transition temperature of the freeze-dried GOS mixtures ( $T_g$ , in °C) was determined by differential scanning



**Fig. 1** Diagram of the experimental approach used in this study and showing the activities measured. (a) Experimental procedures for the production of GOS-containing wastewater extracts. (b) Cell growth and acidifying activity of *L. plantarum* WCFS1 showing when the cells were harvested. (c) Method used for determining the storage stability of *L. plantarum* WCFS1 using the slope value of the linear

regression. (d) Method used for determining the acidifying activity of *L. plantarum* WCFS1 using the time necessary for a pH drop of 1.5 units. C-CW, chickpea cooking water; C-BW, chickpea boiling water; L-CW, lentil cooking water; L-BW, lentil boiling water; FOS, fructooligosaccharide; SUC, sucrose

**Table 1** Carbohydrate composition and  $T_g$  values of the GOS-containing legume extracts, the commercial FOS sample, and Suc used in this work as determined by <sup>a</sup>Martins et al. (2023), <sup>b</sup>Romano et al. (2016), and <sup>c</sup>in this work

		GOS				FOS (Beneo Orafti)	Suc
		C-CW	C-BW	L-CW	L-BW		
% (g/100 g of dry matter)	Monosaccharides	5 <sup>a</sup>	4 <sup>a</sup>	2 <sup>a</sup>	3 <sup>a</sup>	3 <sup>b</sup>	0 <sup>b</sup>
	Disaccharides	18 <sup>a</sup>	18 <sup>a</sup>	4 <sup>a</sup>	4 <sup>a</sup>	5 <sup>b</sup>	100 <sup>b</sup>
	DP=3	23 <sup>a</sup>	21 <sup>a</sup>	11 <sup>a</sup>	10 <sup>a</sup>	25 <sup>b</sup>	0 <sup>b</sup>
	DP=4	14 <sup>a</sup>	14 <sup>a</sup>	15 <sup>a</sup>	13 <sup>a</sup>	29 <sup>b</sup>	0 <sup>b</sup>
	DP≥5	2 <sup>a</sup>	2 <sup>a</sup>	8 <sup>a</sup>	6 <sup>a</sup>	38 <sup>b</sup>	0 <sup>b</sup>
	Total oligosaccharides	39 <sup>a</sup>	37 <sup>a</sup>	34 <sup>a</sup>	29 <sup>a</sup>	92 <sup>b</sup>	0 <sup>b</sup>
$T_g$ (°C)		23.33 <sup>c</sup>	41.00 <sup>c</sup>	47.83 <sup>c</sup>	40.22 <sup>c</sup>	46.92 <sup>b</sup>	40.28 <sup>b</sup>

C-CW chickpea cooking water, C-BW chickpea boiling water, L-CW lentil cooking water, L-BW lentil boiling water, FOS fructo-oligosaccharide, Suc sucrose, DP degree of polymerization

calorimetry (DSC) using a Q100 calorimeter calibrated with indium, lead, and zinc (TA Instruments-Waters LLC, New Castle, DE, USA). Samples were equilibrated at 10% relative humidity (RH) with a saturated solution of NaOH. Equilibration lasted *ca.* 2 weeks. Five milligrams of each sample was weighed in gold capsules and hermetically sealed. An empty pan was used as a reference. The thermal history of each sample was removed, and then the samples were heated from  $-60$  to  $75$  °C with a heating ramp of  $10$  °C/min. Measures were taken in duplicate.

## Production and Storage of *L. plantarum* WCFS1 Concentrates

### Bacterial Strain and Culture Conditions

The strain used in this study was *L. plantarum* WCFS1 (NIZO Food Research B.V., The Netherlands). Twenty-five microliters of stock culture stored at  $-80$  °C was thawed at  $40$  °C for 5 min before inoculating in 10 mL of MRS broth (Scharlau Microbiology; Barcelona, Spain). This pre-culture was incubated at  $37$  °C for 18 h, and then 80  $\mu$ L was used to inoculate a second pre-culture containing 10 mL of MRS broth. The second pre-culture was incubated at  $37$  °C for 10 h, after which 10 mL was used to inoculate a 5-L bioreactor (Biostat® B Type 8,840,326, B. Braun Biotech International GmbH; Melsungen, Germany) containing 4.99 L of culture medium.

The culture medium, sterilized at  $121$  °C for 20 min, was composed of MRS broth supplemented with  $40$  g·L<sup>-1</sup> glucose (ADM; Chicago, IL, USA) to avoid starvation stress caused by the depletion of the carbon source after reaching the stationary growth phase. Fermentation was carried out at  $37$  °C and pH 5.8, which was controlled by the automatic addition of a 20% (w/v) NaOH solution. The stirring was set at 200 rpm to ensure the culture homogenization.

Cell growth and acidifying activity were monitored throughout fermentation. Cell growth was monitored by an

infra-red probe (Excell210; CellID, Roquemaure, France), measuring absorbance at 880 nm. Acidifying activity was determined according to the volume of NaOH solution injected into the bioreactor during fermentation to maintain a controlled pH. The maximum acidifying rate (dv/dt, in mL·min<sup>-1</sup>) was determined as the peak of the first derivative of the NaOH consumption curve. Cells were harvested 3.2 h after reaching the maximum acidifying rate (Fig. 1b), corresponding to the early stationary growth phase. Three fermentations were carried out in order to consider biological replicates.

### Concentration and Protection Conditions

Cell suspensions were concentrated by centrifugation (Avanti® JXN-30 centrifuge, Beckman Coulter; CA, USA) at 15,900 g for 10 min at  $4$  °C. Cell pellets were re-suspended in protective solutions at 23% (w/w) dry matter, using a 1:2 cell pellets-to-protective solution weight ratio. The protective solutions were prepared at 20% (w/w) in  $8.9$  g·L<sup>-1</sup> NaCl and sterilized by autoclaving at  $121$  °C for 20 min. Six protective agents were evaluated: the four GOS-containing wastewater extracts produced as described previously by Martins et al. (2023), a commercial FOS sample (Orafti®P95; Beneo Orafti; Tienen, Belgium), and sucrose (Azucarera; Madrid, Spain) (SUC), whose composition and  $T_g$  values is in Table 1. Freeze-dried cells in the presence of sucrose were included for comparative purposes as they represent the typical formulation for long-term storage of lactic acid bacteria.

### Stabilization and Storage Conditions

Protected cell concentrates were frozen at  $-80$  °C for 24 h in cryotubes. The cryotubes were then transferred to pre-cooled shelves at  $-45$  °C in a pilot-scale freeze-dryer (VirTis Genesis 35 L SQ EL-85, SP Scientific; Warminster, PA, USA). The primary drying was carried out at

a temperature of  $-20\text{ }^{\circ}\text{C}$  and at a chamber pressure of 0.1 mbar and the secondary drying at a temperature of  $25\text{ }^{\circ}\text{C}$ . The freeze-dried samples were stored for 1 week at  $25\text{ }^{\circ}\text{C}$  or for 3 weeks at  $37\text{ }^{\circ}\text{C}$ . Aliquots of the protected cell concentrates were also maintained in cryotubes at  $-80\text{ }^{\circ}\text{C}$  for analyzing the frozen samples.

The process from production to storage of *L. plantarum* WCFS1 concentrates was repeated three times (three biological replicates). In addition, each measurement (culturability, storage stability, acidifying activity, pH, and water content) was measured in triplicate (three technical replicates).

### Biological Activity of *L. plantarum* WCFS1 Concentrates

Biological activity measurements were carried out before and after freezing, after freeze-drying, after 1 week of storage at  $25\text{ }^{\circ}\text{C}$ , and after 2 and 3 weeks of storage at  $37\text{ }^{\circ}\text{C}$ . Before analysis, frozen samples were thawed at room temperature for 5 min, and freeze-dried samples were rehydrated using the same volume of  $8.9\text{ g}\cdot\text{L}^{-1}$  NaCl present before freeze-drying and incubated for 30 min at  $37\text{ }^{\circ}\text{C}$ .

### Culturability Measurements

Cell culturability was determined using the agar plate count method. Fresh, thawed, and rehydrated cell suspensions were serially diluted in  $8.9\text{ g}\cdot\text{L}^{-1}$  NaCl, and 100  $\mu\text{L}$  of the appropriate dilutions was spread into MRS agar plates (VWR International Eurolab; Barcelona, Spain) in triplicate (three technical replicates). Colonies were enumerated (Scan@ 500, Interscience; Saint Nom la Bretèche, France) after incubating the plates for 48 h at  $37\text{ }^{\circ}\text{C}$  in anaerobic conditions. Plates containing between 30 and 300 colonies were kept for cell concentration evaluation. Culturability measurements were expressed in  $\log(\text{CFU}\cdot\text{mL}^{-1})$ .

### Accelerated Storage Stability Test

Storage stability was evaluated from the culturability loss rate during 3 weeks of storage at  $37\text{ }^{\circ}\text{C}$  (Fig. 1c), as previously described (Guerrero Sanchez et al., 2022). For each protective formulation and biological replicate, the logarithmic value of the cell count ( $\log(\text{CFU}\cdot\text{mL}^{-1})$ ) was plotted as a function of the storage time ( $t$ , in days). The experimental values were fitted using the following equation:

$$\text{Cell count}(\log(\text{CFU} \cdot \text{mL}^{-1})) = -K \times t(\text{days}) + b$$

where the inactivation rate constant  $K$  (in  $\log(\text{CFU}\cdot\text{mL}^{-1})\cdot\text{days}^{-1}$ ) refers to the absolute value of the slope

of the linear regression. The lower the absolute value of the slope  $K$ , the higher the storage stability was.

**Acidifying Activity** The Cinac® system (AMS Alliance; Frépillon, France) was used to measure the acidifying activity of the bacterial suspensions according to the procedure described by Spinnler and Corrieu (1989). The measurements were performed in a  $37\text{ }^{\circ}\text{C}$  water bath. The growth medium composed of  $10\text{ g}\cdot\text{L}^{-1}$  glucose and  $10\text{ g}\cdot\text{L}^{-1}$  yeast extract (Condalab; Madrid, Spain) was sterilized at  $121\text{ }^{\circ}\text{C}$  for 20 min before being distributed into 250 mL PYREX® round bottle. Each bottle contained 150 mL of medium and was inoculated with 100  $\mu\text{L}$  of fresh, thawed, or rehydrated cell suspensions diluted 10 times in  $8.9\text{ g}\cdot\text{L}^{-1}$  NaCl. For each sample, the acidifying activity was characterized as the time necessary for a pH drop of 1.5 units (dtpH 1.5, in min). The lower the value of the dtpH 1.5 was, the higher the acidifying activity was (Fig. 1d).

### Chemical and Physical Properties of *L. plantarum* WCFS1 Concentrates

#### pH

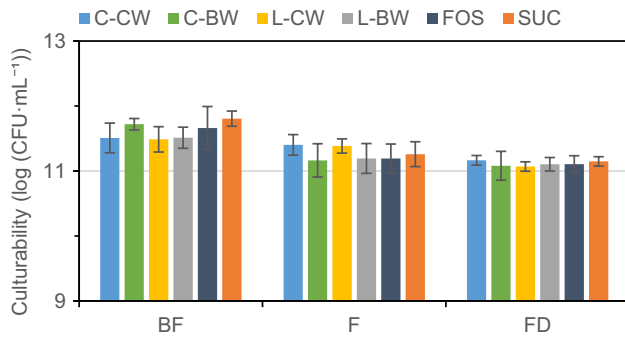
The pH of *L. plantarum* WCFS1 concentrates was measured at room temperature using a portable pH meter (LAQUA PH1100, Horiba Scientific®; Tokyo, Japan), on freeze-thawed and rehydrated freeze-dried samples prepared according to the procedure described above in the section of biological activity (“[Biological Activity of \*L. plantarum\* WCFS1 Concentrates](#)” section).

#### Water Content

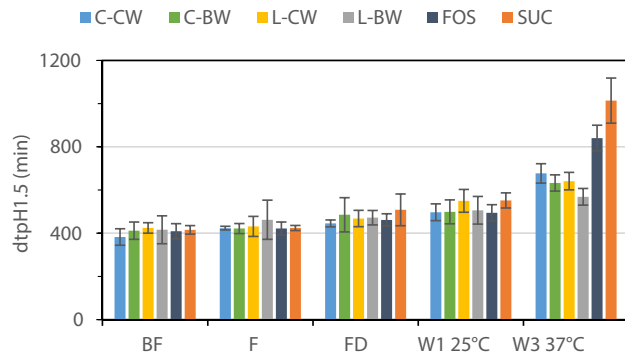
The Karl Fisher titration method was used to measure the moisture content of the samples, using a Metrohm KF 756 apparatus (Metrohm AG; Herisau, Switzerland). At least 20 mg of powder was mixed with 3 mL of dry methanol and titrated with hydranal coulomat AG (Honeywell Fluka; Seelze, Germany) until the end point was reached. Measurements were carried out after freeze-drying.

### Statistical Analyses

The nonparametric Kruskal–Wallis test and the post hoc Conover Iman test with Bonferroni correction were performed using XLSTAT 19.6 (Addinsoft; Paris, France) to compare data concerning biological and physical activities. A significance level of 95% ( $p$ -value  $< 0.05$ ) was considered.



**Fig. 2** Biological activity as determined by the culturability (log (CFU·mL<sup>-1</sup>)) of *L. plantarum* WCFS1 concentrates protected with six different protective formulations at different steps of the production process: before freezing (BF), after freezing (F), and after freeze-drying (FD). C-CW, chickpea cooking water; C-BW, chickpea boiling water; L-CW, lentil cooking water; L-BW, lentil boiling water; FOS, fructo-oligosaccharide; SUC, sucrose



**Fig. 3** Acidifying activity, characterized as the time necessary for a pH drop of 1.5 units (dtpH 1.5, in min), of *L. plantarum* WCFS1 concentrates protected with six different protective formulations at different steps of the production process: before freezing (BF), and after freezing (F), freeze-drying (FD), 1 week of storage at 25 °C (W1 25 °C), and 3 weeks of storage at 37 °C (W3 37 °C). C-CW, chickpea cooking water; C-BW, chickpea boiling water; L-CW, lentil cooking water; L-BW, lentil boiling water; FOS, fructo-oligosaccharide; SUC, sucrose

**Table 2** pH value of *L. plantarum* WCFS1 concentrates protected with six different protective formulations after freezing (F), freeze-drying (FD), 1 week of storage at 25 °C (W1 25 °C), 2 weeks of storage at 37 °C (W2 37 °C), and 3 weeks of storage at 37 °C (W3 37 °C)

Protective formulation	C-CW	C-BW	L-CW	L-BW	FOS	SUC
pH F	4.43 ± 0.11	4.60 ± 0.16	4.49 ± 0.13	4.43 ± 0.16	3.72 ± 0.04	4.05 ± 0.06
FD	4.60 ± 0.16	4.72 ± 0.10	4.57 ± 0.03	4.59 ± 0.06	3.65 ± 0.04	3.98 ± 0.29
W1 25 °C	4.58 ± 0.24	4.71 ± 0.10	4.58 ± 0.15	4.62 ± 0.12	3.73 ± 0.07	3.99 ± 0.23
W2 37 °C	4.55 ± 0.16	4.64 ± 0.09	4.59 ± 0.09	4.55 ± 0.15	3.90 ± 0.03	4.07 ± 0.14
W3 37 °C	4.57 ± 0.21	4.55 ± 0.08	4.53 ± 0.15	4.47 ± 0.13	3.93 ± 0.07	4.08 ± 0.14

Data represented are the average and standard deviation of at least two biological replicates. C-CW chickpea cooking water, C-BW chickpea boiling water, L-CW lentil cooking water, L-BW lentil boiling water, FOS fructo-oligosaccharide, SUC sucrose

## Results and Discussion

### Effect of Protective Formulation on the Survival of *L. plantarum* WCFS1 After Stabilization Processes

Compared to its state before freezing, the biological activity of *L. plantarum* WCFS1 concentrates slightly decreased after the freezing and freeze-drying stages of the production process. This decrease was observed regardless of the protective formulation used, as determined by their culturability and acidifying activity (Fig. 2  $\Delta < 6\%$  and Fig. 3  $\Delta \leq 15\%$ , respectively). No statistical differences in culturability and acidifying activity were observed after these two stages among the protective formulations used (Online Resource 1 and 2). During these processes, microorganisms are exposed to several environmental stresses (thermal, osmotic, mechanical, oxidative) that can cause cell membrane damage, and protein and DNA denaturation, resulting in cell viability and activity loss. However, depending on the bacterial strain, the protective formulation used, and the stress magnitude, the resistance to freezing and freeze-drying processes can strongly differ (Béal & Fonseca, 2015; Fonseca et al., 2015; Tripathi & Giri, 2014). Protective agents play an important role in the conservation of biological activity. It had previously been demonstrated that sucrose, FOS, and GOS are effective protective compounds for lactic acid bacteria during production processes (Romano et al., 2016; Tymczyszyn et al., 2011). In addition, no clear differences in culturability were observed after freeze-drying of *Lactobacillus delbrueckii* ssp. *bulgaricus* CIDCA 333 cells protected with two different GOS preparations (Tymczyszyn et al., 2011). The protective capability of sugars and prebiotics on the stabilization of lactic acid bacteria during their freeze-drying and subsequent storage has been mainly associated with two accepted hypotheses. One of them proposes that sugars can replace the water between lipid headgroups when it is removed during drying, maintaining the membrane integrity. The other one is based on sugars capacity to form glassy matrices in which the high viscosity and low molecular mobility constraints molecular interactions (Crowe et al.,

1988; Golowczyc et al., 2013; Romano et al., 2016; Sosa et al., 2016; Tymczynszyn et al., 2011).

The pH of the *L. plantarum* WCFS1 concentrates was determined after freezing and freeze-drying (Table 2). During these first two stages of the production process, the pH value was maintained. The protective formulations involving FOS and SUC presented the lowest pH (3.8 pH units of average) compared to those involving GOS-containing legume extracts (4.6 pH units of average). During freezing, the hydrogen ions are concentrated as the available liquid water decreases causing an acid shock to the cells. To minimize this shock, the cell concentrates can be neutralized before freezing. However, neutralization of *Bifidobacterium animalis* ssp. *lactis* biomass did not have a positive effect on freeze-drying survival (Saarela et al., 2009). This was also noted in this work, as the differences in pH of the *L. plantarum* WCFS1 concentrates protected with GOS extracts (less acidic) or with FOS/SUC (more acidic) did not result in a significant change in the cells' biological activity as determined by their culturability and acidifying activity after freezing and freeze-drying.

The survival of dried bacteria is affected by moisture content. Removing the whole water from the cells (0% moisture content) during freeze-drying is harmful to survival as it results in damage to cell proteins. Therefore, a certain amount of water (hydration water) must remain in a dehydrated state for a satisfactory survival rate (Zayed & Roos, 2004). However, a high moisture content leads to a decrease in  $T_g$  resulting in a higher  $T-T_g$  value ( $T$  being the storage temperature), which in turn causes a decrease in bacterial viability. When the  $T_g$  is below the storage temperature ( $T-T_g > 0$ ), the samples change from a glassy stable state into a viscous unstable state (rubbery state), where the mobility of molecules increases. A higher molecular mobility leads to loss of bacterial viability and a decrease of the shelf life of the stored powder (Broeckx et al., 2016; Guerrero Sanchez et al., 2022; Passot et al., 2012). Moisture contents of *L. plantarum* WCFS1 concentrates after freeze-drying with the different protective formulations were in the range of 1.82–3.87% (Table 3). Our results are comparable to those reported for *L. plantarum* TISTR 2075 (1.38–3.83% (Sayedboworn et al., 2017)), *Ligilactobacillus salivarius* subsp. *salivarius* (UCC500) freeze-dried with various protective agents (2.8 to 5.6% (Zayed & Roos, 2004)), *L. plantarum*

NCIMB 8826 freeze-dried in alginate microcapsules coated with chitosan (<10% (Albadran et al., 2015)), and *L. delbrueckii* ssp. *bulgaricus* CFL1 freeze-dried in a sucrose matrix (2.5 to 3.7% (Passot et al., 2012)). Therefore, water content in the range of 1.3–5% appears to lead to better bacterial survival after the freeze-drying process. The optimal water content value of a dried bacterial sample is determined by the nature of the protective formulation used and the storage temperature.

### Effect of Protective Formulation on the Survival of *L. plantarum* WCFS1 After Storage

After freeze-drying, protected *L. plantarum* WCFS1 cells were stored at two different temperatures (25 °C and 37 °C) for 7 and 28 days, respectively.

In the case of storage at 25 °C, biological activity slightly decreased for all protective formulations after 1 week of storage compared to the levels observed after the freeze-drying stage ( $\Delta < 2.5\%$  in culturability and  $\Delta < 13.5\%$  in acidifying activity). In addition, no statistical differences were observed among the different protective formulations (Online Resource 1 and 2). After 3 weeks of storage at 37 °C, biological activity abruptly decreased for all protective formulations as compared to that observed after the freeze-drying stage ( $\Delta < 16.5\%$  in culturability and  $\Delta < 52.5\%$  in acidifying activity), and GOS extracts' formulations were significantly better cell protectants than SUC's and FOS' (Online Resource 1 and 2). During storage, the culturability of freeze-dried bacteria tended to decrease. Water content, storage temperature, and exposure to oxygen are some of the factors that can affect the viability of dried microorganisms. Low storage temperatures lead to the highest bacterial survival because in these conditions, chemical reactions responsible for cell damage are slowed down (Foerst et al., 2012; Shu et al., 2018). The biological activity of *L. plantarum* WCFS1 cells decreased with the increasing storage temperature (from 25 to 37 °C). The decrease in viability during storage of freeze-dried lactic acid bacteria has been related to membrane damage caused by lipid oxidation (Albadran et al., 2015; Santivarangkna et al., 2008; Sayedboworn et al., 2019). However, the loss of membrane integrity and lipid oxidation are not the only detrimental reactions which can occur during storage; the drastic loss of culturability during

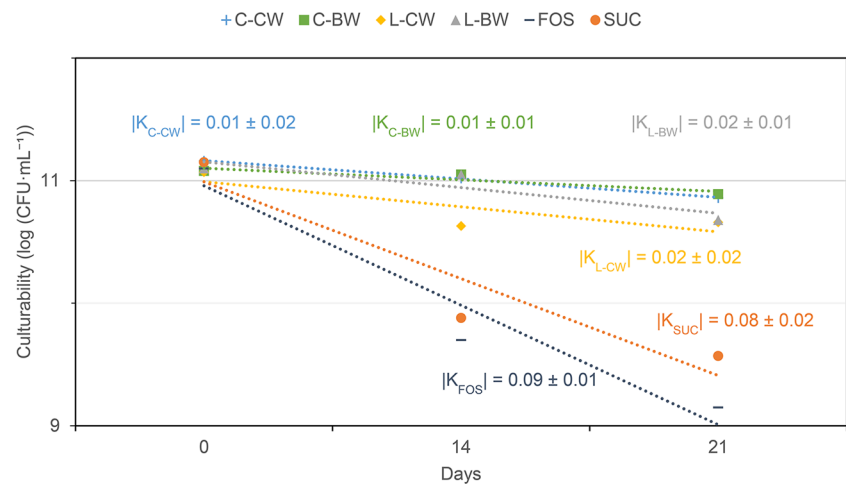
**Table 3** Moisture content (%) of freeze-dried *L. plantarum* WCFS1 concentrates protected with six different protective formulations

Protective formulation	C-CW	C-BW	L-CW	L-BW	FOS	SUC
Moisture content (%)	3.87 ± 0.19	2.92 ± 1.04	1.82 ± 1.20	2.74 ± 1.03	3.30 ± 1.25	3.85 ± 1.43

Data represented are average and standard deviation of one to three biological replicates and at least two technical replicates. No statistical differences (at the 95% confidence level) were observed between all samples (six formulations) after freeze-drying. C-CW chickpea cooking water, C-BW chickpea boiling water, L-CW lentil cooking water, L-BW lentil boiling water, FOS fructo-oligosaccharide, SUC sucrose



**Fig. 4** Culturability ( $\log$  (CFU·mL<sup>-1</sup>)) of *L. plantarum* WCFS1 concentrates protected with six different protective formulations during 3 weeks of storage at 37 °C showing the absolute value of the slope of the linear regression ( $|K|$ ). C-CW, chickpea cooking water; C-BW, chickpea boiling water; L-CW, lentil cooking water; L-BW, lentil boiling water; FOS, fructo-oligosaccharide; SUC, sucrose

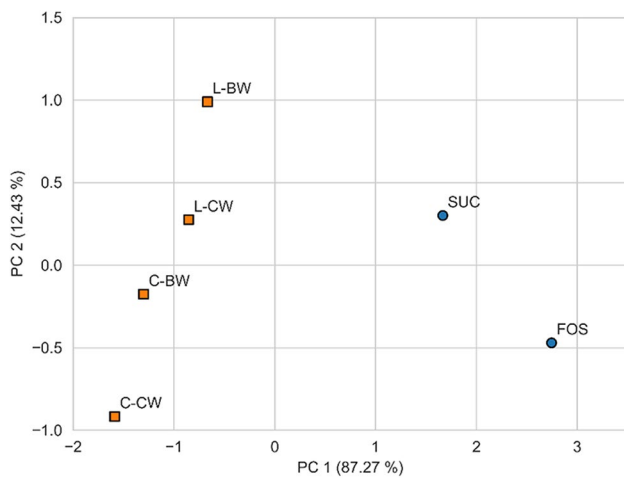


storage at 37 °C of *L. salivarius* CECT5713 was associated with damage to proteins, nucleic acids, and peptidoglycans of the cell wall (Guerrero Sanchez et al., 2023).

With regard to the culturability results, the absolute slope ( $|K|$ ) values were determined, based on the CFU·mL<sup>-1</sup> calculated during the 3 weeks of storage at 37 °C, as depicted in Fig. 4. The lowest  $|K|$  values were obtained for the protective formulations involving GOS-containing legume extracts, showcasing a higher protection of *L. plantarum* cells. The four GOS extract formulations provided the highest protection with similar results, with no statistical differences between legumes (chickpea and lentil) or treatments used to obtain the GOS extracts (cooking and boiling). In addition, no statistical differences between FOS and SUC samples were observed (Online Resource 1).

The acidifying activity results (Fig. 3) were in line with the culturability assays, in which the protective formulations involving FOS and SUC were confirmed to have conferred the lowest protection towards *L. plantarum* cells, by showing very pronounced losses of acidifying activity (with no statistical differences between the two samples). GOS extracts yielded the lowest dtpH 1.5 values, with the most successful protective formulation being L-BW, followed by C-BW, L-CW, and C-CW (Online Resource 2). L-BW and C-BW being the sources of the most successful protective formulations suggest that the boiling treatment used to obtain these GOS extracts is the preferred extraction procedure, providing equal or better results to the cooking counterparts but with less environmental impact, since the soaking of the seeds can be dismissed. The observed results may be explained by a combination of factors. In the present study, average values of  $T-T_g$  were as follows: 13.7 °C (C-CW), -4.0 °C (C-BW), -10.8 °C (L-CW), and -3.2 °C (L-BW), as calculated from  $T_g$  values found in Table 1, with  $T=37$  °C, i.e., storage temperature. Examples of  $T_g$  curves obtained for each wastewater extract are shown in Online Resource 3. Tymczynszyn et al. (2012) obtained similar

results of  $T_g$  for commercial GOS at 11% RH. All wastewaters obtained  $T_g$  above 37 °C (except for C-CW), which can be useful for these formulations to help the preservation of the bacterial structure (Oluwatosi, et al. 2022; Lee et al., 2020).  $T_g$  of commercial FOS is 46.92 °C, while  $T_g$  of sucrose is 40.28 °C (Table 1) (Martins et al., 2023; Romano et al., 2016), results that are similar to those obtained for the wastewaters. The  $T_g$  results of the present study contribute to the understanding of the correct preservation of the bacteria achieved by the use of legume wastewaters; however, they do not explain the differences observed in terms of culturability, with respect to the other protective samples. Some authors suggest that the presence of mono- and polysaccharides could be useful in protective formulations (Oluwatosi, et al. 2022; Romano et al., 2016, 2021). In previous work, starch was found in great amounts in the produced GOS extracts (as determined by HPLC-RI and colorimetric tests with iodine, Martins et al., 2023), whereas it is not present in neither the FOS nor SUC formulations (Martins et al., 2023). Li et al. (2016) used gelatinized starch for the entrapment of lactic acid bacteria, finding that this material protected against different conditions. The starch is not the most studied material for its protective and encapsulant properties. However, it would be interesting to evaluate the usefulness of this component in the wastewaters of legumes, since they constitute a big part of these foods. Polysaccharides have been shown to increase *L. plantarum* WCFS1's resistance to freeze-drying by improving cell membrane integrity synergistically with low molecular weight cryoprotectants (Wang et al., 2021). Jawan et al. (2022) also observed that combinations of cryoprotectants, including galactose and trehalose, increased the survivability of *Lactobacillus delbrueckii* ssp. *lactis* Gh1 during freeze-drying and storage at different temperatures. However, in the same study, the complex mixture of skim milk did not fare so well in the bacterial preservation. In contrast, Zayed and Roos (2004) reported that the stabilization of *L. salivarius* subsp. *salivarius* (UCC 500) was



**Fig. 5** PCA performed using the culturability, acidifying activity, and pH data of the samples after 3 weeks in storage at 37 °C. C-CW, chickpea cooking water; C-BW, chickpea boiling water; L-CW, lentil cooking water; L-BW, lentil boiling water; FOS, fructo-oligosaccharide; SUC, sucrose

enhanced by the combination of various cryoprotective compounds, such as the skim milk mixture. The protective effect in that case was attributed to skim milk containing buffering agents such as proteins and minerals, besides cryoprotective compounds. These compounds could possibly also be found in GOS extracts from the lixiviation of the legumes during soaking and cooking (Serventi, 2020), which may explain the higher pH values observed during the whole production process, including storage (Table 2). Principal component analysis (PCA) was performed with Python software using the culturability, acidifying activity, and pH data of the samples after 3 weeks in storage at 37 °C. The results show the separation of two groups along the PC1 axis, one with the GOS wastewaters and another with FOS and SUC (Fig. 5). This separation is influenced by the pH of the protective formulations and explains the wastewater effectiveness in protecting the microorganisms in the accelerated storage test.

GOS are composed of a variable number of galactose units, within 2 and 10. The type of linkage between units varies according to their origin and obtaining process. Plant-based GOS are  $\alpha$ -GOS and are important components of seeds. The prebiotic effect of  $\alpha$ -GOS is mainly associated with tri- and tetrasaccharides (Martins et al., 2019). In addition to the prebiotic properties, the ability of GOS to act as protectants has been reported. In this work, the four GOS-containing wastewater legume extracts appeared as better protective formulations than fructo-oligosaccharides and sucrose for stabilizing *L. plantarum* WCFS1 during storage at 37 °C (Figs. 3 and 4). *L. plantarum* CIDCA 83114 spray-dried with protective formulations containing GOS and maltodextrin allowed the recovery of 93% microorganisms; in contrast, only 64% microorganisms were recovered when

no GOS were included in the formulation (Sosa et al., 2016). *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 protected with a formulation including C-BW wastewater exhibited a good resistance to freeze-drying and storage stages (Passot et al., 2024). Protective formulations with different GOS compositions were also demonstrated to be highly efficient in the preservation of *L. delbrueckii* ssp. *bulgaricus* CIDCA 333 both after freeze-drying and storage at 5 and 25 °C (Tymczyszyn et al., 2011). The efficiency of GOS as protectants could be related to their ability to immobilize cells in a glassy matrix, which reduces molecular mobility and consequently slows down degradation reactions, and to their enough disaccharides content, useful for stabilizing and thus preserving bacterial membranes (Passot et al., 2024; Torres et al., 2011; Tymczyszyn et al., 2012). In addition to their proven efficacy, the use of  $\alpha$ -GOS from legumes' wastewaters to stabilize probiotic cultures is attractive because they may be safely incorporated into food products without raising health concerns regarding lactose intolerance in consumers, as is the case of  $\beta$ -GOS that are obtained from lactose, or other milk-derived products (Jawan et al., 2022). According to the Global Aquafaba Market Outlook (2023 to 2033) report by Persistence Market Research (Persistence Market Research, 2023), in 2022, worldwide sales of aquafaba accounted for 38.8 million US\$, with an expected growth in CAGR between 2023 and 2033 of 13.1%. The main cause behind economic growth is the increasing interest in vegan products, namely egg substitutes. The report explains how aquafaba is set to become a viable alternative to egg products when preparing vegan versions of meringues, waffles, and pancakes. The environmental concerns, along with dietary tendencies and increased interest in functional foods, brew favorable conditions for the incorporation of GOS wastewaters and stabilized lactic acid bacteria in novel food products with health benefits.

## Conclusions

In the present study, the stability of *Lactiplantibacillus plantarum* WCFS1 with the addition of different protective molecules (GOS extracts from chickpeas and lentils, fructo-oligosaccharides, and sucrose) was evaluated. The biological activity of the bacteria decreased after freezing and freeze-drying, with no influence of the protective molecule used. However, after storage at 37 °C for 3 weeks, wastewaters from legumes' preparation containing GOS were the most effective protective compounds towards *L. plantarum* WCFS1, proving the initial hypothesis correct. The culturability studies showed no significant differences between the legumes, or cooking procedures; however, the sample with the boiling wastewater obtained from cooking without soaking of lentils showed the best acidifying

activity. Our results suggest the boiling method is economically and environmentally advantageous, conferring the best protection for the lactic acid bacterium, with decreased costs and time, with the dismissal of the soaking procedure of the seeds. The protective capacity of the GOS wastewaters appears to be explained by a combination of physical–chemical factors intrinsic to these mixtures, namely their buffering capability, the increased  $T_g$  values, and the presence of the carbohydrates of various sizes, including the polysaccharide starch, in contrast with the reference materials used for comparison. The increased shelf-life of probiotic cultures is a serious concern, with economic and environmental impact. Prebiotic and probiotic mixtures' incorporation in food products could represent an interesting application for these substances, thus improving the consumers' health through their diet, besides nutraceutical supplementation.

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**Author Contribution** G.N.M, M.G.S, A.D.C, and S.C are responsible for the experimental work and methodology. G.N.M, M.G.S, and F.F did the conceptualization. G.N.M. and M.G.S. did the data curation and writing of the original draft. F.F, A.G.-Z, and P.C.C did the supervision, the review of the manuscript, and the funding acquisition.

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**Data Availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Declarations

**Competing Interest** The authors declare no competing interests.

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