International Journal of Food Microbiology 426 (2025) 110908

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Review

# Functional characterization of the riboflavin-overproducing and dextran-producing *Weissella cibaria* BAL3C-5 C120T strain for the development of biofortified plant-based beverages

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ARTICLE INFO

*Keywords:*
Lactic acid bacteria
Vitamin $B_2$
Dextran
Functional drinks
Biofortification
Rheology

ABSTRACT

Riboflavin (vitamin $B_2$) is essential for human beings and it has to be provided by healthy nutrition. The use of fermentation with riboflavin-overproducing lactic acid bacteria (LAB) represents an ideal strategy to generate, by *in situ* biofortification, functional drinks. These beverages can positively contribute to consumer health and address nutritional deficiencies. In the present work, the functional capabilities of *Weissella cibaria* BAL3C-5 C120T for riboflavin-overproduction and dextran-production during fermentation of oat-, rice-, soybean- and almond-based drinks have been evaluated. It was confirmed that the strain was capable of producing riboflavin and dextran in the analysed drinks. This property was especially pronounced in the oat-based drink, where after 24 h of fermentation the strain was able to increase riboflavin and dextran levels up to 3.4 mg/L and 3.2 g/L, respectively. Moreover, under optimized conditions the strain was able to enrich the fermented oat-based drinks with the prebiotic oligosaccharide panose (up to 6.6 g/L). In addition, in the oat-based drinks BAL3C-5 C120T showed a good pH-lowering ability (from 7.0 to 3.8) as well as a high 80 % cell viability after one month of storage. Rheological analysis of the resulting fermented oat-based beverages revealed a thixotropic structure related to a gel-like behaviour which was not observed in the non-fermented control drinks. In summary, these results confirmed the unique characteristics of *W. cibaria* BAL3C-5 C120T strain for the development of biofortified and functional plant-based beverages with improved nutritional and rheological properties. Analysis of the BAL3C-5 C120T strain survival under gastrointestinal conditions and its autoaggregation properties, also indicated its potential use as a probiotic delivered in an oat-based fermented beverage. In this context, this study also promotes the utilization of *W. cibaria* species in health and food industries where it has not yet been used as a starter or adjunct culture.

## 1. Introduction

Functional foods are defined as food or drinks containing one or several components that affect multiple biological functions, promoting general well-being and reducing the risk of suffering diseases (Nazir et al., 2019). In addition, they are a strategy to alleviate nutritional deficiencies, preventing the development of nutrition-related disorders and improving physical and mental state. In this sense, current trends in food science and microbiology move towards *in situ* biofortification of potentially functional foods with various essential metabolites such as

*Abbreviations:* LAB, lactic acid bacteria; RF, riboflavin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; Dsr, dextransucrases; MRSG, Man Rogosa Sharpe broth containing 2% glucose; MRSS, Man Rogosa Sharpe broth containing 2% sucrose; RAM, riboflavin assay medium; CFU, colony-forming units; HPLC, high performace liquid chromatography; GC-MS, gas chromatography–mass spectrometry; PBS, phosphate-buffered saline; EPS, exopolysaccharide; H-B, Herschel-Bulkley.

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https://doi.org/10.1016/j.ijfoodmicro.2024.110908
Received 14 June 2024; Received in revised form 23 August 2024; Accepted 5 September 2024
Available online 7 September 2024

vitamins, minerals and others (Nazir et al., 2019; Ofori et al., 2022). Furthermore, the greater consumer awareness of the development of foods known as "clean-label" products with lower content of artificial additives, as well as healthier alternatives for example low-sugar, low-fat, or gluten-free products, is increasingly noticed (Wakeel et al., 2018).

To face these challenges, the utilization of functional metabolite-producing lactic acid bacteria (LAB) is a very promising strategy. Among others, the production of dextran-type exopolysaccharides and vitamin $B_2$ (riboflavin, RF) stand out (Díaz-Montes, 2021; Levit et al., 2021). Concerning the dextrans, they are α-glucans formed by a main chain of D-glucopyranosyl residues with α-(1,6) linkages and varying percentages of branches with α-(1,4), α-(1,3) or α-(1,2) bonds (Zannini et al., 2016). They are synthesized in a reaction catalysed by dextransucrases (Dsr, glycoside hydrolase GH 70 family) by hydrolysis of the sucrose disaccharide generating fructose and glucose, and by transferring this latter monosaccharide to the growing chain of the polymer. Dextrans can act as new hydrocolloids with many rheological properties depending on their molecular weight and branching, improving the texture and structure of various foods (*e.g.* in the formulation of gluten-free bakery or low-fat dairy products) (Lynch et al., 2018; Werning et al., 2022). Also, *in situ* production of dextran has been investigated in non-dairy beverages *e.g. Liquorilactobacillus hordei* TMW 1.1907 was used to produce cloudy dextrans at high levels up to 8.5 g/L in apple or grape juices (Eckel et al., 2019). Moreover, the postbiotic high molecular weight dextrans produced by LAB have several beneficial properties for human health as prebiotic (Kim et al., 2022), antiviral (Nácher-Vázquez et al., 2015), anti-inflammatory (Zhou et al., 2022), immunomodulatory (Zarour et al., 2017), antioxidant and hypocholesterolemic agents (Mohd Nadzir et al., 2021).

RF is one of the essential micronutrients established by the World Health Organization (WHO) as an indicator to assess development, growth and nutritional status (WHO, 1967). It is the precursor of the cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which are essential in various oxidation-reduction processes and have an essential role in cellular energy metabolism (Levit et al., 2021). Mainly, RF is present in foodstuffs of animal origin and in lower amounts in vegetables and cereal- or plant-based products. Dietary recommended values by the EFSA range from 0.4 mg/day for breastfed infants to 1.6 mg/day for healthy adults. For pregnant and lactating women, additional requirements are needed, and values of 1.9 and 2.0 mg/day, respectively, are considered (EFSA, 2017). The role of RF has been linked to the prevention of a wide range of diseases like, anaemia, migraine, cancer, oxidative stress, hyperglycaemia and hypertension (Said and Ross, 2012; Udhayabanu et al., 2018). Thus, RF deficiency, known as ariboflavinosis, whose symptoms may appear with an intake of <0.2–0.3 mg/day, influences iron absorption, metabolism of other vitamins, tryptophan metabolism, mitochondrial dysfunction, brain dysfunction, skin disorders, migraines, circulatory problems and digestive disorders (Suwannasom et al., 2020; Thakur et al., 2017). Photosensitizing properties of RF make it interesting also as an antiviral and antimicrobial agent (Farah et al., 2022).

Although RF is present in a wide variety of foods, important deficiency has been observed worldwide, in both developed and developing countries due to an inadequate diet (Plantone et al., 2021). In this context, vitamin $B_2$ biofortified foods by RF-overproducing LAB have attracted great attention compared to chemically synthesized RF, as it represents a more natural, consumer-friendly and more sustainable alternative (Revuelta et al., 2017). In addition, RF-overproducing genetically modified microorganisms still represent a great challenge for the industry mainly because of consumer or regulatory concerns related to genetic engineering (Zhang et al., 2022). In this context, the use of selected LAB for biofortification of various foods has been successfully confirmed using *Lactiplantibacillus plantarum* (Yépez et al., 2019), *Limosilactobacillus fermentum* (Russo et al., 2014), *Limosilactobacillus reuteri* (Spacova et al., 2022) or *Weissella cibaria* (Hernández-Alcántara et al., 2022) strains. Actually, the ability to increase RF concentration in food matrices that lack or have minimal concentrations of this vitamin is of interest for food and health industries. Thus, the biofortification of plant-based products represents a very interesting approach for the development of potential functional beverages. This is because they constitute an important part of the diet in certain populations, and in recent years it is becoming a boom, considering these as a healthy alternative to dairy products (Drewnowski et al., 2021).

Previously, we have selected by treatment with the toxic RF analogue roseoflavin, a range of spontaneous RF-overproducing *W. cibaria* strains carrying mutations in the regulatory region of the *rib* operon, which encodes the enzymes involved in the RF biosynthetic pathway (Diez-Ozaeta et al., 2023). The strain of interest *W. cibaria* BAL3C-5 C120T was able to increase 70 times the production levels of its parental strain BAL3C-5, and presented a constitutive RF production with a deregulated expression of the *rib* operon. Whole genome sequencing of both mutant and parental strains revealed a single difference which coincided with the C120T mutation at the regulatory region of the *rib* operon (Diez-Ozaeta et al., 2023). To the best of our knowledge, this was the first time that a single mutation was certified as responsible for the overproducing phenotype in a LAB strain. In addition, we have validated the capability of the strain to enrich by fermentation with dextran (≈1 g/100 g), and RF (350–150 μg/100 g) gluten-free-doughs, and confirmed a safety assessment of the strain (Russo et al., 2024). Therefore, in this work with the aim to develop new plant-based functional drinks and expanding the potential utility of *W. cibaria* BAL3C-5 C120T, we have evaluated the behaviour of this strain and that of its parental BAL3C-5 during fermentation of different commercial vegetable drinks, taking into account the low concentration of RF that they present. In addition to their RF overproducing ability and their metabolic performance, the detection of their dextran production capability and the connected rheological properties of the resulting beverages have been also analysed. Finally, in order to confirm the suitability of the BAL3C-5 C120T strain as a potential starter and probiotic culture, its resistance to simulated gastrointestinal conditions and its autoagglutination capability were tested.

## 2. Material and methods

### 2.1. Bacterial strains and growth conditions

*W. cibaria* BAL3C-5 and BAL3C-5 C120T strains, designated as parental wild-type and mutant strains, respectively, were used in this study. The BAL3C-5 strain was previously isolated from rye sourdough (Llamas-Arriba et al., 2021), and its derivative, the spontaneous mutant RF-overproducing BAL3C-5 C120T strain, that was previously selected upon BAL3C-5 strain treatment with roseoflavin (Diez-Ozaeta et al., 2023). The bacteria were grown at 30 °C and propagated in the liquid RF assay medium, which is a semidefined broth lacking RF (RAM, Difco, Le Pont de Claix, France), and the de Man, Rogosa and Sharpe rich medium lacking dextrose (Condalab, Torrejón de Ardoz, Spain) supplemented with either 2 % sucrose (MRSS) or 2 % glucose (MRSG).

### 2.2. Analysis of stability of the BAL3C-5 C120T RF-overproducing phenotype

*W. cibaria* BAL3C-5 C120T was cultured in RAM medium at 30 °C for >100 generations. Levels of RF production expressed as fluorescence were determined as indicated in Section 2.4 and cell viability was analysed by plate counting every 14 generations in order to confirm the stability of the RF-overproducing phenotype. The experiments were performed in triplicate.

### 2.3. Plant-based beverage fermentations

Both strains were inoculated in commercial plant-based beverages. These matrices included oat (Alitey oat beverage), soy (Carrefour Soy

beverage), almond (Ecomil almond-based drink) and rice (Yosoy rice-based drink) beverages. The nutritional profile of the different plant-based alternatives is shown in Table 1. Overnight cultures of the *W. cibaria* strains grown in MRSS were centrifuged at 9300 ×*g* for 10 min and the cells resuspended in fresh medium to give an optical density at a wave length of 600 nm ($OD_{600\ nm}$) of 0.1. Then, cultures were grown to an $OD_{600\ nm}$ of 1 (≈5 × $10^8$ colony-forming units (CFU)/mL), centrifuged as mentioned above and resuspended in the corresponding plant-based beverage to give an initial cell density of approximately 5 × $10^7$ CFU/mL. All beverages were supplemented with 5 % sucrose. Fermentations were carried out for 24 h at 30 °C. At the time points 0 h and 24 h, samples were collected for RF and dextran quantification, as well as for pH and cell viability determination. Measurements of pH were performed using the Crison pH/mV-meter 501 and quantification of CFU/mL were carried out by plating, on MRS agar, by serial dilutions of cultures in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO$, and 1.47 mM $KH_2PO$) pH 7.0.

The following fermentations of oat-based beverage were performed as above but with different sugars supplementation. Fermentations were performed without sugar addition, with 2.5 % maltose plus 2.5 % sucrose supplementation, and with 5 % sucrose supplementation. After fermentation for 24 h, beverages were stored at 4 °C for 4 weeks. Samples were taken at the time point 0 h and after 24 h of fermentation, and every 7 days during the storage period. All assays were performed in triplicate. Samples were analysed for RF and dextran quantification, pH and cell viability determination, and characterization of rheological properties of fermented beverages.

### 2.4. Analysis of fermentation products (RF, dextran, sugars and metabolites)

RF quantification was performed through fluorescence detection by microplate reader and high-performance liquid chromatography (HPLC). Before RF analysis slight pre-treatment of samples was performed. For non-fermented control beverages, coagulation was chemically induced by addition of 1 M HCl until pH 4.0 was reached. Then, non-fermented and fermented samples were centrifuged at 12000 ×*g* for 10 min. Supernatants were taken and the RF present in them was detected and quantified by a fluorescence detection using a Varioskan Flask System (Thermo Fisher Scientific, Waltham, MA, USA) microplate reader, following the method previously described prior, and after HPLC fractionation (Mohedano et al., 2019). Briefly, aliquots of 200 μL in triplicate were analysed in a sterile 96-well polystyrene flat bottom plate (Corning, Maine USA). Fluorescence was measured using an excitation wavelength of 440 nm and detection of emission wavelength at 530 nm. RF concentration was calculated using a calibration curve.

HPLC analysis was performed using Shimadzu-Prominence chromatograph (Shimadzu, Duisburg, Germany) equipped with a fluorescence detector RF 10A XL (Shimadzu). A EC 250/4.6 Nucleosil 120-5 C18 column (Macherey-Nagel, Düren, Germany) and a TRC-160K1 precolumn (Teknokroma, Barcelona, Spain), both thermostated at 40 °C, were used. Measurements were carried out under isocratic conditions with a flow rate of 0.8 mL/min, and a mobile phase consisting of 0.083 M sodium acetate and methanol (60:40, v/v) at pH 5. The injection volume was 20 μL and the elution time 12 min. Identification of RF was performed by comparison of its retention time with that of pure standard (98 %, Sigma-Aldrich, Gillingham, England), and quantification was carried out by an external calibration.

**Table 1**
Nutritional profile of plant-based beverages used in this study (per 100 mL).

| | Oat beverage | Soy beverage | Almond beverage | Rice beverage |
|---|---|---|---|---|
| Energy (kJ) | 194 | 138 | 145 | 221 |
| Energy (kCal) | 46 | 33 | 35 | 52 |
| Fat (g) | 0.8 | 1.8 | 1.9 | 1 |
| Saturated fatty acids (FA) (g) | 0.2 | 0.3 | 0.3 | 0.1 |
| Monounsaturated FA (g) | 0.3 | Not listed | Not listed | 0.3 |
| Polyunsaturated FA (g) | 0.3 | Not listed | Not listed | 0.6 |
| Carbohydrates (g) | 8.1 | 1 | 3.2 | 10 |
| Sugars (g) | 5.4 | 0.7 | 0.3 | 4 |
| Proteins (g) | 1.3 | 3.2 | 1 | 0.3 |
| Salt (g) | 0.07 | 0.06 | 0.14 | 0.07 |

The concentration of sugars initially present in the beverages and of the metabolites generated during fermentation was determined by gas chromatography–mass spectrometry (GC–MS) analysis of 100 μL of beverage supernatant samples, as previously described (Besrour-Aouam et al., 2021). Myo-inositol was used as internal standard, and quantification of the compound's concentration was performed according to peak area, corrected with the response factors calculated using the internal standard and the software GC-ChemStation Rev. E.02.00 (2008) from Agilent (Palo Alto, CA, USA).

Soluble dextran present in the beverage supernatants was quantified as previously described for bread samples (Hernández-Alcántara et al., 2022) through quantification of the isomaltose generated by the polymer hydrolysis, with the following modifications. Briefly, to convert dextran into isomaltose, 750 μL of each sample supernatant was treated with 150 μL of dextranase solution containing 0.18 g of *Chaetomium erraticum* dextranase (Sigma-Aldrich, Darmstadt, Germany). Samples were incubated at 30 °C for 18 h, centrifuged at 12000 ×*g* for 10 min, and supernatants filtered by using a 0.22 μm filter. Finally, aliquots were stored at −20 °C until further analysis by GC–MS as indicated above. Three replicates for each condition tested were analysed.

### 2.5. Rheological properties of oat-based fermented beverages

The rheological characterization of fermented and non-fermented control oat beverages was performed using a stress controlled rotational rheometer Physica MCR 101 (Anton Paar, Graz, Austria) in both continuous and oscillatory modes. Rheological analyses were performed at the initial time point, at 24 h of fermentation, and throughout one month of refrigerated storage, with evaluations conducted on a weekly basis.

Continuous flow analysis was carried out at 5 °C using a concentric cylinder geometry, CC17/T200 (Rotor Diameter = 16.7 mm, and Length = 25 mm, and Cup Diameter = 18 mm), for control beverage, and a cone-plate system CP25 (25 mm diameter, 1° cone angle and 48 μm gap) for fermented beverages. Shear stress and apparent viscosity were determined as a function of shear rate in three steps: 1) range from 0.1 $s^{-1}$ to 100 $s^{-1}$, 2) maintaining 100 $s^{-1}$ for 20 s, and 3) decreasing finally the shear rate from 100 $s^{-1}$ to 0.1 $s^{-1}$.

Flow curves, data of shear stress ($\tau$)-shear rate ($\dot{\gamma}$), were fitted by the power law-Ostwald de Waele model (Eq. (1)), and the Herschel–Bulkley model (Eq. (2)), respectively,

$$\tau = K_p \dot{\gamma}^{n_p} \quad (1)$$

where $K_p$ is the consistency coefficient (Pa·$s^n$), $n_p$ is the pseudoplasticity index (dimensionless), that reflect the closeness to Newtonian flow. When the magnitude of $n < 1$, the fluid is shear-thinning, and when $n >$ 1, the fluid is shear thickening in nature.

$$\tau = \tau_y + K_{H-B} \dot{\gamma}^{n_{H-B}} \quad (2)$$

where $K_{H-B}$ is the consistency coefficient (Pa·$s^n$), $n_{H-B}$ is the pseudoplasticity index (dimensionless). $\tau_y$ is the yield stress (Pa).

The thixotropy property was investigated by loop test analysis (Barnes, 1997) programmed as described above including three shear steps. This test allowed the study of the breakdown of the structure at increasing shear rates and the subsequent recovery during the down-ramp. In the case of thixotropic fluids, the recovery of the structure is slowed down and the initial viscosity is not expected to be reached. As a

result, the area within the hysteresis loop, A, (in Pa $s^{-1}$), is dimensionally the "energy" relative to the volume of the sheared sample, indicating the energy involved in thixotropic structure breakdown.

The viscoelastic properties were investigated by oscillatory shear mode, using the same geometry described above. The temperature was maintained at 5 °C. The analysis was performed under linear viscoelastic conditions (in the linear viscoelastic region LVR), previously evaluated by strain sweep testing at a fixed angular frequency (1 rad/s). The LVR strain selected was approximately 1 %, since a linear range between 0.1 and 10 % strain had been determined. The storage modulus (G′) and loss modulus (G″) were analysed as a function of angular frequency in the range 0.1–10 rad/s.

### 2.6. Survivability in the simulated gastrointestinal conditions

Simulated gastric and intestinal fluids were used to analyse the ability of *W. cibaria* strains to resist gastrointestinal tract conditions. Late-exponential-growth cultures were centrifuged at 8000 ×*g* for 10 min, washed twice with PBS and resuspended at $1 \times 10^8$ CFU/mL in 35 mL of the corresponding assay medium, either saline solution (0.9 % NaCl) or oat-based beverage. Also, the LAB survival was evaluated in the 24 h fermented oat-based beverage without further bacterial inoculation. The aim of testing in oat-based beverages was to evaluate the protective effect of the matrix itself (with or without fermentation) on bacterial viability. The three distinct matrices were evaluated for the gastric simulation assay, a saline solution, the oat-based beverage and the 24 h fermented oat-based beverage. The pH of each matrix was adjusted to 2.5 with 1 M HCl and 875 μL of a pepsin (at 100,000 U/mL) solution was added to the 35 mL treated samples to give a final concentration of 2500 U/mL of the enzyme. Suspensions were incubated at 37 °C for 90 min with agitation to simulate peristalsis. Samples were taken at time points 0 min and 90 min. Afterwards, to mimic the intestine phase, the pH was first increased with 2.5 M NaOH until pH 4.0, and then 15 mL of a solution containing 3 % bile salts (Sigma-Aldrich) and 1 % pancreatin (Sigma-Aldrich) dissolved in 0.1 M $NaHCO_3$ (pH 7.0) were added to the gastric solution. Suspensions were incubated as above for 180 min, and samples were taken at 0 min, 90 min and 180 min. Finally, 100 μL of the samples were plated in appropriate dilutions on MRS agar and incubated at 30 °C for 24 h. Cell viability was expressed as CFU/mL.

### 2.7. Evaluation of autoaggregation

Bacterial suspensions were grown overnight in MRS broth and subsequently harvested by centrifugation at 8000 ×*g* for 8 min at room temperature. The cells were then washed twice with PBS (pH 6.6) and adjusted with the same buffer to an $OD_{600\ nm}$ of 0.5 (AO). Each bacterial suspension was incubated at 30 °C, and auto-aggregation values were measured at 3, 6, and 24 h (At). Auto-aggregation values were calculated using the following formula:

$$A = (1–At/A0) \times 100\%$$

### 2.8. Statistical analysis

All measurements were analysed with one-way ANOVA. A $p$ value of $\leq 0.05$ was considered significant. When ANOVA tests were significant, mean pairwise comparisons were computed with a Tukey's test ($\alpha = 0.05$). All analyses were performed with the R software version 4.3.1 (R Core Team, 2023).

## 3. Results and discussion

### 3.1. Stability of the RF-overproducing phenotype

Food biofortification entails the supplementation of the food with specific nutrients, by fermentation of the food matrices or exogenous addition, with the aim of increasing the nutritional value and strengthening both human health and development. Considering the high adaptability of LAB for industrial food fermentation and the ability of specific strains to synthesize RF (RF-producing LAB), they are considered good candidates to develop RF-rich foodstuffs instead of both chemical and transgenic approaches. However, RF productivity obtained from fermentations with wild-type strains is generally low.

In this context, we selected for testing in commercial drinks the *W. cibaria* BAL3C-5 C120T strain, which is able to produce around 7 mg/L of RF in a medium lacking this compound, and capable of synthesizing the vitamin constitutively independently of the presence of RF or FMN in the growth medium (Diez-Ozaeta et al., 2023). There was the possibility that a no complex spontaneous mutational event (a change of T by C) could revert BAL3C-5 C120T to the RF wild type phenotype, provided that the only genetic different between this strain and its parental wild type strain is a punctual mutation at the regulatory region of the *rib* operon. Thus, prior to the testing of the BAL3C-5 C120T in beverages, it was necessary to determine if the property of producing high levels of RF was stable. Therefore, the mutant strain was grown for >100 generations in RF-free RAM medium, and the free RF present in the culture supernatants as well as bacterial growth were measured. The results presented in Fig. 1 showed no significant changes in the levels of the RF produced by the bacteria during the continuous growth, confirming that the RF-overproducing phenotype was stable for at least 100 generations.

### 3.2. RF-overproducing phenotype confirmed in plant-based beverages

In plant-based beverages there is absence or only low concentration of RF. Thus, with the aim of developing new fermented foodstuff, the performance of the RF-overproducing BAL3C-5 C120T, in comparison with that of its parental RF-producing BAL3C-5 (wild-type) strain was evaluated in commercial plant-based beverages. As controls, drinks prior to LAB addition were used. Also, the capability of these LAB to produce dextran was investigated. Given that LAB produce dextran using sucrose as substrate, the drinks were supplemented with 5 % sucrose. After 24 h of fermentation at 30 °C, the concentration of RF and dextran was determined in the inoculated samples of oat-, rice-, soy- and almond-based commercial beverages, as well as the pH-lowering capacity and cell viability of the LAB strains (Fig. 2). The experimental design is depicted in Fig. 2A.

With regard to the RF levels (Fig. 2B), in the case of the wild-type strain, no statistically significant RF increase was observed after fermentation, whereas in rice-based drink a 2.8-fold increase was detected in comparison with the non-fermented control. By contrast, BAL3C-5 C120T significantly increased RF concentration in all tested beverages (Fig. 2B), compared with the concentrations of the vitamin detected in the control non-fermented beverages and those drinks fermented with BAL·C-5. Thus, we observed an increase of: 24.7-fold in oat-based, 8.7-fold in rice and soy-based and 8.1-fold in almond-based compared to their corresponding beverage baseline (Fig. 2B). As stated above, the highest RF value was observed in oat-based beverage, where BAL3C-5 C120T was able to raise RF content from 0.11 mg/L to almost 3 mg/L. Similarly, other research studies have successfully tested the application of different LAB strains for drinks biofortification. Thus, *L. plantarum* strains have been used to increase the concentration of RF in a soy-based drink to 0.7–1.86 mg/L (Juarez del Valle et al., 2016), or in the range of 0.5–1.5 mg/L in a kefir-like beverage (Yépez et al., 2019).

On the bases of the results presented here and the previous knowledge, it could be stated that among the plant-based drink tested here, the oat-based beverage was the best matrix for RF biofortification by the *W. cibaria* strain BAL3C-5 C120T and that the levels of RF (2.73 mg/L) reached in the oat-based drink fermented with this strain were superior to that previously described for other drinks fermented by LAB.

Furthermore, in this work, both tested strains were able to produce 600–800 mg/L of dextran in oat-based beverage, as well as 85–100 mg/L

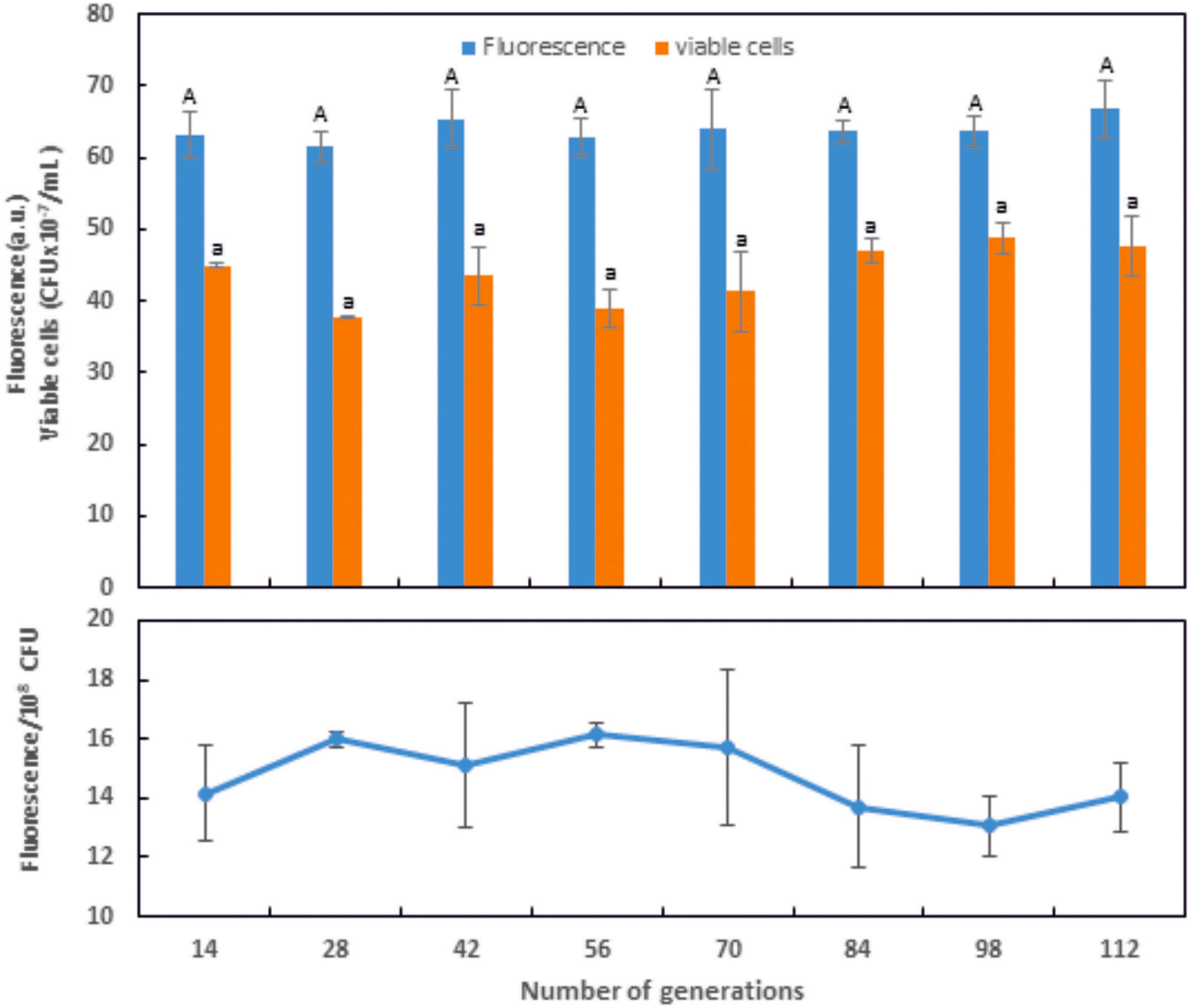


**Fig. 1.** Riboflavin (RF) produced by BAL3C-5 C120T strain after successive generations. RF concentration (dotted line) and population density (bars) were measured every 14 generations in the RF-free RAM medium (A). (B) RF fluorescence per $1 \times 10^8$ CFU.
The statistical analysis with one-way ANOVA did not reveal differences neither for the counts (CFU/mL) or the RF fluorescence.

and 50–70 mg/L in rice- and soy-based drinks, respectively (Fig. 2C). Dextran was not detected in the fermented almond-based drink or in any of the control non-fermented plant-based beverages. Thus, the oat-based drink was the most interesting matrix for dextran biofortification by both LAB. Previously, the use of LAB has been considered a promising strategy for *in situ* production of exopolysaccharide (EPS) in a wide range of foodstuff. Among others, some *Leuconostoc*, *Weissella*, *Pediococcus* strains as well as some lactobacilli have been reported as interesting vehicles for homopolysaccharides (dextran, levan, and/or β-glucan) fortification of fermented milks, plant- and fruit-based juices, or grain-based foodstuff (Hamet et al., 2015; Han et al., 2014; Hernández-Alcántara et al., 2022; Juvonen et al., 2015; Pérez-Ramos et al., 2017; Song, 2013). In these cases, in addition to improve the technological and organoleptic properties, the EPS produced by LAB have been related to multiple biological activities, such as antioxidant, prebiotic, anti-inflammatory, cholesterol lowering capacities and health benefits (Bhat and Bajaj, 2018; Dinić et al., 2018; Farinazzo et al., 2020; Pan et al., 2020; Zannini et al., 2016). Moreover, in the present study, not only the ability of BAL3C-5 C120T to overproduce RF, but also its capacity to increase dextran concentration in plant-based drinks has been confirmed. Beside the evaluation of dextran production, the levels of sugars and some of their metabolites present in the drinks were determined (Table 2).

In all control drinks, in addition to the added sucrose (≈200 mM), fructose (up to 4.9 mM in the rice-based drink) and glucose (up to 259 mM and 347 mM in oat- and rice-based drinks) were detected. Moreover, along with the rice-based drink, the oat-based beverage was the highest in carbohydrates content, and only in these two types of beverages was detected maltose (94 mM and 83 mM, respectively) and maltotriose (9228 mM and 272 mM, respectively). As expected, both LAB showed a similar fermentation pattern. Production of lactic acid (up to ≈415 mM in soy-based drink) was detected as well as an increase of the fructose concentration, presumably generated by the *W. cibaria* Dsr hydrolytic activity of sucrose, concomitant with dextran production. Accordingly, the greatest increase in fructose (from 1.9 mM to ≈30 mM) was observed in the fermented oat-based drinks by the two LABs, accompanied by the highest decrease in sucrose concentration from 192 mM to ≈78 mM (Table 2) and by the highest yield in dextran production (12 mM, Table 2 and Fig. 2C) were detected. In addition, glucose was not metabolized in the oat-based drink, whereas maltotriose (273 mM) was apparently consumed during fermentation, and this fact could be decisive to understand the better behaviour observed for the BAL3C-5 C120T strain in the oat drink. It is also necessary to emphasize that in the case of the oat drink, the production of panose after fermentation was observed.

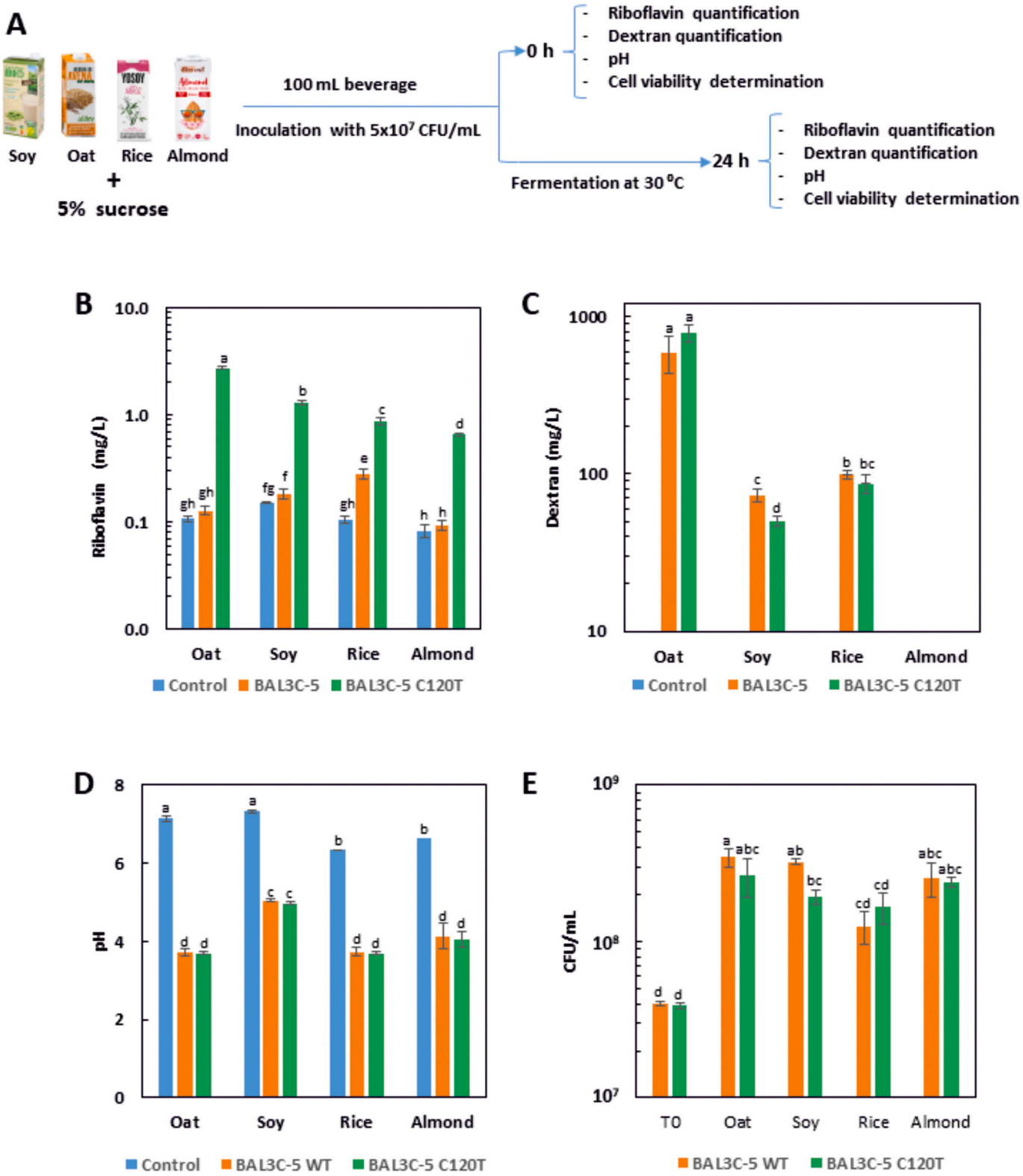


**Fig. 2.** Analysis of plant-based drinks fermented with *W. cibaria* strains. The experimental design is shown (A). In the lower part the concentration of RF (B) and dextran (C), as well as the values of pH (D) and cell viability (E) after 24 h of fermentation of the vegetable drinks with either BAL3C-5 or BAL3C-5 C120T strains are depicted. The different letters indicate statistically significant differences with a two-ways ANOVA analysis.

Panose is a trisaccharide constituted by a maltose unit and a glucose unit through a α-1,6 glycosidic bond. This trisaccharide has been described as a prebiotic candidate, by confirming its bifidogenic effect *in vitro* (Mäkeläinen et al., 2009).

When the pH (Fig. 2D) and the cell viability (Fig. 2E) were evaluated after 24 h of fermentation, a good performance of the BAL3C-5 C120T similar to that of its parental BAL3C-5 strain was observed. These results supported that the RF-overproducing phenotype had no negative effect on the fermentative capability of the strain. Oat-based beverage suitability was once again confirmed, since after fermentation, the drink

**Table 2**
Metabolic analysis of each plant based-beverage before and after fermentation with the corresponding *W. cibaria* strain.

| | Sucrose (mM) | Fructose (mM) | Dextran (mM) | Glucose (mM) | Lactic acid (mM) | Maltose (mM) | Maltotriose (mM) | Panose (mM) |
|---|---|---|---|---|---|---|---|---|
| Oat Control | 192.43 ± 10.41$^{a}$ | 1.95 ± 0.03$^{b}$ | – | 258.57 ± 12.8$^{a}$ | – | 82.68 ± 6.14$^{a}$ | 271.97 ± 56.16$^{a}$ | – |
| Oat+BAL3C-5 | 72.8 ± 8.53$^{b}$ | 27.43 ± 4.66$^{a}$ | 10.93 ± 2.85$^{a}$ | 266.86 ± 19.24$^{a}$ | 132.33 ± 17.99$^{a}$ | 74.42 ± 9.86$^{a}$ | – | 0.33 ± 0.08$^{a}$ |
| Oat+BAL3C-5 C120T | 83.55 ± 5.53$^{b}$ | 34.72$^{b}$ ± 8.05$^{a}$ | 12.44 ± 1.87$^{a}$ | 263.08 ± 1.97$^{a}$ | 80.78 ± 12.85 $^{a}$ | 42.39 ± 1.29$^{b}$ | – | 0.53 ± 0.25$^{a}$ |
| Soy Control | 205.85 ± 0.63$^{a}$ | 0.74 ± 0.01$^{b}$ | – | 3.3 ± 0.08$^{a}$ | 0.5 ± 0.02$^{b}$ | – | – | – |
| Soy+BAL3C-5 | 206 ± 17.91$^{a}$ | 13.01 ± 0.46$^{a}$ | 1.36 ± 0.13$^{a}$ | 1.8 ± 0.35$^{b}$ | 425.18 ± 28.91$^{a}$ | – | – | – |
| Soy+BAL3C-5 C120T | 193.55 ± 0.54$^{b}$ | 12.97 ± 0.54$^{a}$ | 0.92 ± 0.06$^{a}$ | 1.62 ± 0.08$^{b}$ | 405.12 ± 23.06$^{a}$ | – | – | – |
| Rice Control | 228.61 ± 59.74$^{a}$ | 4.88 ± 0.02$^{b}$ | – | 347.41 ± 81.47$^{a}$ | – | 94.2 ± 16.02$^{a}$ | 9228.24 ± 439.37$^{a}$ | – |
| Rice+BAL3C-5 | 174.88 ± 5.17$^{a}$ | 7.73 ± 0.48$^{a}$ | 1.36 ± 0.13$^{a}$ | 295.81 ± 9.92$^{a}$ | 78.38 ± 7.03$^{a}$ | 90.69 ± 1.12$^{a}$ | 4899.34 ± 639.07$^{b}$ | – |
| Rice+BAL3C-5 C120T | 182.9 ± 3$^{a}$ | 7.88 ± 1.19$^{a}$ | 1.12 ± 0.22$^{a}$ | 313.86 ± 4.59$^{a}$ | 63.57± 15.28$^{a}$ | 88.12 ± 0.8$^{a}$ | 4230.02 ± 387.62$^{b}$ | – |
| Almond Control | 224.98 ± 26.64$^{a}$ | 0.81 ± 0.03$^{b}$ | – | 1.88 ± 1.08$^{a}$ | 0.43 ± 0.61$^{b}$ | – | – | – |
| Almond+BAL3C-5 | 242.38 ± 44.35$^{a}$ | 1.61 ± 0.47$^{b}$ | – | 0.42± 0.17$^{b}$ | 77.51 ± 15.08$^{a}$ | – | – | – |
| Almond+BAL3C-5 C120T | 236.23 ± 20.67$^{a}$ | 2.87 ± 0.13$^{a}$ | – | 0.36 ± 0.01$^{b}$ | 59.73 ± 7.71$^{a}$ | – | – | |

Fermentations were performed during 24 h with either BAL3C-5 C120T or BAL3C-5. Then, samples were used to quantitatively identify sugars and metabolites by GC–MS analysis. Samples of the unfermented drinks were used as control. Data are presented as mean ± standard deviation. The different letters among the same matrix indicate statistical significant differences with one-way ANOVA analysis.

presented the highest pH decrease and the highest counts increase compared to the rest of the matrices (Fig. 2D). Lowering of the pH was significant after fermentation by BAL3C-5 C120T in oat-, soy-, almond- and rice-based beverages (down to 3.72, 5.05, 4.14 and 3.74, respectively) and almost equal results were obtained for the parental strain. It must be stated that pH-lowering properties are of great relevance mainly for the safety and antimicrobial properties of the final product. Regarding viable cells, all beverages showed an increase of around 10-fold, from an initial population of 3–4 × $10^{7}$ CFU/mL to 1–3 × $10^{8}$ CFU/mL, although a slightly higher increase was detected in the oat-based drink (Fig. 2E). All in all, it was confirmed that strain BAL3C-5 C120T was capable of synthesizing high levels of RF and dextran in the plant-based beverages tested, the most significant performance being in the oat beverage. Nowadays, plant-based products have a high consumption among the population and they have enormous potential in the coming years as an alternative to dairy products. In addition, they contain a very low RF concentration, therefore the development of these types of beverages *in situ* biofortified with this vitamin is of great interest for the general population and for the food industry.

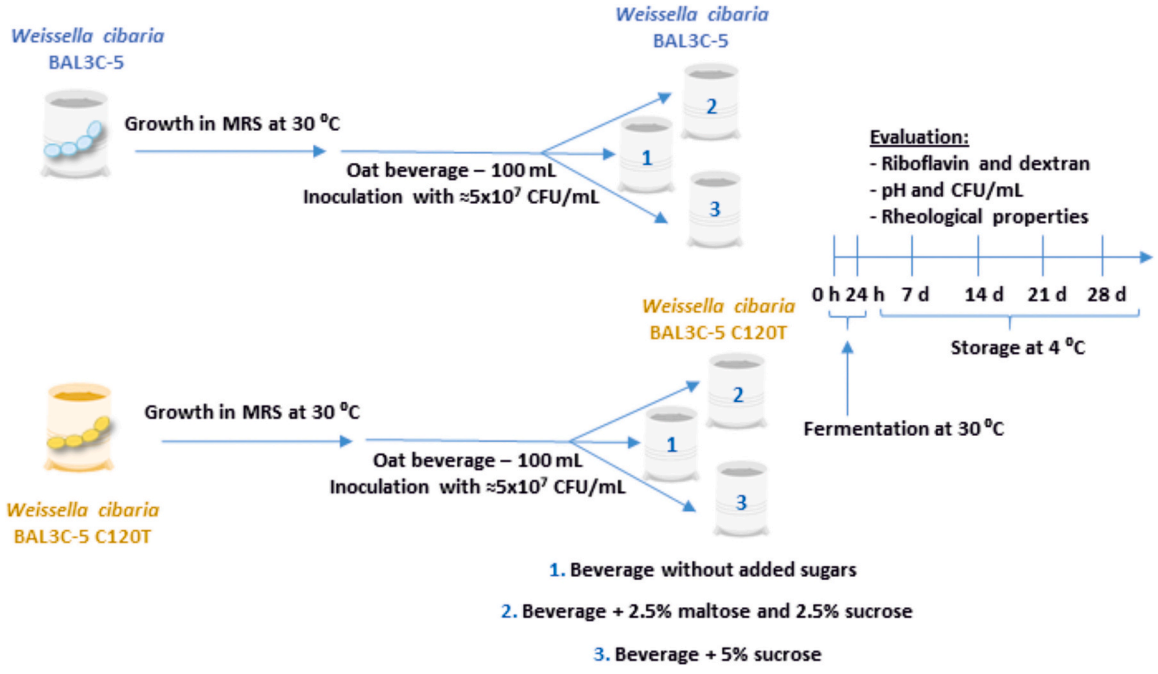


**Fig. 3.** Schematic representation of the methodology used for the fermentation of oat-based beverages by the *W. cibaria* strains. The different steps of the assays as well as the analysis preformed are indicated.

### 3.3. In situ biofortification of oat-based beverage and characterization of their rheological properties

Since oat-based beverage resulted in the most promising matrix, it was decided to further investigate its fermentation with the aim of developing a new biofortified drink with new rheological properties. The LAB Dsr can utilise, in conjunction with sucrose, maltose as donor substrate but not as acceptor for further polymer elongation. Thus, the increase of maltose concentration in the drink can also induce a higher production of the panose detected in this work (Table 2) as previously described (Fernandes and Rodrigues, 2007). Moreover, the increase of sucrose concentration prior fermentation could result in a higher yield of dextran. Therefore, the influence of addition of sucrose or sucrose plus maltose was evaluated according to the protocol depicted in Fig. 3, comparing the behaviour of both LAB strains in oat-based drinks: (i) without added sugars, (ii) supplemented with 2.5 % maltose plus 2.5 % sucrose and (iii) supplemented with 5 % sucrose. The fermentations were performed during 24 h at 30 °C and drinks were stored during 28 days at 4 °C. The drinks were evaluated for RF and dextran production as well as for the levels of viable. The levels of various compounds, pH, cells viability and rheological characteristics after fermentation and at the end of the storage period under refrigeration were analysed (Fig. 4).

#### 3.3.1. Significant increase of RF content in oat-based beverages

The RF analysis (Fig. 4A) confirmed once again the RF-overproducing capacity of BAL3C-5 C120T compared with that of BAL3C-5. In the three conditions tested, the levels of RF after 24 h fermentation with BAL3C-5 C120T were similar: 3.40 mg/L, 3.23 mg/L or 3.16 mg/L were detected, respectively, for the drinks with no-added sugars, supplemented with either 2.5 % of each sugar or 5 % sucrose (Fig. 4A). In addition, in the best case (without sugar addition), a 30-fold increase with respect to the baseline (from 0.11 mg/L to 3.40 mg/L). Thus these results showed that sugar supplementation was not necessary to improve the behaviour and production of RF and that these supplementations do not have a significant negative effect in the vitamin production. Hence, it was also a positive result concerning the nutritional profile of the beverage. After 24 h, in the three fermentation conditions, RF levels remain stable after 4 weeks under refrigeration, and the resulting beverage showed a potential shelf-life stability of 28 days, a very important aspect with regard to the functional potential of the beverage. In the case of the parental strain, the values remained in the same range as the baseline. Recently, it was reported the high RF overproduction capacity of spontaneous *L. reuteri* mutant (Spacova et al., 2022). Although it was stated that in culture medium it was able to produce around 18 mg/L, RF overproduction in food matrices is generally presented as the total fluorescence intensity of the beverages without determining the actual concentration of RF available. In the present work, a 30-fold increase compared to the baseline was observed, an increase higher than those previously described in food biofortification using LAB (Juarez del Valle et al., 2016; Russo et al., 2014; Yépez et al., 2019). Thus, to confirm the suitability of direct measurement of RF fluorescence in oat-based drink, the fluorescence of the samples was measured prior to and after HPLC fractionation. The results revealed almost identical values by the two methods used in all the drinks tested (Supplementary Fig. S1). Consequently, it is possible to perform accurate determinations of RF concentration in oat-based beverages by the simple method of direct fluorescent measurement in a Varioskan microplate type reader.

One 200 mL glass of this beverage would mean an intake of approximately 0.7 mg/day. This means that the recommended daily amounts of RF (0.4–2 mg/day depending on the population group and condition) would be satisfied in a great extent within the framework of a complete and healthy diet. It is necessary to highlight that in recent years a nutritional deficiency in essential micronutrients such as vitamins and minerals has been observed due to high calorific dietary patterns in the USA, Canada, and European countries (Cashman, 2018; Mantadakis, 2020; Ofori et al., 2022). Moreover, hidden hunger, also known as micronutrient deficiency, is a very common feature in developing countries due to over-consumption of staple foods as a consequence of the difficulty to access adequate foods due to poor economic and political status (Gödecke et al., 2018).

Proper dietary patterns as well as development of new functional foods may be considered to improve general health status in both developed and developing countries. One promising vehicle to overcome this problem is plant-based products, since they represent a high percentage of the diet in many populations. Among them, plant-based beverages are increasingly popular as dairy alternatives (McClements et al., 2019; Montemurro et al., 2021). However, it is necessary to ensure that the new alternatives are not nutritionally inferior to dairy products, and that they present an adequate nutritional profile. In a recent study, it was observed that only 16 % of plant-based beverages out of a total of 641 products had RF, and none of them fulfilled the 15 % of the daily value recommended to be established as “Source of”, which represents 0.09 mg/100 mL (Drewnowski et al., 2021). In the present study, the RF increase up to 3.4 mg/L was obtained, almost triplicating the ≈1.2 mg/L value commonly observed in milk, which is considered one of the main dietary sources of RF (Drewnowski et al., 2021). Hence, it is correct to state that resulting oat-based beverages are an improved source of RF compared to the current dairy and non-dairy products available on the market.

Regarding to the pH-lowering property, the high fermentative power of the BAL3C-5 C120T strain was highlighted as the pH was lowered to approximately 3.8 in all cases (Fig. 4B). This characteristic remained stable during the storage time. This is a very important criterion to ensure the safety and microbiological stability of the drink as well as to increase the shelf life of the product. In the case of the cell viability, after 24 h of fermentation the population increased >10-fold, reaching $10^8$–$10^9$ CFU/mL. After 7 days in refrigeration, the population remained at 100 % of the initial inoculum and after 4 weeks in storage, around 80 % of the population was preserved in all cases (Fig. 4B). Population stability is another feature to highlight in order to enhance the possible probiotic character of the strains, ensuring high counts throughout the storage period (Montemurro et al., 2021).

The plant-based beverages are considered a healthy substitute of dairy, with low concentration of saturated fats and cholesterol. The main factors currently driving their growing consumption are an increased awareness of health benefits, increasing number of people intolerant or/and allergic to dairy, or increased gravitation towards vegan lifestyle. In this sense, the growing popularity of plant-based beverages constitute an interesting strategy to develop new functional foodstuff. In the present study, the ability of BAL3C-5 C120T strain for RF biofortification of oat-based (and others) beverages was confirmed. This fact is of great relevance, since this type of beverage naturally does not present RF or in a very low concentration, and the chemical addition of RF is generally carried out. Moreover, it must be highlighted the relevance of RF due to its antioxidant, antiaging, anti-inflammatory and anticancer properties. Indeed, recently multiple research studies have been done on the framework of RF and the prevention of multiple health disorders (Farah et al., 2022; Revuelta et al., 2017; Suwannasom et al., 2020; Thakur et al., 2017).

#### 3.3.2. Production of dextran and prebiotic oligosaccharides in the oat-based beverages

Therefore, we wanted to determine in the three fermentation condition analysed, the production of dextran by the two LAB after the 24 h fermentation period and at the end of the fermented drinks storage at 4 °C (28 days) (Fig. 5A). No production was detected in the control drinks without LAB addition (Fig. 5A). In the case of drinks supplemented with 5 % sucrose, both LAB produced high levels of dextran during the fermentation period. The levels reached with BAL3C-5 (4.5 g/L) and BAL3C-5 C120T (3.3 g/L), were also 10–fold and 13–fold higher than that detected in the corresponding fermented drinks without

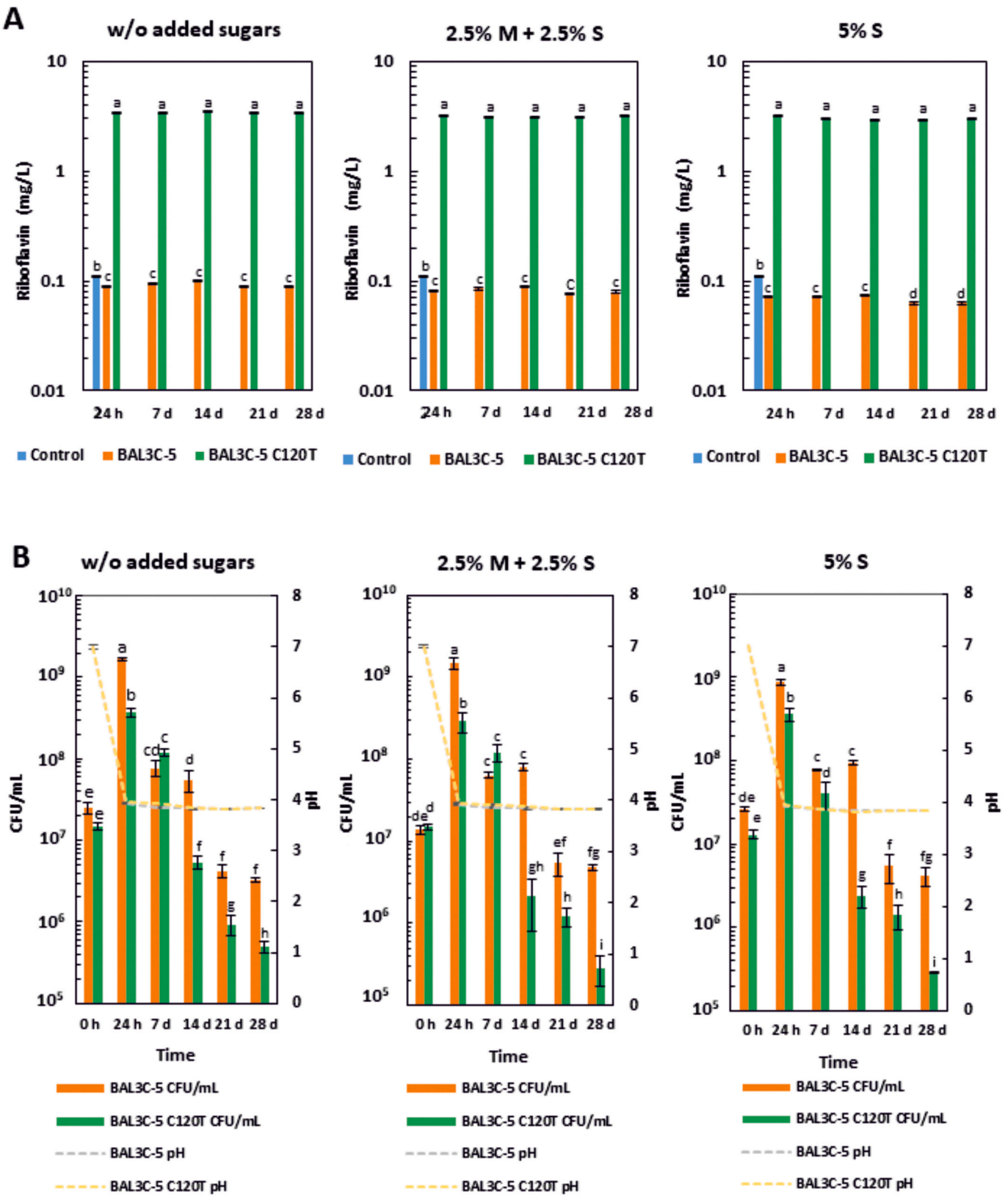


**Fig. 4.** Analysis of oat-based drinks fermentation. (A) oat-based beverages with no added (w/o) sugars, with 2.5 % maltose (2.5 % M) plus 2.5 % sucrose (2.5 % S) or with 5 % (5 %) of sucrose, after 24 h fermentation with either BAL3C-5 or BAL3C-5 C120T strains and during storage times (7 d, 14 d, 21 d and 28 d). (B) The same evaluation for evolution of pH and cell viability represented as CFU/mL for the corresponding fermentation conditions, without sugars, 2.5 % of each sugar, or 5 % of sucrose. Also, initial values of CFU/mL and pH (0 h) are depicted. The different letters indicate statistical significant differences detected with mixed ANOVA analysis.

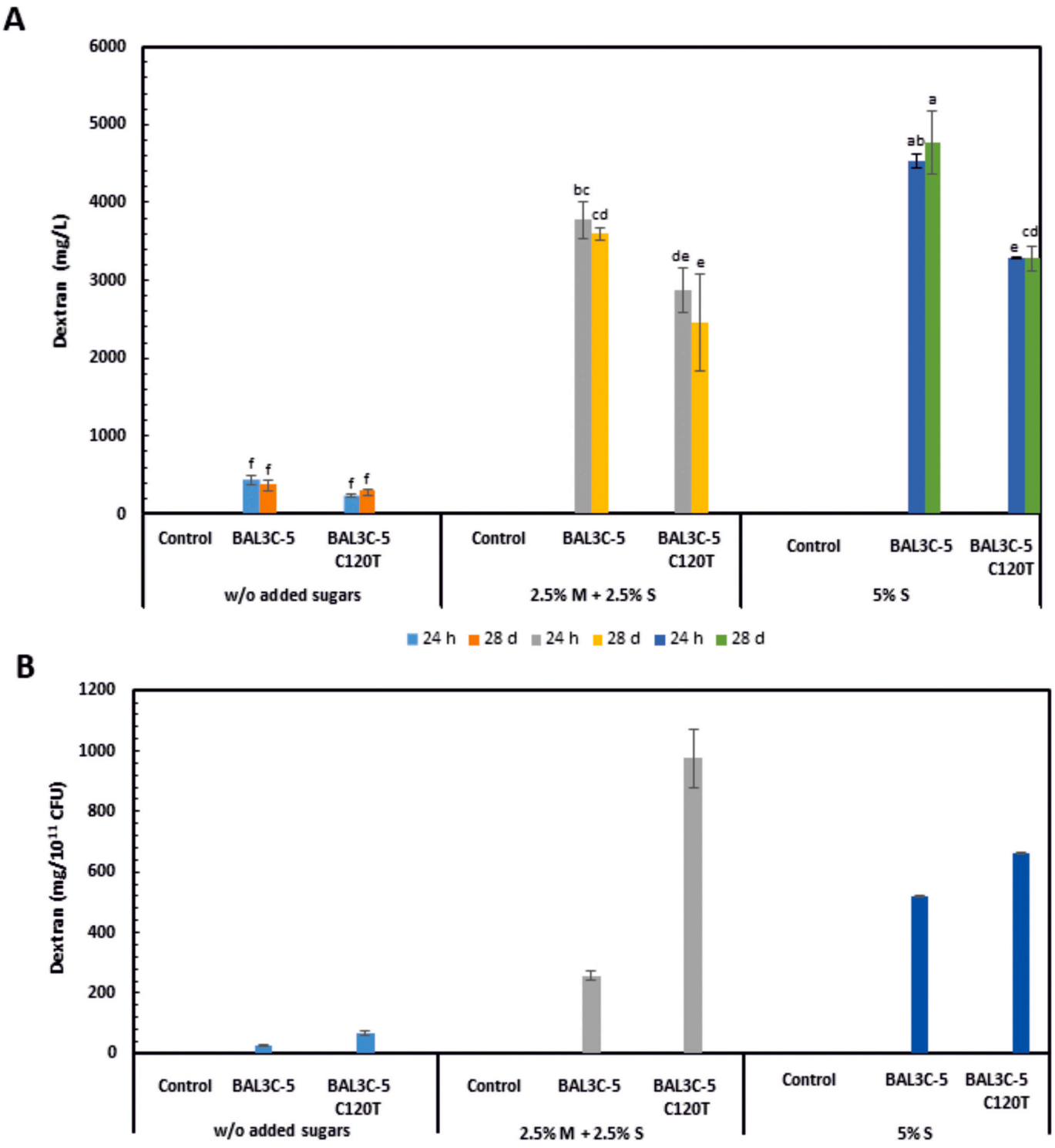


**Fig. 5.** Detection of dextran production in oat-based drinks fermented with the *W. cibaria* strains. (A) The levels of the homopolysaccharide were measured after 24 h fermentation with either BAL3C-5 or BAL3C-5 C120T strains and after 28 days of storage at 4 °C. The values obtained are represented in (A). The different letters indicate statistically significant differences with one-way ANOVA analysis. In (B) the dextran production per $1 \times 10^{11}$ CFU is depicted.

sugar addition. Also, the supplementation with 2.5 % sucrose plus 2.5 % maltose provided similar results as that obtained with 5 % sucrose, although slightly lower dextran levels were observed. It has to be stated that after fermentation the CFU/L of the BAL3C-5 strain were always higher than that obtained with the BAL3C-5 C120T strain (Fig. 4B). Therefore, when the dextran produced by $10^{11}$ CFU was estimated, it was clear that BAL3C-5 C120T was a better producer than its parental strain, since levels of 977 mg *vs* 257 mg and 663 mg *vs* 519 mg under supplementation with 2.5 % sugars and 5 % sucrose, respectively, were detected. (Fig. 5B). Consequently, the results obtained revealed that improving fermentation conditions to support better growth of BAL3C-5 C120T should result in an optimization of this strain as a dextran producer.

In addition, measurement of dextran levels after fermented drinks storage for 28 days under refrigeration conditions revealed no statistically significant decrease (Fig. 5A). Therefore, it seems that it remains

stable at a high molecular weight without further hydrolysis. This is important for the postbiotic effect of the dextran as an immunomodulator, since only high molecular weight dextran produced by LAB have this effect (Zarour et al., 2017). Furthermore, *in vitro* and *in vivo* studies performed with fish models, support that dextrans from LAB have an antiviral activity due to a stimulation of the innate immune system by induction of production of interferons (IFN-1 and IFN-γ) (Nácher-Vázquez et al., 2015). The innate immune responses plays an essential role in primary defense against pathogens and for example, this is one of the starting points for immune evasion by SARS-CoV-2 (Silva et al., 2022). Moreover, old people are subject to immunosenescence and this is one of the reasons why they are very sensitive to SARS-CoV-2 infections. Therefore, drinks enriched with dextran could have healthy benefits for the elderly population reinforcing their defences against viral infections.

Analysis of the sugars metabolism (Table 3) revealed that, as expected, the fermentation conditions affected not only the production of dextran but also that of panose. Addition of 2.5 % maltose in conjunction with 2.5 % sucrose was the best condition to increase panose production by the two LAB. The highest level was that produced by the wild- type strain (20 g/L, 42.2 mM), and also high levels were detected with BAL3C-5 C120T (6.6 g/L, 25.8 mM). Moreover, in drinks fermented by either LAB in the mentioned supplementation, it was detected an increase of around 15-fold in comparison with the levels of the prebiotic trisaccharide produced in the absence of sugar addition. Finally, in the presence of 5 % sucrose a 6–9-fold increase of panose concentration over the basal levels was detected. Consequently, the results presented above support that BAL3C-5 C120T and its parental strain can be used to generate a novel oat-based functional beverages fortified with dextran and panose.

The plant-based beverage market is increasingly growing, with a compound annual growth rate of 6.7 % during the period 2020–2027, which means a growth from USD 14.46 billion in 2019 to USD 24.30 billion by 2027 (Fior Markets, 2020). In this context, it seems that the use of BAL3C-5 C120T to generate a new type of functional oat-based fermented drink biofortified with RF, the prebiotic panose and the postbiotic dextran has industrial interest. Moreover, this new functional beverage is useful for vegetarian, vegan, milk intolerant and elderly populations and should be successful in this important market.

### 3.3.3. *Rheological properties in fermented oat-based beverages*

The oat-based drinks experienced a visible change in structure and texture (see Supplementary Fig. S2) presumably as a result of acidification during the fermentation process. As in the case of milk, coagulation of the beverages occurred after reaching the isoelectric point of the main oat proteins (oat globulins), due to the acidification of the medium. Furthermore, the microorganisms themselves, as well as their metabolites (*e.g.* dextran), can influence the aggregation of proteins. They can even interact with other components of the matrix, giving rise to structural changes. Fig. 6 illustrates the rheological characteristics of these samples in terms of the flow behaviour and the viscoelastic response, which is the subject of discussion below.

The non-fermented control beverage exhibited Newtonian flow behaviour, that is, the viscosity was independent of the applied shear rate. The behaviour became non-Newtonian when the control drink was acidified and chemically coagulated, revealing a change in fluid structure. These changes were even more significant in the case of the beverages fermented with the mutant and parental strains, as evidenced from Fig. 6A and B.

The characterization of the non-Newtonian behaviour was quantified by fitting the shear stress-shear rate flow curves using both the Herschel-Bulkley (H-B) model (Eq. (2)) and the power law model (Eq. (1)); the corresponding parameters are included in Table 4. The H-B model demonstrated an excellent fit to non-Newtonian behaviour, including the lower shear rate regime where plastic behaviour was observed. A yield stress, $\tau_y$, the stress that must be exceeded for flow to occur, was calculated. This critical value represents the stress below which the material behaves as a solid, absorbing the strain energy without flowing. As already mentioned, unlike the Newtonian control beverage with $\tau_y = 0$ and $n = 1$, the rest of the samples were yield stress fluids. Above the yielding regime, once flow is initiated, a general shear thinning behaviour (index $n < 1$) was observed for all the samples, as the viscosity decreased as the shear rate increased.

In addition to the yield stress characterization, a detailed investigation of the evolution of the structure as a function of shear and time was of interest. In many fluids, viscosity is independent of time and depends solely on the shear rate and temperature. However, in the case of some highly concentrated dispersions, the viscosity does not achieve a steady state during experimental time. Instead, its value depends on the stabilization of the internal network structure, which is broken by shear forces and will need a period of time to be rebuilt. This leads to a time-dependent shear thinning behaviour, known as thixotropic behaviour. The property of thixotropy can be easily identified in Fig. 6C and D, as the down and up ramp flow curves had a different trajectory and formed a hysteresis loop. The calculated area within the hysteresis loop, A, (in Pa $s^{-1}$) represents the energy consumed in the breakdown of the structure and defines the magnitude of the thixotropy (values of *A* are given in Table 4).

**Table 3**
Influence of sugar addition on sugars metabolism of oat-based beverages fermented with *W. cibaria* strains.

| Sample | Sucrose (mM) | Fructose (mM) | Dextran (mM) | Glucose (mM) | Lactic acid (mM) | Maltose (mM) | Maltotriose (mM) | Panose (mM) |
|---|---|---|---|---|---|---|---|---|
| w/o Control | 6.51 ± 0.1$^{a}$ | 3.52 ± 0.49$^{a}$ | – | 132.22 ± 4.18$^{a}$ | – | 167.32 ± 4.76$^{a}$ | 108.37 ± 2.79$^{a}$ | – |
| w/o BAL3C-5 | – | 6.17 ± 0.44$^{b}$ | 2.41 ± 0.35$^{a}$ | 165.01 ± 2.01$^{a}$ | 8.06 ± 0.34 $^{a}$ | 129.94 ± 1.62$^{b}$ | 63.81 ± 2.10$^{c}$ | 2.80 ± 0.29$^{a}$ |
| w/o BAL3C-5 C120T | 0.52 ± 0.03$^{b}$ | 5.73 ± 0.17$^{b}$ | 1.35 ± 0.12 $^{b}$ | 139.82 ± 5.74$^{a}$ | 6.65 ± 0.53$^{a}$ | 115.03 ± 3.96$^{b}$ | 84.24 ± 4.25$^{b}$ | 1.78 ± 0.19$^{a}$ |
| 2.5 % M + 2.5 % S Control | 86.35 ± 21.3$^{a}$ | 4.56 ± 0.55$^{a}$ | – | 161.07 ± 29.48$^{a}$ | – | 205.30 ± 10.941$^{a}$ | 62.23 ± 1.08$^{b}$ | – |
| 2.5 % M + 2.5 % S BAL3C-5 | 4.31 ± 1.46$^{c}$ | 79.33 ± 1.50$^{b}$ | 20.95 ± 1.28$^{a}$ | 144.85 ± 4.94$^{a}$ | 7.57 ± 0.02$^{a}$ | 232.71 ± 5.53$^{a}$ | 61.74 ± 1.08$^{b}$ | 42.14 ± 0.22$^{a}$ |
| 2.5 % M + 2.5 % S BAL3C-5 C120T | 37.98 ± 4.65$^{b}$ | 48.75 ± 1.07$^{b}$ | 15.92 ± 1.57$^{a}$ | 142.03 ± 18.51$^{a}$ | 7.18 ± 0.33$^{b}$ | 222.38 ± 2.33$^{b}$ | 84.24 ± 4.25$^{a}$ | 25.85 ± 0.33$^{b}$ |
| 5 % S Control | 144.44 ± 30.53$^{a}$ | 5.8 ± 0.91$^{a}$ | – | 222.72 ± 0.29$^{a}$ | – | 169.05 ± 2.60$^{a}$ | 61.14 ± 0.62$^{a}$ | – |
| 5 % S + BAL3C-5 | 53.37 ± 3.75$^{b}$ | 83.4 ± 0.71$^{c}$ | 25.14 ± 0.46$^{a}$ | 167.9 ± 1.78$^{b}$ | 8.33 ± 0.47$^{a}$ | 75.92 ± 3.70$^{b}$ | 55.48 ± 0.62$^{b}$ | 25.11 ± 0.41$^{a}$ |
| 5 % S+ BAL3C-5 C120T | 76.82 ± 25.29$^{b}$ | 63.97 ± 2.98$^{b}$ | 17.49 ± 0.06$^{b}$ | 162.46 ± 5.26$^{b}$ | 6.86 ± 2.81$^{b}$ | 76.45 ± 1.81$^{b}$ | 36.18 ± 1.89$^{b}$ | 10.92 ± 3.89$^{b}$ |

Fermentations were performed during 24 h with either BAL3C-5 C120T or BAL3C-5 as well as in the absence of LAB (Control): without sugar addition (w/o) or in the presence of either 2.5 % maltose plus 2.5 % sucrose (2.5 % M + 2.5 % S) or 5 % sucrose. Data are presented as mean ± standard deviation. The different letters indicate statistical significant differences with one-way ANOVA analysis.

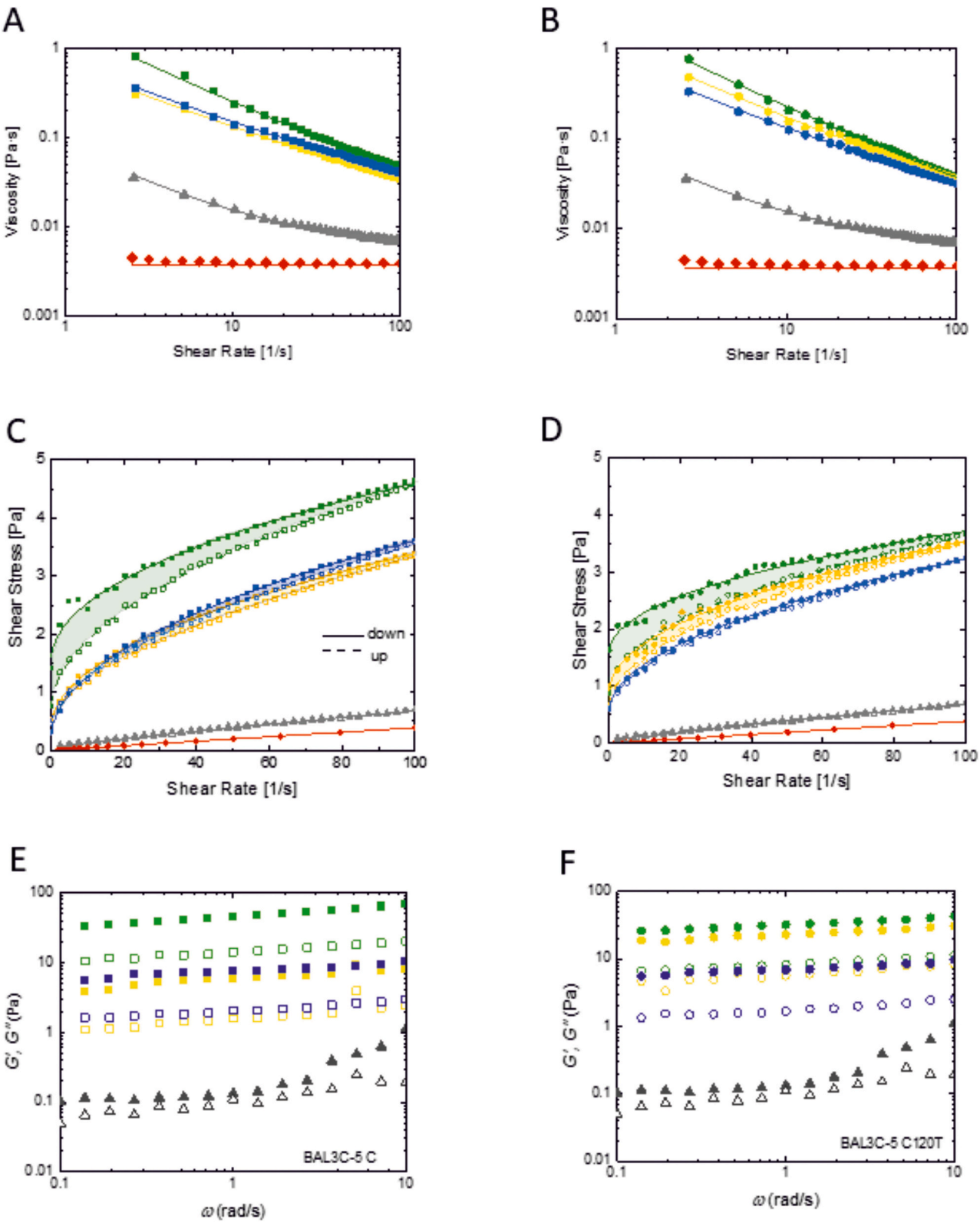


**Fig. 6.** Rheological behaviour of oat-based drinks fermented with *W. cibaria* strains. Non-fermented beverages (Δ and ⋄) and beverages fermented with BAL3C-5 C120T (○) and BAL3C-5 (□) strains under the different conditions are displayed: control beverage (symbols in red), the pH-controlled beverage (symbols in grey), and the fermented beverages: (w/o) sugars (symbols in green), 2.5 % sugars (symbols in yellow), and 5 % sucrose (symbols in blue). (A) and (B) The pseudoplastic behaviour or shear thinning flow is characterized by a viscosity which decreases as the shear rate increases. (C) and (D) The thixotropy is identified by the presence of a hysteresis loop in the down and up ramp flow curves. (E) and (F) The viscoelastic behaviour is studied by the dependence with frequency of the storage modulus, G′, (filled symbols) and the loss modulus, G″, (empty symbols). "Gel" behaviour ($G' > G''$) characterizes all the samples.

**Table 4**
Rheological analysis of fermented oat-based beverages.

| | $n_p$ | $K_p$ (Pa·s$^n$) | $R^2$ | $\tau_y$ (Pa) | $n_{H\text{-}B}$ | $K_{H\text{-}B}$ (Pa·s$^n$) | $R^2$ | $A$ (Pa s$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Control | 1.01 | 0.003 | 0.996 | – | 1.01 | 0.003 | 0.996 | – |
| Control pH | 0.74 | 0.02 | 0.992 | 0.07 | 0.88 | 0.01 | 0.998 | 2 |
| BAL3C-5 C120T | | | | | | | | |
| w/o sugars | 0.22 | 1.34 | 0.992 | 1.51 | 0.46 | 0.27 | 0.989 | 28 |
| 2.5 % M + 2.5 % S | 0.36 | 0.64 | 0.997 | 0.52 | 0.48 | 0.33 | 0.997 | 14 |
| 5 % S | 0.43 | 0.45 | 0.996 | 0.49 | 0.49 | 0.29 | 0.998 | – |
| BAL3C-5 | | | | | | | | |
| w/o sugars | 0.29 | 1.28 | 0.992 | 1.48 | 0.47 | 0.35 | 0.990 | 33 |
| 2.5 % M + 2.5 % S | 0.48 | 0.73 | 0.999 | 0.49 | 0.60 | 0.18 | 0.999 | 12 |
| 5 % S | 0.48 | 0.73 | 0.998 | 0.47 | 0.58 | 0.23 | 0.998 | 13 |

Fermentations were performed with either BAL3C-5 C120T or BAL3C-5 as well as in the absence of *W. cibaria* strains: without sugar addition or in the presence of either 2.5 % maltose plus 2.5 % sucrose or 5 % sucrose. Control beverages were fermented in the absence of LAB addition prior (control) or after chemical acidification (control pH). The flow ($n_p$) and consistency index ($k_p$) were obtained from Power law model (Eq. (1)). The yield stress ($\tau_y$), flow ($n_{H\text{-}B}$), and consistency index ($k_{H\text{-}B}$) were obtained from Herschel-Bulkley model (Eq. (2)). Thixotropy parameter, $A$, corresponds to the area enclosed under the up and down ramp flow curves. All corresponding data were obtained from 24 h fermented beverages.

The rheological parameters that describe the flow curve, yield stress, $\tau_y$, consistency index, K, and pseudoplasticity index, n, as well as the thixotropy property, are indicative of the degree of complexity and strength of the fluid structure. It is therefore, generally assumed, that these values will increase for more complex molecular structures. In fact, considering the dextran-producing phenotype of the two *W. cibaria* strains, the potential network formed by the EPS together with other components of the beverage, which is disrupted when applying a high shear rate, has been reported in other studies (Juvonen et al., 2015; Zannini et al., 2016), and could explain the high values for the complex behaviour of yielding, shear thinning and thixotropic nature of these fluids. Thus, the control beverages showed a low value of these flow parameters, due to the low level of interactions between constituents, while more structured fermented beverages showed a significant increase, even higher in the case of the beverage without added sugars. It is noteworthy that fermented structures with added sugars have lower yield stress values compared to beverages without added sugars, demonstrating superior capacity for viscosity recovery. In contrast, beverages without sugar exhibited higher hysteresis loops and thixotropic indices. This indicates that the obtained structures, with high viscosity at rest, would necessitate a longer recovery period to rebuild.

Indeed, when viscosity was evaluated for a determined shear rate of 100 s$^{-1}$, the increase was significant after fermentation, increasing from 3 mPa·s (control beverage) to >30 mPa·s for the different fermentation conditions (Table 5). The chemically acidified control beverage also presented an increase in viscosity compared to the non-fermented control, although much lower than the viscosity observed after fermentation. It should also be noted that the behaviour and viscosity of the resulting beverages remained stable during the storage period, that is, the structure of the drink was not compromised during this period (data not shown).

The linear viscoelastic moduli of the samples, storage modulus G′, and loss modulus G″ are presented in Fig. 6E and F. Generally, viscoelastic fluids in the linear region are typically time or frequency dependent due to the microstructure's response to flow. At short times (high frequencies), the structures cannot respond quickly, resulting in an elastic response (G′ > G″). Conversely, at much longer experimental times (low frequencies), the material can adjust continuously, allowing for flow and the observation of a viscous effect (G″ > G′). Therefore, the behaviour at different frequency scales is viscoelastic. The essential difference between the time-frequency dependence observed in linear viscoelastic experiments and the time-dependent flow behaviour corresponding to the thixotropic event, is that in the linear region the structure responds but remains unchanged, whereas during continuous flow the structure breaks down by deformation. As illustrated in Fig. 6E and F, the viscoelastic behaviour of the fermented beverages was observed to be essentially elastic (G′ > G″) in the frequency scale of the experimental measurements. This response, which is characteristic of a

**Table 5**
Analysis of viscosity of oat-based fermented drinks.

| | Viscosity (mPa·s) | | | | |
|---|---|---|---|---|---|
| | Time | Unfermented | Fermented | | |
| | | | w/o sugars | 2.5 % M + 2.5 % S | 5 % S |
| Control | 24 h | 3.01 ± 0.05 | – | – | – |
| Control pH | 24 h | 8.26 ± 0.01 | – | – | – |
| BAL3C-5 C120T | 24 h | – | 36.95 ± 0.35 | 33.35 ± 2.76 | 32.85 ± 0.78 |
| | 14 d | – | 34.50 ± 1.70 | 33.25 ± 0.92 | 33.00 ± 0.14 |
| | 28 d | – | 35.90 ± 2.12 | 32.10 ± 1.13 | 29.80 ± 0.00 |
| BAL3C-5 | 24 h | – | 49.35 ± 4.17 | 34.70 ± 1.13 | 38.50 ± 2.69 |
| | 14 d | – | 41.15 ± 0.35 | 35.40 ± 0.99 | 39.40 ± 5.09 |
| | 28 d | – | 39.05 ± 0.92 | 32.30 ± 0.92 | 36.75 ± 0.21 |

Fermentations were performed for 24 h with either BAL3C-5 C120T or BAL3C-5: without sugar addition (w/o sugars) or in the presence of either 2.5 % maltose plus 2.5 % sucrose (2.5 % M + 2.5 % S) or 5 % sucrose (5 % S). Control beverages were non fermented and analysed prior (control) or after chemical acidification (control pH). Viscosity values, for a shear rate of 100 s$^{-1}$ were determined for all beverage samples after 24 h of fermentation. In addition, viscosity of the bacterial fermented beverages was analysed during a storage period (14 d and 28 d) under refrigeration. Data are presented as mean ± standard deviation.

"gel behaviour", would result from the aggregation or physical cross-linking of protein molecules into a three-dimensional solid-like network. The differing values of the elastic modulus observed among the fermented beverages could be attributed to variations in the dimensions of the network and/or interactions between components. Consequently, it is concluded that a strong elastic behaviour (modulus independent of frequency) characterizes the fermented beverages, due to the network's ability to block the flow, even at low frequencies. In contrast, the observed behaviour of chemically acidified beverages, with a similar contribution of both moduli (G′ ≈ G″), is indicative of a lower elastic character. This is in contrast to the non-fermented beverage, for which no elasticity can be measured, as the beverage exhibited newtonian behaviour, a typical viscous response.

In summary, fermentation of oat-based drinks with BAL3C-5 and BAL3C-5 C120T strains resulted in the formation of well-structured fluids, identified by a strong elastic "gel behaviour" (G′ > G″). The observed response is likely due to the formation of a solid-like three-dimensional network, which could be driven by complex interactions

between the EPS, protein network, and other components of the beverage. Moreover, these fermented structures exhibited high sensitivity to shear, displaying extreme shear thinning flow with a yield stress. Additionally, a time-dependent flow behaviour was identified in the fermented beverages without added sugars, where the thixotropy index was clearly relevant. These complex behaviours are in contrast to the elastic ($G' \approx G''$) but weak shear thinning and non-thixotropic behaviour of the non-fermented beverage and the viscous newtonian behaviour of the chemically acidified beverage.

Dextran has water binding ability and it is known that its addition improves the rheological properties of fermented dairy products (including acidified milk gels), reducing syneresis and enhancing creaminess and viscosity (Mende et al., 2013). In addition, it has been shown that dextran stabilizes milk proteins, apparently through a type of depletion flocculation mechanism, improving the texture of the dairy product (Pachekrepapol et al., 2014). Therefore, it is feasible that the rheological properties detected in the oat-based drinks fermented with the *Weissella* strains are mainly due to the levels of dextran (3.5–4.5 g/L) produced by them. Nevertheless, even if other factors contribute to the rheological changes in the fermented drinks, it seems that the oat-based beverage generated by BAL3C-5 C120T fermentation, in addition to its functional properties, has good rheological properties.

### 3.3.4. Survival of BAL3C-5 C120T under simulated gastrointestinal conditions

Once the capacity of the BAL3C-5 C120T strain for RF and dextran biofortification was confirmed in plant-based beverages, it was necessary to analyse its potential for *in vivo* applications targeting the gastrointestinal system, as a preliminary assay for the potential of this strain as a probiotic bacterium. When simulating the gastrointestinal conditions, three matrices were used: a saline solution and the oat beverage, both inoculated with 5–8 $\times$ $10^8$ CFU/mL, and the oat beverage fermented for 24 h, which contained a LAB population of 5 $\times$ $10^8$ CFU/mL after fermentation (Fig. 7). The protective effect of the food matrix was evident. After 90 min in simulated gastric conditions, in the saline solution the population drastically decreased to 2 $\times$ $10^4$ CFU/mL, while in the inoculated and fermented oat beverages, the population remained approximately in the same order (2–4 $\times$ $10^8$ CFU/mL) and 2-log lower (5–6 $\times$ $10^6$ CFU/mL), respectively. After 180 min under simulated intestinal conditions, the same pattern was repeated. A final drop of up to 4–3 $\times$ $10^3$ CFU/mL was observed in the saline solution compared to a drop up to 5–3 $\times$ $10^5$ CFU/mL for the two different

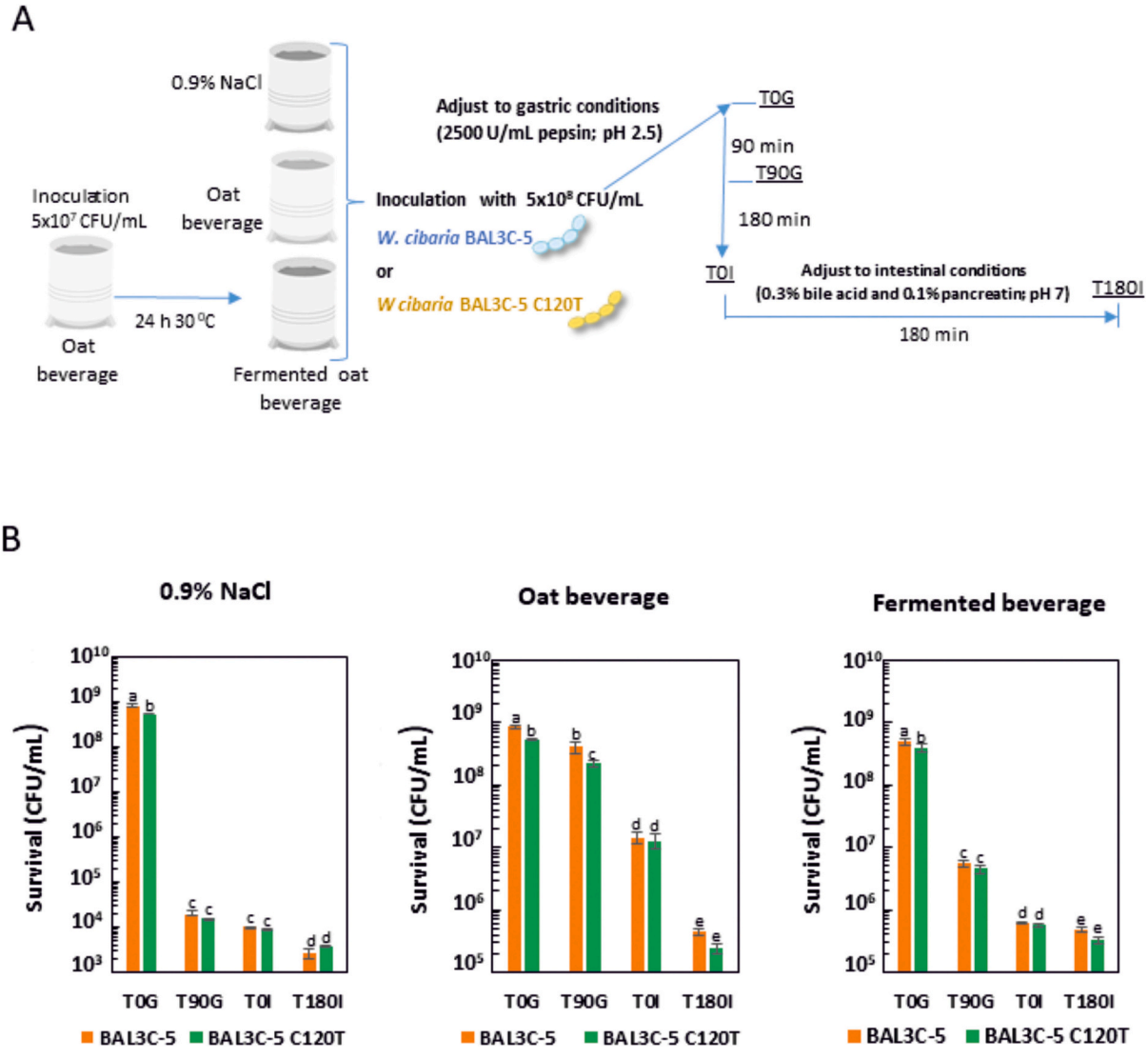


**Fig. 7.** Tolerance of the *W. cibaria* strains to gastrointestinal conditions. (A) Scheme of the protocol followed to evaluate the resistance of the BAL3C-5 or BAL3C-5 C120T strains to the simulated gastrointestinal conditions. (B) Analysis of the survival of the strains under the different matrices evaluated: saline solution, oat beverage and 24 h fermented oat beverage. Evaluation was performed prior (T0G) and after exposure to gastric (T0G and T90G) and intestinal (T0I and T180I) stresses. The different letters indicate statistically significant differences with one-way ANOVA.

conditions with the oat-based drink (Fig. 7). Although a significant viability drop was observed, a high population was still detectable after the assay. In this sense, the ability of the BAL3C-5 C120T strain to resist gastrointestinal conditions is relevant, since it could facilitate the *in situ* production of RF in the digestive system, improving its bioavailability, facilitating its absorption and benefiting the intestinal microbiota. However, it is necessary to investigate the adhesion capacity in intestinal cells as well as the capacity to produce RF under these conditions. In fact, only strains that present confirmed gastrointestinal stability and biological safety can be proposed as probiotics (de Melo Pereira et al., 2018; George Kerry et al., 2018).

Another essential property of potential probiotics is their ability to self-aggregate, a way of colonizing different environments as well as a strategy for recognition, communication, and survival that allows them to resist and remain in the gastrointestinal tract. In addition, this capacity can also have a barrier effect on potential pathogens, preventing their colonization (Del Re et al., 2000; Krausova et al., 2019; Saito et al., 2019). The autoaggregation capacity increased during the incubation time for both LAB strains. The autoaggregation values ranged from 19 % after 3 h to 26 % after 6 h. Furthermore, both strains exhibited a high autoaggregation after 24 h incubation with values around 80 % (Fig. 8). These results agree with those reported by other research studies, where autoaggregation capacities of *Lactobacillus* and *Bifidobacterium* probiotic strains were evaluated (Abouloifa et al., 2020; Cizeikiene and Jagelaviciute, 2021). In this context, another trait that may influence gut colonization, and thus probiotic characteristics of current strains, is the dextran production capacity. Although in the present study the relationship between the dextran-producing capacity and the adhesion capacity of the strains has not been evaluated, different studies have confirmed that the dextran-producing capacity facilitates gut colonization, stabilizes bacterial biofilm, enhances microbial adhesion and autoaggregation capacities, and increases tolerance to environmental stresses (Deng et al., 2020; Tuo et al., 2013).

## 4. Conclusions

In the present work, the functional characterization of *W. cibaria* BAL3C-5 C120T has confirmed that it is a microorganism useful for food biofortification due to its RF-overproducing phenotype. Its usage to ferment oat-based drinks seems to be a good approach for covering the daily recommended RF values through the development of novel functional foods with enhanced RF content. In addition, production of dextran by *W. cibaria* BAL3C-5 C120T can provide new rheological properties to the oat-based drink. Moreover, the biofortification of oat-based drinks by BAL3C-5 C120T with the postbiotic dextran and the prebiotic panose will increase the functionality of the beverage. BAL3C-5 C120T showed a good survival in the oat-based drink after storage and under *in vitro* simulated gastrointestinal conditions, together with a high auto aggregation capability. Therefore, BAL3C-5 C120T could be used as probiotic bacteria delivered in the functional oat-drink, if their RF-overproducing and dextran-producing capabilities are exerted in the digestive tract. However, although promising results have been observed *in vivo* studies are still needed to confirm its probiotic potential.

## Funding

This research was funded by the Spanish Ministry of Science, Innovation and Universities, [grant RTI2018-097114-B-I00], CSIC [grant COOPA20488] and the Basque Government [grants IT1662-22 and PIBA_2020_1_0032]. I.D.O. is the beneficiary of a postdoctoral grant Margarita Salas by UPV-EHU [MARSA21/25] in the framework of the requalification of the Spanish university system funded by the European Union -NextGenerationEU. P.R. is the beneficiary of a grant by the University of Milan in the framework of "Linea 2—A. 2022".

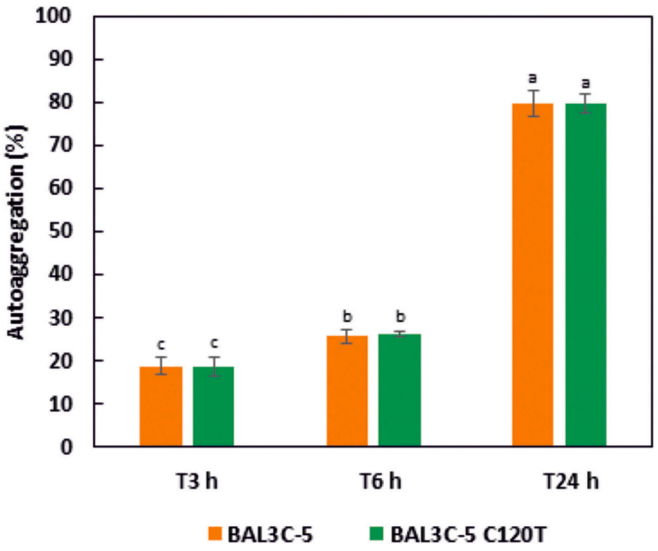


**Fig. 8.** Autoaggregation of the *W. cibaria* strains. The aggregation capacity of BAL3C-5 and BAL3C-5 C120T strains was evaluated after 3 h, 6 h and 24 h incubation. The different letters indicate statistically significant differences with one-way ANOVA.

## CRediT authorship contribution statement

**Iñaki Diez-Ozaeta:** Writing – original draft, Investigation, Data curation. **Irati Berasarte:** Methodology. **Ahmed Fouad Zeid:** Investigation. **Mercedes Fernández:** Investigation. **Pasquale Russo:** Writing – review & editing, Methodology, Conceptualization. **Paloma López:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization. **Mª. Teresa Dueñas:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Mari Luz Mohedano:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

## Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2024.110908.

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