Valle-Vargas *et al. AMB Express* (2025) 15:20
https://doi.org/10.1186/s13568-024-01810-4

AMB Express

ORIGINAL ARTICLE Open Access

Improving the survival under gastric conditions of a potential multistrain probiotic produced in co-culture

Marcelo Fernando Valle-Vargas[1], Yesica Vanesa Rojas-Muñoz[2], Ruth Yolanda Ruiz-Pardo[1], Luisa Villamil-Díaz[1] and María Ximena Quintanilla-Carvajal[1*]

Abstract
Process and culture medium composition in bioreactor could be optimized in order to find the best conditions that improve survival of probiotic microorganism under exposure to gastric conditions such low pH and bile salts. Therefore, this study aimed to optimize agitation, yeast extract, and di-sodium phosphate (Na_2HPO_4) concentration to improve the survival under gastric conditions of a multistrain consortium produced in a laboratory bioreactor. Viability, survival low pH (3.00), bile salt tolerance, and antagonistic activity against the pathogen *Streptococcus agalactiae* were evaluated. As the main result, a high concentration of di-sodium phosphate (2.63% w/v) increased the viability of *L. lactis* A12 (9.05 to 9.46 Log_{10} CFU/mL) and *Priestia* species (0.00 to 6.88 Log_{10} CFU/mL), survival to pH 3.00 (60 to 93%), survival of bile salts (58– 93%) antagonistic activity (8.74 to 15.56 mm), and final pH of culture medium (4.34 to 6.95). Optimal conditions that improved probiotics characteristics were 150 RPM, 0.83% w/v yeast extract, and 2.63% w/v Na_2HPO_4. Co-culture of *L. lactis* A12 with *Priestia* species improved significantly ($p < 0.05$) the antagonistic activity (10.41 mm) against *S. agalactiae* compared to mono-culture (7.70 mm). Our results suggested that was possible to produce a potential multistrain preparation in a lab bioreactor with high viability of *L. lactis* A12 (9.33 Log_{10} CFU/mL), high survival to gastric conditions (> 85%), and with antagonistic activity against fish pathogen. This preparation could be used as a feed additive intended for fish nutrition.

Keywords *L. lactis*, *Priestia*, Bioreactor, Antagonistic activity, Probiotic, Co-culture

Introduction

Probiotics are viable microorganisms that administered in adequate amounts confer health benefits to the host (World Health Organization: Food and Agriculture Organization of the United Nations 2006). In 2021, the world probiotic market for animal feed was valued at US 4.9 billion, and it is estimated to reach US 7.78 billion by 2030 (https://www.verifiedmarketresearch.com/product/probiotics-in-animal-feed-market/). Probiotics represent a wide group of bacteria, mostly lactic acid bacteria (LAB), other Gram-positive bacteria like *Bacillus* spp., and yeast like *Saccharomyces cerevisiae* (Merrifield and Carnevali 2014; Sahandi et al. 2019). In aquaculture, specifically

*Correspondence:
María Ximena Quintanilla-Carvajal
maria.quintanilla1@unisabana.edu.co
[1]Grupo de Investigación en Procesos Agroindustriales (GIPA), Doctorado en Biociencias, Facultad de Ingeniería, Universidad de La Sabana, Campus del Puente del Común, Km. 7, Autopista Norte de Bogotá. Chía, Cundinamarca, Colombia
[2]Grupo de Investigación en Procesos Agroindustriales (GIPA), Facultad de Ingeniería, Universidad de La Sabana, Campus del Puente del Común, Km. 7, Autopista Norte de Bogotá. Chía, Cundinamarca, Colombia

Springer Open

© The Author(s) 2025. **Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

fish species, probiotics administration improves growth performance, nutrient digestibility, immunomodulation, microbiota modulation, resistance against pathogens, etc. (Ringø et al. 2018). For utilization of probiotics as feed additives, they must demonstrate certain properties such as acid and bile tolerance, non-hemolytic, antibacterial activity, susceptibility to drugs, adhesion, film formation, among others (Chauhan and Singh 2019).

In vitro assessment of microorganisms with probiotic potential have showed positive results at test tube or Erlenmeyer level (Reda et al. 2018; Kaktcham et al. 2019; Kuebutornye et al. 2020), however, it is necessary to produce potential probiotic in laboratory bioreactor to increase biomass production (Makowski et al. 2017; Norizan et al. 2020; Galante et al. 2023) and maintain probiotic characteristics (Aragón-Rojas et al. 2018). Probiotics manufacturing involves several steps such as (a) selection of microorganism strains, (b) fermentation, and (c) drying (Fenster et al. 2019). The present research is focused in the second one (b). Fermentation at the same time involves the selection of growth media, growth conditions, and the type of fermentation (solid or liquid state fermentation) (FAO 2016). Production of probiotics can be carried out from Erlenmeyer flask to bioreactors (laboratory, pilot, and industrial scale) (Makowski et al. 2017) to obtain biomass and/or compounds such as bacteriocins, enzymes, low molecular compounds, etc. (Mora-Villalobos et al. 2020). In bioreactors, parameters such as pH, temperature, gas flow, gas concentration (O_2. N_2, and CO_2), aeration, and nutrient addition can be controlled. These parameters as well as medium components and their concentration can affect probiotic metabolism (da Rosa et al. 2019). Several authors have reported the use of bioreactors for the production of probiotic biomass and metabolites (Jangra et al. 2016; Makowski et al. 2017; Malvido et al. 2019; Norizan et al. 2020; Akdoğan and Çelik 2021; Verma et al. 2023), however, probiotic characteristics like resistance to acidic environments, tolerance to bile salts, and antimicrobial activity have not been widely evaluated through an experiment design. There is a study by Aragón-Rojas et al. (2018) who evaluated viability, and tolerance to acidic and bile salt environment of *Lactobacillus fermentum* K73 produced in a culture medium composed by sweet whey and yeast extract. These authors reported that medium component concentration and process parameters (pH and agitation) affected probiotic characteristics.

Our research group in previous studies has reported the isolation of three bacteria (*Lactococcus lactis* A12, *Priestia megaterium* M4, and *Priestia* sp. M10) (Melo-Bolívar et al. 2019). These probiotic consortiums presented non-hemolytic activity, resistance to acidic environments, tolerance to bile salts, antibacterial activity, etc. (Melo-Bolívar et al. 2022). Then, this consortium improves growth performance and immunological parameters, as well as resistance against the pathogen *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*) fingerlings in an *in vivo* trial (Melo-Bolívar et al. 2023b).

Our first study, aimed to design an agro-industrial by-product-based culture media using whey, sugarcane molasses, and palm kernel cake as components to produce *L. lactis* A12, *P. megaterium* M4, and *Priestia* sp. M10 in monoculture conditions (Valle Vargas et al. 2024b). Then, a second study aimed to produce a potential multistrain (*L. lactis* A12, *P. megaterium* M4, and *Priestia* sp. M10) preparation in co-culture conditions using agro-industrial by-products-based culture medium in a 250 mL flask Erlenmeyer (Valle-Vargas et al. 2023). This culture medium was composed of whey, sugarcane molasses, and palm kernel cake. Also, yeast extract and di-sodium phosphate were used as nitrogen source and buffering agent, respectively, which represent 99.41% of the total cost of the culture medium. In both research, *L. lactis* A12 presented similar (Valle Vargas et al. 2024b) or higher (Valle-Vargas et al. 2023) bacterial growth under optimal conditions compared to BHI broth.

L. lactis and *Bacillus* species has been used in aquaculture, especially in finfish species such as tilapia, carp, and rohu, among others (Melo-Bolívar et al. 2021). *L. lactis* is a bacteria with demanding nutrient requirements for optimal growth. Since most *L. lactis* strains lack of capacity for amino acid synthesis, it is necessary nitrogen sources in the culture medium (Cano-Lozano et al. 2022). In the same way, phosphorous compounds are important for *L. lactis* growth (Costas Malvido et al. 2018), as well as, buffering agents for maintaining pH within the optimal range (5.8– 6.5), which improve nutrient consumption (Malvido et al. 2019). *Priestia* species, some strains are former *Bacillus* including *Bacillus megaterium* according to the new genera proposed by Gupta et al. (2020). These species grow well on routine media such as nutrient broth and trypticase soy agar, among others (Vos et al. 2009). Previous studies have used by-products such as glycerol and mushrooms wastewater (rich in polysaccharides) as carbon sources for production of *Priestia megaterium* in pH ranges between 5.5 -8.0 (Huang et al. 2018; Gómez Cardozo et al. 2020).

The production of biomass faces several challenges such as the design of low-cost culture medium that meet microorganism nutrient requirements (Acosta-Piantini et al. 2023; Valle-Vargas et al. 2023), find optimal process conditions (Balkhis-Ibrahim et al. 2010; Manzoor et al. 2017; Papizadeh et al. 2020), and the preservation of probiotic characteristics (Aragón-Rojas et al. 2018). Among process parameters, agitation play a key role in mass and heat transfer phenomena because it accelerates the transfer of nutrients from the medium to bacterial cells

(Mustafa et al. 2019), however, this could cause hydrodynamic stress that affects the kinetics and biological activity of bacteria cells (Cano-Lozano et al. 2022).

Since agitation and medium components could affect probiotic metabolism (Aragón-Rojas et al. 2018; Valle-Vargas et al. 2023; Valle Vargas et al. 2024b), it is necessary to evaluate these parameters using response surface methodology (RSM) design and its effect on probiotic characteristics such as resistance to an acidic environment, tolerance to bile salts, and antibacterial activity against *S. agalactiae*, which not been widely reported, and more especially in fish nutrition. Therefore, the aim of this study was to optimize agitation, yeast extract, and di-sodium phosphate concentration to improve the survival under gastric conditions of a probiotic a multistrain preparation produced in a laboratory bioreactor.

Materials and methods

Ethical statement

The project followed the Colombian national government's regulations. The Permit for accessing genetic resources was issued by the Colombian Ministry of Environment Number 117 (Otrosí 4) on the 8th of May 2018 for five years.

Microorganisms

L. lactis A12, *P. megaterium* M4, and *Priestia* sp. M10 were isolated from a competitive exclusion bacterial culture derived from gut microbiomes of Nile tilapia (*Oreochromis niloticus*) specimens reared on aquaculture farms in Colombia in a previous study of Melo-Bolívar et al. (2019). Then, the three isolated strains were assessed for their probiotic potential and sequenced its whole genome to identified them (Melo-Bolívar et al. 2022). Bacteria were deposited under code A12 (*L. lactis* A12), M4-MR4 (*Priestia megaterium* M4), and M10-MR10 (*Priestia* sp. M10) in the Chilean Collection of Microbial Genetic Resources (CChRGM) at the Instituto de Investigaciones Agropecuarias (INIA, Chillan, Chile). This institute is registered in the World Data Centre for Microorganisms (WDCM) with registration number 1067. Microorganisms and activation protocol are reported in previous study of Valle-Vargas et al. (2023).

The bacteria were selected based on the previous study of Melo-Bolívar et al. (2023a) who evaluated the effect the of monostrain and multistrain combinations of the three bacteria (*L. lactis* A12, *P. megaterium* M4, and *Priestia* sp.M10) on the specific growth rate (SGR) and the antibacterial activity against fish pathogen *S. agalactiae* and *Aeromonas hydrophila*. They found that a combination of 61% *L. lactis* A12, 23% *Priestia* sp. M10, and 16% v/v *P. megaterium* M4 exhibited higher SGR and antibacterial activity than probiotic bacteria alone.

General description of probiotic bacteria

L. lactis is spherical or ovoid-shaped cells and occur singly or in chains. It is Gram-positive, facultatively anaerobic, non-motile, and non-spore-forming (Mills et al. 2011). *Priestia* species (some strains are former *Bacillus*) are normally a rod-like bacteria, straight or slightly curved, occurring singly and in pairs, some in chains, and occasionally as long filaments. It is Gram-positive, mainly aerobic, and spore forming bacterium (Vos et al. 2009).

Preparation of culture medium and fermentation conditions

Culture medium and fermentations conditions used in these study is described by Valle-Vargas et al. (2023). Bioreactor conditions were set agitation speed (according to experiment design), temperature (28 °C), and incubation time (17 h). The initial cell concentrations of *L. lactis* A12 and *Priestia* species were 8.01 ± 0.00 and 6.21 ± 0.04 Log_{10} CFU/mL, respectively. Finally, after the process finished, samples of the final culture medium with grown probiotic bacteria were taken to evaluate final cell concentration (Log_{10} CFU/mL), survival to acid pH (Log_{10} CFU/mL), survival to bile salt (Log_{10} CFU/mL), antagonistic activity against *S. agalactiae* (inhibition zone, mm), and pH as response variables according to the methodology described by Valle-Vargas et al. (2023).

For the assessment of the viability of probiotic bacteria, the drop plate method was used (Valle Vargas et al. 2024a). 100 µL of the sample were transferred to 900 µL of Phosphate Buffer Solution (PBS). Then, tenfold seria dilutions were made. 20 µL of each dilution was dropped onto the surface of TSA and allowed to dry. Finally, the agar plates were incubated at 28 °C for 24 h. After 24 h, colonies were counted in each plate, and the final cell concentration was expressed as Log_{10} CFU/mL. The differentiation of *L. lactis* A12 and *Priestia* species was based on colonies morphology. *L. lactis* A12 colonies were white, punctiform, flat, and smooth with regular edge. *Priestia* species colonies were beige, round, convex, and smooth with regular edge. *P. megaterium* M4 and *Priestia* sp. M10 presented similar morfology for that reason were counted together and expressed as *Priestia* species.

Experimental design in a 1.7 L BioFlo III bioreactor

In a previous study, an optimal culture medium composition to produce potential probiotic bacteria in co-culture conditions was achieved (Valle-Vargas et al. 2023). These conditions were whey (1.00% w/v), sugarcane molasses (0.50% w/v), and yeast extract (1.50% w/v). PKC was added at 0.77% w/v. However, it was necessary to evaluate the probiotic characteristics of these bacteria in co-culture conditions in a lab bioreactor. For this purpose, a Box-Behnken design (BBD) was used to optimize yeast

extract (% w/v), di-sodium phosphate (% w/v), and agitation speed (RPM) that maximizes response variables.

The BBD was built using the statistical software Design Expert (Stat-Ease Inc., Minneapolis, MN, U.S.A). The design consisted of 17 runs, with 5 replicates at central points is shown in Table 1. The selected factors were agitation speed (100– 200 RPM), yeast extract (0.50–1.50% w/v), and di-sodium phosphate, Na_2HPO_4 (0.25–2.63% w/v). Yeast extract and di-sodium phosphate were selected as factors because have been reported that these components affect bacterial metabolism (Aragón-Rojas et al. 2018; Costas Malvido et al. 2018) and in our recent study (Valle-Vargas et al. 2023) were the most expensive components of culture medium. Agitation was chosen due to is related to heat and mass transfer phenomena involved in the fermentation process (Mustafa et al. 2019).

Optimization and validation

The best combination of component concentration and agitation speed in the culture medium that maximized the viability of probiotic bacteria in co-culture conditions and improved probiotic characteristics were achieved using the desirability function (Aragón-Rojas et al. 2018; Valle-Vargas et al. 2023). Validation of response variables was performed at optimal conditions. The error percentage of predicted and experimental data at the optimal conditions was calculated. Validation runs were performed in triplicates. Homogeneity of variance, normality, and independence of data were checked.

Antagonistic activity of *L. lactis* A12 in mono- and co-culture conditions

Under optimal conditions, *L. lactis* A12 was grown in monoculture and co-culture conditions. Antagonistic activity was carried out according to methodology described above.

Antagonistic activity of mono- and co-culture fermentations were compared using a two-tailed *t -test* with an alpha level of 0.05. Also, the homogeneity of variance for *t-test* was confirmed with a *F- test* (alpha level of 0.05).

Results

Model fitting of Box Behnken design

Response variables data of the Box Behnken design for probiotics characteristics are shown in Table 1. Response variables data were fitted to a quadratic model and ANOVA were carried out with a significance level of 0.05 (Table 2). Model selections were made considering the lowest *p*-value for the mixture and process factors and the corrected Akaike information criterion (AIC_c) (data not shown). Also, model reduction for the Viability of *L. lactis* A12 and survival to bile salts were made to improve statistical parameters. ANOVA assumptions such as homoscedasticity, normality, and data independence were verified using residuals vs. predicted, normal probability (%) vs. residuals, and residuals vs. run number plots, respectively.

All models explained more than 90% of the total variability of the response according to their R^2 and R^2 adjusted values. Lack-of-Fit presented a non-significant *p-value*, indicating that these models fit appropriately the experimental data. Adequate precision is another

Table 1 BBD with experimental results

Run	A: Agitation (RPM)	B:Yeast extract (%w/v)	C: Di-sodium phosphate (%w/v)	*L. lactis* A12 (Log_{10} CFU/mL)	*Priestia* species (Log_{10} CFU/mL)	Survival of *L. lactis* A12 to pH 3.00 (Log_{10} CFU/mL)	Survival of *L. lactis* A12 to bile salts (Log_{10} CFU/mL)	Antagonistic activity (mm)	Final pH
1	150	1.00	1.44	9.36 ± 0.06	6.74 ± 0.02	5.72 ± 0.10	7.62 ± 0.05	13.48 ± 2.66	6.39 ± 0.01
2	150	1.00	1.44	9.35 ± 0.01	6.60 ± 0.02	5.57 ± 0.23	7.50 ± 0.03	12.54 ± 3.59	6.39 ± 0.01
3	200	1.00	2.63	9.34 ± 0.06	6.81 ± 0.03	6.60 ± 0.15	7.65 ± 0.04	12.28 ± 2.56	6.80 ± 0.00
4	200	1.50	1.44	9.24 ± 0.09	6.87 ± 0.06	7.19 ± 0.04	7.57 ± 0.02	8.64 ± 1.12	6.42 ± 0.02
5	150	0.50	2.63	9.22 ± 0.00	6.31 ± 0.06	6.63 ± 0.09	7.62 ± 0.03	15.56 ± 1.37	6.76 ± 0.01
6	100	1.00	0.25	9.08 ± 0.02	0.00 ± 0.00	5.30 ± 0.17	4.76 ± 0.07	11.76 ± 1.94	4.45 ± 0.02
7	150	1.50	0.25	9.11 ± 0.05	0.00 ± 0.00	7.16 ± 0.07	5.99 ± 0.05	12.85 ± 2.80	4.55 ± 0.01
8	150	1.00	1.44	9.32 ± 0.02	6.80 ± 0.02	5.52 ± 0.17	7.41 ± 0.03	14.06 ± 0.00	6.33 ± 0.02
9	100	0.50	1.44	9.36 ± 0.01	6.80 ± 0.01	5.08 ± 0.07	7.50 ± 0.03	9.93 ± 1.95	6.37 ± 0.01
10	150	1.00	1.44	9.38 ± 0.01	6.76 ± 0.05	5.51 ± 0.23	7.39 ± 0.09	13.01 ± 0.67	6.22 ± 0.00
11	200	0.50	1.44	9.32 ± 0.01	6.81 ± 0.01	5.65 ± 0.13	7.71 ± 0.04	11.54 ± 2.12	6.07 ± 0.01
12	150	1.00	1.44	9.42 ± 0.09	6.88 ± 0.02	5.45 ± 0.07	7.60 ± 0.07	13.77 ± 0.41	6.22 ± 0.01
13	100	1.50	1.44	9.34 ± 0.03	6.82 ± 0.02	5.43 ± 0.09	6.78 ± 0.07	10.07 ± 0.72	6.41 ± 0.01
14	150	1.50	2.63	9.39 ± 0.06	6.80 ± 0.11	7.88 ± 0.03	6.98 ± 0.08	9.20 ± 2.36	6.95 ± 0.01
15	150	0.50	0.25	9.06 ± 0.04	0.00 ± 0.00	5.84 ± 0.04	5.24 ± 0.07	11.45 ± 0.00	4.34 ± 0.00
16	100	1.00	2.63	9.46 ± 0.01	6.73 ± 0.02	6.33 ± 0.05	7.91 ± 0.04	10.00 ± 0.00	6.61 ± 0.03
17	200	1.00	0.25	9.05 ± 0.02	0.00 ± 0.00	7.14 ± 0.06	6.43 ± 0.05	11.64 ± 2.39	4.49 ± 0.01

Table 2 ANOVA and statistical parameters for probiotic characteristics

Model term	*p– value*					
	***L. lactis* A12**	***Priestia* species**	**Survival to pH 3.00**	**Survival to bile salts**	**Antagonistic activity**	**Final pH**
Model	0.0019	< 0.0001	< 0.0001	< 0.0001	0.0010	< 0.0001
A: Agitation	0.1048	0.6688	< 0.0001	0.0028	0.2376	0.8470
B: Yeast extract	0.4708	0.1120	< 0.0001	0.2230	0.0037	0.0336
C: Na_2HPO_4	0.0001	< 0.0001	0.0063	< 0.0001	0.7265	< 0.0001
AB	0.6071	0.8620	0.0142	0.1865	0.0494	0.1868
AC	0.4453	0.7290	0.0037	0.0013	0.1032	0.5018
BC	0.3157	0.0629	0.8541	0.0085	0.0005	0.9274
A^2	-	0.1465	0.2024	-	0.0003	0.6107
B^2	0.1407	0.7322	0.0026	0.1220	0.0054	0.5195
C^2	0.0160	< 0.0001	< 0.0001	< 0.0001	0.6807	< 0.0001
Lack-Of-Fit	0.1235	0.3640	0.0552	0.0520	0.3951	0.2273
Fitting parameters						
R^2	0.9094	0.9994	0.9787	0.9761	0.9486	0.9940
R^2 adjusted	0.8188	0.9986	0.9514	0.9523	0.8825	0.9862
Adeq Precision	9.7058	82.6347	18.7972	20.4803	13.1761	31.0682

Table 3 Percentage of contribution (%) of model term

Model term	***L. lactis* A12**	***Priestia* species**	**Survival to pH 3.00**	**Survival to bile salts**	**Antagonistic activity**	**Final pH**
A	–	–	22.22	5.37	–	–
B	–	–	22.49	–	13.33	0.60
C	55.50	63.61	4.52	55.44	–	82.81
AB	–	–	3.20	–	4.13	–
AC	–	–	5.57	6.89	–	–
BC	–	–	–	3.58	26.92	–
A^2	–	–	–	–	32.77	–
B^2	–	–	6.37	–	11.59	–
C^2	24.58	36.15	31.80	23.76	–	15.58

statistical parameter, which measures the signal-to-noise ratio. Values higher than 4.0 are desirable. Adeq precision values for all response variables were higher than 4.0. This indicates that models can be used to navigate the experiment design space.

For each model, percentage contribution (PC) was calculated for all terms by dividing the sum of squares (SS) of each term by the total sum of squares (data not shown) (Barón et al. 2021). This parameter was calculated to determine the contribution of each significant term ($p < 0.05$) to the response variable (Table 3). Equations (1)– (6) show the fitted models for each response variable expressed in terms of actual components and factor levels (Valle-Vargas et al. 2023)

$$L.\,lactis A12\,(Log_{10}CFU/mL) = 8.8104 + 0.0004 \times [A] + 0.4042 \times [B] + 0.3814 \times [C] - 0.0006\,[AB] - 0.00 \times [AC] + 0.0504 \times [BC] - 0.174 \times \left[B^2\right] - 0.0898 \times \left[C^2\right] \quad (1)$$

$$Priestia species\ (Log_{10}CFU/mL) = -1.3892 - 0.0111 \times [A] - 0.0600 \times [B] + 9.5783 \times [C] + 0.0004 \times [AB] + 0.0003 \times [AC] + 0.2059 \times [BC] + 0.0000 \times \left[A^2\right] - 0.0770 \times \left[B^2\right] - 2.4428 \times \left[C^2\right]$$

(2)

$$Survival\ to\ pH\ 3.00\,(Log_{10}CFU/mL) = 4.6332 + 0.0238 \times [A] - 3.9017 \times [B] - 0.6304 \times [C] + 0.0119 \times [AB] - 0.00 \times [AC] - 0.0294 \times [BC] - 0.0001 \times \left[A^2\right] + 1.6370 \times \left[B^2\right] + 0.6456 \times \left[C^2\right]$$

(3)

$$Survival\ to\ bile\ salts\,(Log_{10}CFU/mL) = 1.9670 + 0.0119 \times [A] + 1.1351 \times [B] + 4.3858 \times [C] + 0.0058 \times [AB] - 0.0081 \times [AC] - 0.5840 \times [BC] - 0.6758 \times \left[B^2\right] - 0.6154 \times \left[C^2\right]$$

(4)

$$Antagonistic\ activity\,(mm) = -16.0944 + 0.2720 \times [A] + 17.2531 \times [B] + 1.4061 \times [C] - 0.0304 \times [AB] + 0.0101 \times [AC] - 3.2605 \times [BC] - 0.0008 \times \left[A^2\right] - 4.9640 \times \left[C^2\right] + 0.0946 \times \left[C^2\right]$$

(5)

$$Final\ pH = 4.1936 - 0.0009 \times [A] - 0.5354 \times [B] + 2.3032 \times [C] + 0.0031 \times [AB] + 0.0006 \times [AC] - 0.0084 \times [BC] - 0.0000 \times \left[A^2\right] + 0.1400 \times \left[B^2\right] - 0.4908 \times \left[C^2\right]$$

(6)

A: agitation (RPM), B: yeast extract (% w/v), and Na_2HPO_4 (% w/v).

Effect of independent variables on probiotic characteristics

Figure 1 shows the contour plots for probiotics characteristics and desirability. Viability of *L. lactis* A12 ranged from 9.05 to 9.46 Log_{10} CFU/mL. As shown in Fig. 1a, high cell counts of *L. lactis* A12 was achieved at high concentrations of Na_2HPO4 (> 1.44% w/v). In the same way, *Priestia* species viability (0.00– 6.88 Log_{10} CFU/mL) was higher when Na_2HPO_4 concentration was higher than 1.44% w/v, while the concentration of 0.25% w/v did not promote *Priestia* growth (Fig. 1b).

Figure 1c and 1d show the contour plots for survival to the acid environment (pH 3.00) and bile salts (pH 7.00). Survival of *L. lactis* A12 to pH 3.00 ranged between 5.08 and 7.88 Log_{10} CFU/mL representing bacterial reductions between 6.08– 39.23%. From Fig. 1c, it can be observed that higher agitation speed and Na_2HPO_4 concentration improves the survival of *L. lactis* under acidic conditions. Also, it can be observed that a low Na_2HPO_4 concentration (0.25% w/v), *L. lactis* A12 shows a high viability after exposure to low pH (3.00) with survival percentage close to 88%. Figure 1d shows that increasing yeast extract concentration (1.50% w/v) and agitation (200 RPM) results in a higher survival of *L. lactis* A12 (93.92%). In our study, *Priestia* species showed low survival (< 3.00 Log_{10} CFU/mL) after exposure to acidic environment and bile salts.

Figure 1e and 1f represent the contour plot of the survival of *L. lactis* A12 to bile salts (0.30% w/v) at pH 7.00. In both Figures, it can be observed that high concentration of Na_2HPO_4 and higher speed of agitation increases the survival of *L. lactis* A12 from 4.76 to 7.91 Log_{10} CFU/mL.

Antagonistic activity (AA) against *S. agalactiae* expressed as an inhibition zone contour plot is shown in Fig. 1g and 1h. AA values ranged from 8.64 to 15.56 mm. Figure 1g shows that antagonistic activity increased from 11 to 13 mm when agitation increased from 100 to 150 RPM, and then AA decreased when agitation was set to 200 RPM. In Fig. 1h, AA was improved when Na_2HPO_4 was used at a higher concentration (2.63%w/v). In both interactions, yeast extract concentration between 0.50 and 1.10% w/v improved AA.

The final pH of the culture medium varied between 4.34 (0.25%w/v) and 6.95 (2.63% w/v). In Fig. 1i, it can be observed that a higher concentration of Na_2HPO_4 resulted in a higher final pH (> 6.00).

Optimization and validation of optimal conditions

The desirability function was used to optimize the bioreactor conditions to maximize the probiotic characteristics. Desirability value increased with high concentration of Na_2HPO_4, high agitation, and low concentration of yeast extract. The optimal conditions for achieving a desirability value of 0.804 were: 150 RPM, 0.83% w/v yeast extract, and 2.63% w/v Na_2HPO_4. Desirability values higher than 0.7 indicate a good optimization of experimental data. Table 4 shows the predicted and experimental values of probiotic characteristics under optimal conditions. Experimental errors for all probiotic characteristics are shown in Table 3. Experimental error values lower than 10% indicates that the desirability function was a useful statistical tool for the optimization of bioreactor conditions.

Finally, antagonistic activity against *S. agalactiae* was significantly higher ($p < 0.05$) when *L. lactis* A12 was grown in presence of *Priestia* species (10.41 ± 1.47 mm) compared to *L. lactis* A12 alone (7.77 ± 1.19 mm).

Discussion

L. lactis A12 and *Priestia* species showed an increase in their viable cell concentration with the presence of high concentration of di-sodium phosphate (≥ 1.44%w/v) regardless of agitation levels. This could be related to different reasons. An increase in the concentration of Na_2HPO_4 induces a higher pH value (6.07—6.95), which is within the optimal pH value (5.8– 6.5) for nutrient consumption by *L. lactis* (Malvido et al. 2019). During fermentation, *L. lactis* species reduce pH (lower than 5.0) by producing lactic acid which reduce bacteria metabolism without affecting bacteria growth until pH is around 4.0 (Andersen et al. 2009). It has been reported that *Bacillus* species had grown in pH range between 5.5—8.0 (Huang et al. 2018; Gojgic-Cvijovic et al. 2019; Gómez Cardozo et al. 2020).

Our finding is in accordance with the results reported by Malvido et al. (2019), who supplemented a whey-based medium with MRS components (except glucose and tween 80) at 25, 50, 75, 100, and 125% (0.50 to 2.50 g/L of Na_2HPO_4) of their standard concentrations in the commercial medium. They found that an increase from 25 to 125% resulted in a higher biomass concentration of *L. lactis* after 24 h of fermentation.

Another reason could be associated that phosphate plays a key role in bacterial metabolism. A higher content of di-sodium phosphate implies a higher content of phosphate in the culture medium. Studies have revealed that extracellular phosphate has an important role in the regulation of metabolism and use of glucose by lactic acid bacteria (Levering et al. 2012). The presence of phosphate may stimulate sugar uptake and induce a higher concentration of fructose 1,6– biphosphate that allows bacterial cells to metabolize glucose most efficiently, resulting in the improvement of the growth of *L. lactis*. On the other hand, low concentration of phosphate could lead to inhibition of glycolysis (Costas Malvido et al. 2018). Similarly,

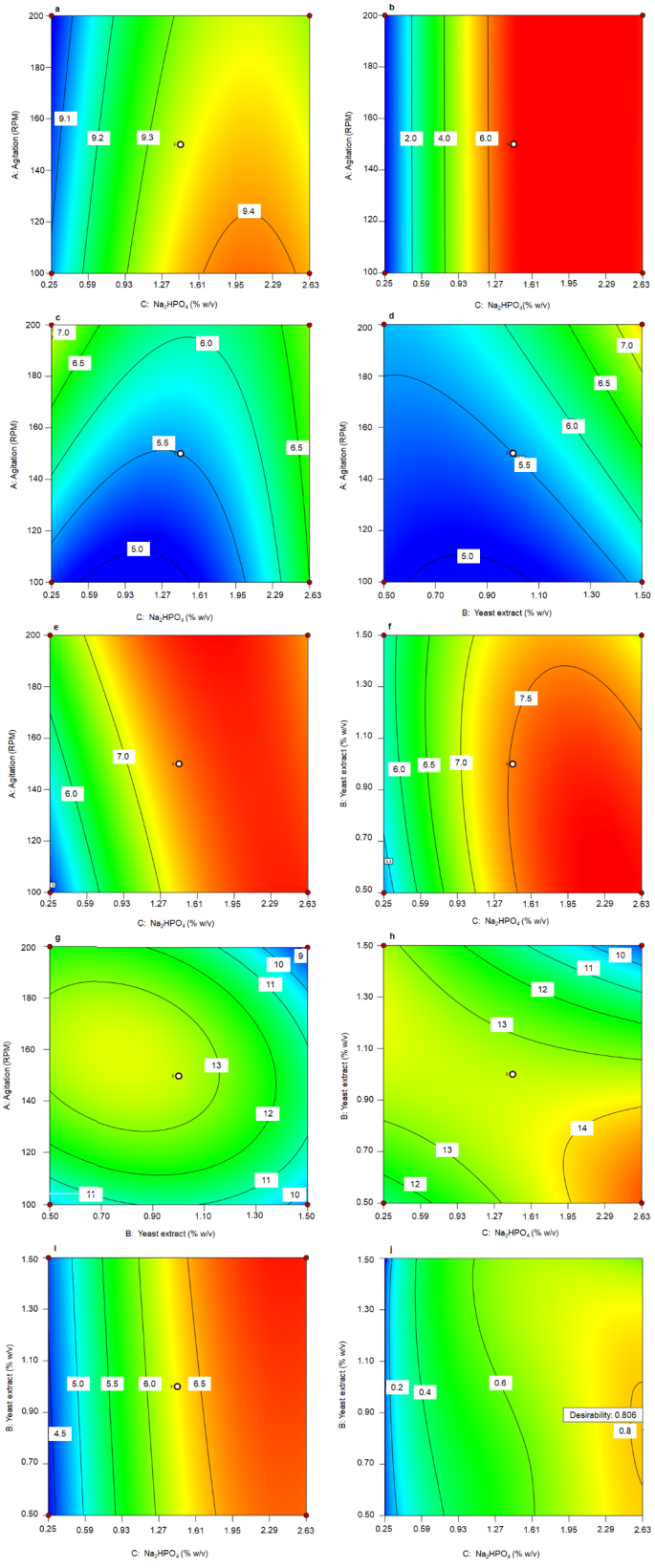

Fig. 1 Contour plots of viability of (**a**) *L. lactis* A12, (**b**) *Priestia* species, (**c** and **d**) survival to pH 3.00, (**e** and **f**) survival to bile salts, (**g** and **h**) antagonistic activity, (**i**) final pH, and (**j**) desirability

Table 4 Validation of optimal conditions

Probiotic characteristics	Predicted value	Observed value	Experimental error (%)
Viability of *L. lactis* A12 Log_{10} CFU/mL)	9.36	9.33 ± 0.08	0.32
Viability of *Priestia* species (Log_{10} CFU/mL)	6.56	6.61 ± 0.05	0.76
Survival to pH 3.00 (Log_{10} CFU/mL)	6.58	7.11 ± 0.06	8.05
Survival to bile salts (Log_{10} CFU/mL)	7.75	7.51 ± 0.06	3.09
Antagonistic activity (mm)	14.25	14.01 ± 0.69	1.68
Final pH	6.75	6.77 ± 0.01	0.29

Costas Malvido et al. (2018) supplemented a whey-based medium with KH_2PO_4 to evaluate probiotic biomass and nisin production by *L. lactis* CECT 539. They found that probiotic biomass increased (from 0.550 to 0.707 g/L) when total phosphorus content increased from 0.240 to 0.480 g/L.

Accordingly, Balkhis Ibrahim et al. (2010) found similar results to those reported in our study. They evaluated the influence of stirring speed, temperature, and carbon and nitrogen sources on the growth of *L. lactis* was investigated. The agitation speed was assessed within the range of 50 to 250 RPM. In line with our results, their findings demonstrated that the highest cell concentration (3.22 g/L) was achieved at 27 °C with a stirring speed of 100 RPM. However, cell biomass concentartion did not change dramatically over the agitation interval evaluated, similar to our results were *L. lactis* A12 viability was not affected by agitation speed. A previous study has shown that when bacteria is not under growth-limiting conditions, *L. lactis* maintains consistent growth characteristics, regardless of the agitation speed (Singh et al. 2015).

Survival after exposure to gastric conditions of *Priestia* species could not being improved by varying process parameters in the proposed experimental design. *Bacillus* species are known for their ability to withstand stressful conditions such low pH and bile salts (Bahaddad et al. 2023), however, survival under these conditions may vary among species. The survival of *L. lactis* A12 under acid (pH 3.00) and bile salts (0.30%w/v) conditions in our study could be associated to the different mechanisms that probiotic bacteria (especially lactic acid bacteria) have to resist low pH environments such as adaptive response, pH homeostasis, restriction of proton permeation, consumption of proton, etc. (Guan and Liu 2020). For probiotic bacteria grown with 1.44 and 2.63% w/v Na_2HPO_4, the final pH was higher than 6.3 which may allow the bacteria to maintain pH homeostasis by regulating the inside and outside pH in acidic conditions (Padan et al. 2005). It has been reported that the intracellular pH of *L. lactis* decrease more than one unit from 7.2 (at an extracellular pH_E of 6.5) to 6.0 at pH_E 4.8 during glucose consumption (Andersen et al. 2009). Probiotics must be bile-tolerant to colonize the intestine. Bile salts are known as biological detergents that emulsify and solubilize lipids including those present in the membrane cell causing cell death (Koskenniemi et al. 2011). *L. lactis* A12 survival to bile salts could be mediated by several mechanisms that have been reported in the literature. One is the homeostasis in an alkaline environment since a higher extracellular pH resulted (4.50– 6.80) in a higher intracellular pH (6.0– 7.2) and this must be close to the alkaline conditions of the bile salt assay (pH_E 7.00) in the present study (Padan et al. 2005). The second mechanism could be related to the ability of probiotic bacteria to produce bile salt hydrolase that modifies and inactivates bile salts. Other strategies of probiotic bacteria to withstand bile salts is by the production of protective protein or changes in cell wall structure (Kudo and Sasaki 2019).

Similarly, Aragón-Rojas et al. (2018) evaluated the effect of process parameters (pH and agitation) and composition (whey and yeast extract) of a culture medium containing *L. fermentum* K73 produced in a lab-stirred bioreactor to improve stress resistance. They found that a high concentration of yeast extract, neutral pH (7.00), and high agitation (300 RPM) improved bacteria viability under acidic (pH 3.00) and bile salts (0.3% w/v and pH 7.00) conditions.

In our research, the final medium containing probiotic bacteria showed mainly antagonistic activity, since cell-free supernatant did not show antibacterial activity against *Streptococcus agalactiae* in preliminary assays (data not shown). It has been reported that bacteriocin and bacteriocin-like peptide production can be triggered when probiotic bacteria are in presence of target microorganisms (Kiousi et al. 2023). Yi et al. (2018) reported that cell suspension of *Bacillus velezensis* JW presented antimicrobial activity against five pathogens including *A. hydrophila* and *S. agalactiae* using the well diffusion method. On the other hand, Fredua-Agyeman et al. (2023) reported that *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Bifidobacterium bifidum* individually inhibited the growth of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* in co-culture conditions.

Agitation and yeast extract supplementation played a key role in improving antagonistic activity, finding maximum values at 150 RPM and 0.70– 0.90%w/v. Agitation speed higher than 150 RPM caused the decrease of the antagonistic activity. A possible explanation is that increasing agitation speed could result in hydrodynamic stress (Cano-Lozano et al. 2022) caused by the shear rate that may affect gene expression reducing bacteriocin activity by lactic acid bacteria (Abbasiliasi et al. 2017). Similarly, Abbasiliasi et al. (2016) evaluated the effect of three parameters (inoculum size, temperature, and agitation speed) on the antimicrobial activity of cell-free

supernatants from *Pediococus acidilactici* Kp10 cultures. They found that antimicrobial activity increased from 0 to 100 RPM, and then decreased from 100 to 200 RPM.

Also, the interaction to yeast extract and Na_2HPO_4 influenced antagonistic activity. Yeast extract is source of amino acids or peptides that could act as inducers or precursors for production of bacteriocins by lactic acid bacteria (Abbasiliasi et al. 2017). In our research, the concentration of Na_2HPO_4 (2.63% w/v) presented the highest antagonistic activity. Stoyanova and Levina (2006) reported that the increase of K_2HPO_4 (from 0.5 to 2.0%) and yeast autolysate (from 35 to 70 mg) improved nisin production from 1150 to 4100 UI/mL by recombinant strain *L. lactis* subsp. *lactis* F-116. In contrast, Costas Malvido et al. (2018) reported that nisin production increased when total phosphorous (TP) content in whey-based culture increased from 0.24 to 0.43 g/L, however, when TP content increased from 0.43 to 0.63 g/L, nisin production decreased. These authors associated this behavior with the increase of TP content inhibited nisin production.

The final pH of the culture medium was affected by the concentration of Na_2HPO_4 which also worked as a buffering agent. Higher concentrations of Na_2HPO_4 (1.44 to 2.63% w/v) resulted in higher pH values (6.07- 6.95). An increase in Na_2HPO_4 improved the buffering capacity of the culture medium that attenuated pH reduction caused by organic acids produced by *L. lactis*. Similarly, Malvido et al. (2019) supplemented whey-based media with nutrients from a commercial medium (0.05– 0.25%w/v Na_2HPO_4) and found that a higher content of Na_2HPO_4 improved buffering capacity resulting in a higher biomass and nisin production, however, final pH was lower than 5.00 at the end of the fermentation process. It is necessary to highlight that the maximum Na_2HPO_4 concentration used in this study was 0.25% w/v which is the lowest level of Na_2HPO_4 used in our research. Additionally, the presence of yeast extract in the culture medium could prevent pH reduction (GAUDREAU et al. 1997).

Finally, under optimal conditions, *L. lactis* A12 grown in co-culture presented higher antagonistic activity ($p < 0.05$) than *L. lactis* A12 in monoculture. This finding is similar to the study of Zhang et al. (2018) who evaluated the effect of pure and mixed fermentation of *Lactobacillus reuteri* and *Bacillus subtilis* on biomass production and antimicrobial activity against pathogen. They found that the presence of *Bacillus subtilis* in the mixed fermentation improved viability of *L. reuteri* ($p < 0.05$) and its antimicrobial activity against *Escherichia coli* ($p < 0.05$). This behavior could be related that in the co-culture fermentations, one strain (ind*uctor strain*) induces the production of antimicrobial compounds by other strain which results in improving the production of bacteriocin with antibacterial activity against pathogens (Gutiérrez-Cortés et al. 2018). Bacteria can grow in naturally occurring communities and artificial or synthetic co-cultures. The latter are assembled for two reasons: improve functionalities of both strain and seek the production of a specific end product. The positive interactions during co-culture conditions are related to commensalism, cooperation, syntropy, and mutualism (Canon et al. 2020). According to our results the type of interaction that could be associated it is commensalism since *L. lactis* A12 improved it antagonistic activity in the presence of *Priestia* species without affecting viability.

It was possible to produce a multistrain probiotic in an agro-industrial by-products-based culture medium under bioreactor conditions. It was found that di-sodium phosphate played a key role in improving viability, survival to low pH environment, presence of bile salts, and antagonistic activity. Optimal conditions of agitation (150 RPM), yeast extract (0.83% w/v), and di-sodium phosphate (2.63% w/v) were achieved. Eventhough Priestia species did not survive to gastric conditions, their presence under optimal conditions improved significantly the antagonistic activity of *L. lactis* A12.

Our results suggested that future work must be carried out including the encapsulation of this multistrain probiotic to improve survival of *Priestia* species under gastric conditions, produce *L. lactis* A12 in a dried presentation to assess their *in vitro* and *in vivo* probiotic potential for future use in fish feed.

Acknowledgements
MFV-V thanks Ministerio de Ciencia, Tecnología e Innovación– Minciencias for funding his doctoral studies through Convocatoria de doctorado nacional para profesores de IES Nº 909 de 2021 and Universidad de La Sabana for granting the award Distinción Carlos Jordana.

Author contributions
MFV-V: conceptualization, formal analysis, investigation, methodology, validation, visualization data, writing– original draft, and writing– review & editing. YVR-M: writing– original draft. RYR-P: conceptualization, resources, and writing– review & editing. LMV-D: conceptualization, resources, and writing– review & editing. MXQ-C: conceptualization, methodology, resources, and writing– review & editing. All authors read and approved the manuscript.

Funding
This research was founded with the Project "Implementation of biotechnological tools and recirculation systems to achieve the sustainability of tilapia farming as a productive strategy for food safety and security in La Guajira", BPIN code 2020000100487; funded through Call No. 6 of the General System of Royalties—R&D fund—for the formation of a list of eligible research and development project proposals for the advancement of knowledge and creation. Also, MXQ-C, RYR-P, and LMV-D provided financial support through the projects ING– 156- 2015, ING-181–2016, and INGPHD-6–2017, respectively, which Universidad de La Sabana funded.

Data availability
The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate.
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest
The authors declare no conflict of interest.

Received: 21 May 2024 / Accepted: 12 December 2024
Published online: 06 February 2025

References

Abbasiliasi S, Tan JS, Kadkhodaei S, Nelofer R, Tengku Ibrahim TA, Mustafa S, Ariff AB (2016) Enhancement of BLIS production by *Pediococcus acidilactici* kp10 in optimized fermentation conditions using an artificial neural network. RSC Adv 6:6342–6349. https://doi.org/10.1039/C5RA22879D

Abbasiliasi S, Tan JS, Tengku Ibrahim TA, Bashokouh F, Ramakrishnan NR, Mustafa S, Ariff AB (2017) Fermentation factors influencing the production of bacteriocins by lactic acid bacteria: a review. RSC Adv 7:29395–29420. https://doi.org/10.1039/C6RA24579J

Acosta-Piantini E, Rodríguez-Díez E, Chavarri M, López-de-Armentia I, Villaran MC, Lombraña JI (2023) Preparation of Hydrolyzed Sugarcane Molasses as a Low-Cost Medium for the Mass Production of Probiotic *Lactobacillus paracasei* ssp. *paracasei* F19. Separations 10:33. https://doi.org/10.3390/separations10010033

Akdoğan M, Çelik E (2021) Enhanced production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) biopolymer by recombinant *Bacillus megaterium* in fed-batch bioreactors. Bioprocess Biosyst Eng 44:403–416. https://doi.org/10.1007/S00449-020-02452-Z/TABLES/5

Andersen AZ, Carvalho AL, Neves AR, Santos H, Kummer U, Olsen LF (2009) The metabolic pH response in *Lactococcus lactis*: An integrative experimental and modelling approach. Comput Biol Chem 33:71–83. https://doi.org/10.1016/j.compbiolchem.2008.08.001

Aragón-Rojas S, Ruiz-Pardo RY, Hernández-Sánchez H, Quintanilla-Carvajal MX (2018) Optimization of the production and stress resistance of the probiotic *Lactobacillus fermentum* K73 in a submerged bioreactor using a whey-based culture medium. CyTA - Journal of Food 16:1064–1070. https://doi.org/10.1080/19476337.2018.1527785

Bahaddad SA, Almalki MHK, Alghamdi OA, Sohrab SS, Yasir M, Azhar EI, Chouayekh H (2023) *Bacillus* Species as Direct-Fed Microbial Antibiotic Alternatives for Monogastric Production. Probiotics Antimicrob Proteins 15:1–16. https://doi.org/10.1007/s12602-022-09909-5

Balkhis Ibrahim S, Abdul Rahman A, Mohamad R, Abdul Rahim R (2010) Effects of agitation speed, temperature, carbon and nitrogen sources on the growth of recombinant Lactococcus lactis NZ9000 carrying domain 1 of aerolysin gene. Afr J Biotechnol 9:5392–5398

Barón RD, Valle-Vargas MF, Quintero-Gamero G, Quintanilla-Carvajal MX, Alean J (2021) Encapsulation of citrulline extract from watermelon (*Citrullus lanatus*) by-product using spray drying. Powder Technol 385:455–465. https://doi.org/10.1016/j.powtec.2021.03.014

Cano-Lozano JA, Villamil Diaz LM, Melo Bolivar JF, Hume ME, Ruiz Pardo RY (2022) Probiotics in tilapia (*Oreochromis niloticus*) culture: Potential probiotic *Lactococcus lactis* culture conditions. J Biosci Bioeng 133:187–194. https://doi.org/10.1016/J.JBIOSC.2021.11.004

Canon F, Nidelet T, Guédon E, Thierry A, Gagnaire V (2020) Understanding the Mechanisms of Positive Microbial Interactions That Benefit Lactic Acid Bacteria Co-cultures. Front Microbiol 11. https://doi.org/10.3389/fmicb.2020.02088

Chauhan A, Singh R (2019) Probiotics in aquaculture: a promising emerging alternative approach. Symbiosis 77:99–113. https://doi.org/10.1007/s13199-018-0580-1

Costas Malvido M, Alonso González E, Outeiriño D, Pérez Guerra N (2018) Production of a highly concentrated probiotic culture of *Lactococcus lactis* CECT 539 containing high amounts of nisin. 3 Biotech 8:292. https://doi.org/10.1007/s13205-018-1318-8

da Rosa LM, Koerich DM, Della Giustina SV (2019) Bioreactors Operating Conditions. In: Essentials in Fermentation Technology. pp 169–212

FAO (2016) Probiotics in animal nutrition– Production, impact and regulation, FAO Animal. FAO, Rome, Italy

Fenster K, Freeburg B, Hollard C, Wong C, Laursen RR, Ouwehand AC (2019) The production and delivery of probiotics: A review of a practical approach. Microorganisms 7. https://doi.org/10.3390/microorganisms7030083

Fredua-Agyeman M, Stapleton P, Gaisford S (2023) Growth assessment of mixed cultures of probiotics and common pathogens. Anaerobe 84:102790. https://doi.org/10.1016/j.anaerobe.2023.102790

Galante N, Palavecino Prpich N, Campos C, Cayré ME, Castro M (2023) Alternative culture medium design for biomass production of autochthonous meat starter *Latilactobacillus sakei* sp. *sakei* ACU-2. Sci Rep 13:5205. https://doi.org/10.1038/s41598-023-29502-2

Gaudreau H, CHAMPAGNE CP, GOULET J, CONWAY J, (1997) Lactic Fermentation of Media Containing High Concentrations of Yeast Extracts. J Food Sci 62:1072–1075. https://doi.org/10.1111/j.1365-2621.1997.tb15040.x

Gojgic-Cvijovic GD, Jakovljevic DM, Loncarevic BD, Todorovic NM, Pergal MV, Ciric J, Loos K, Beskoski VP, Vrvic MM (2019) Production of levan by *Bacillus licheniformis* NS032 in sugar beet molasses-based medium. Int J Biol Macromol 121:142–151. https://doi.org/10.1016/j.ijbiomac.2018.10.019

Gómez Cardozo JR, Velasco Bucheli R, Marín Pareja N, Ruíz Villadiego OS, Correa Londoño GA, Mora Martínez AL (2020) Fed-batch production and characterization of polyhydroxybutyrate by *Bacillus megaterium* LVN01 from residual glycerol. Dyna (Medellin) 87:111–120. https://doi.org/10.15446/dyna.v87n214.83523

Guan N, Liu L (2020) Microbial response to acid stress: mechanisms and applications. Appl Microbiol Biotechnol 104:51–65. https://doi.org/10.1007/s00253-019-10226-1

Gupta RS, Patel S, Saini N, Chen S (2020) Robust demarcation of 17 distinct Bacillus species clades, proposed as novel *Bacillaceae* genera, by phylogenomics and comparative genomic analyses: description of *Robertmurraya kyonggiensis* sp. nov. and proposal for an emended genus *Bacillus* limiting it only to the members of the Subtilis and Cereus clades of species. Int J Syst Evol Microbiol 70:5753–5798. https://doi.org/10.1099/ijsem.0.004475

Gutiérrez-Cortés C, Suarez H, Buitrago G, Nero LA, Todorov SD (2018) Enhanced Bacteriocin Production by *Pediococcus pentosaceus* 147 in Co-culture With *Lactobacillus plantarum* LE27 on Cheese Whey Broth. Front Microbiol 9. https://doi.org/10.3389/fmicb.2018.02952

Huang J, Zhang D, Ou Y, Zhang G, Zheng L, Lin L, Ye X, Zhu X, Pan Y (2018) Optimization of cultural conditions for *Bacillus megaterium* cultured in agaricus bisporus industrial wastewater. Biomed Res Int 2018. https://doi.org/10.1155/2018/8106245

Jangra M, Belur PD, Oriabinska LB, Dugan OM (2016) Multistrain probiotic production by co-culture fermentation in a lab-scale bioreactor. Eng Life Sci 16:247–253. https://doi.org/10.1002/elsc.201500069

Kaktcham PM, Tchamani Piame L, Sandjong Sileu GM, Foko Kouam EM, Temgoua JB, Zambou Ngoufack F, de Lourdes P-C (2019) Bacteriocinogenic *Lactococcus lactis* subsp. *lactis* 3MT isolated from freshwater Nile Tilapia: isolation, safety traits, bacteriocin characterisation, and application for biopreservation in fish pâté. Arch Microbiol 201:1249–1258. https://doi.org/10.1007/s00203-019-01690-4

Kiousi DE, Efstathiou C, Tzampazlis V, Plessas S, Panopoulou M, Koffa M, Galanis A (2023) Genetic and phenotypic assessment of the antimicrobial activity of three potential probiotic lactobacilli against human enteropathogenic bacteria. Front Cell Infect Microbiol 13. https://doi.org/10.3389/fcimb.2023.1127256

Koskenniemi K, Laakso K, Koponen J, Kankainen M, Greco D, Auvinen P, Savijoki K, Nyman TA, Surakka A, Salusjärvi T, de Vos WM, Tynkkynen S, Kalkkinen N, Varmanen P (2011) Proteomics and Transcriptomics Characterization of Bile Stress Response in Probiotic *Lactobacillus rhamnosus* GG. Mol Cell Proteomics 10:S1–S18. https://doi.org/10.1074/mcp.M110.002741

Kudo H, Sasaki Y (2019) Intracellular pH Determination for the Study of Acid Tolerance of Lactic Acid Bacteria. pp 33–41

Kuebutornye FKA, Lu Y, Abarike ED, Wang Z, Li Y, Sakyi ME (2020) In vitro Assessment of the Probiotic Characteristics of Three *Bacillus* Species from the Gut of Nile Tilapia, *Oreochromis niloticus*. Probiotics Antimicrob Proteins 12:412–424. https://doi.org/10.1007/s12602-019-09562-5

Levering J, Musters MWJM, Bekker M, Bellomo D, Fiedler T, de Vos WM, Hugenholtz J, Kreikemeyer B, Kummer U, Teusink B (2012) Role of phosphate in the central metabolism of two lactic acid bacteria– a comparative systems biology approach. FEBS J 279:1274–1290. https://doi.org/10.1111/j.1742-4658.2012.08523.x

Makowski K, Matusiak K, Borowski S, Bielnicki J, Tarazewicz A, Maroszyńska M, Leszczewicz M, Powałowski S, Gutarowska B (2017) Optimization of a Culture Medium Using the Taguchi Approach for the Production of Microorganisms

Active in Odorous Compound Removal. Appl Sci 7:756. https://doi.org/10.3390/app7080756
Malvido MC, González EA, Bazán Tantaleán DL, Bendaña Jácome RJ, Guerra NP (2019) Batch and fed-batch production of probiotic biomass and nisin in nutrient-supplemented whey media. Braz J Microbiol 50:915–925. https://doi.org/10.1007/s42770-019-00114-1
Manzoor A, Qazi JI, Haq IU, Mukhtar H, Rasool A (2017) Significantly enhanced biomass production of a novel bio-therapeutic strain *Lactobacillus plantarum* (AS-14) by developing low cost media cultivation strategy. J Biol Eng 11. https://doi.org/10.1186/s13036-017-0059-2
Melo-Bolívar JF, Pardo R, Junca RY, Sidjabat H, Cano-Lozano HE, Yolanda R, Junca H, Sidjabat HE, Cano-Lozano JA, Villamil Díaz LM, (2022) Competitive Exclusion Bacterial Culture Derived from the Gut Microbiome of Nile Tilapia (Oreochromis niloticus) as a Resource to Efficiently Recover Probiotic Strains: Taxonomic, Genomic, and Functional Proof of Concept. Microorganisms, (2022) Vol 10. Page 1376(10):1376. https://doi.org/10.3390/MICROORGANISMS10071376
Melo-Bolívar JF, Pardo RYR, Quintanilla-Carvajal MX, Díaz LE, Hume ME, Villamil L (2023a) Development of a Nile tilapia (*Oreochromis niloticus*) gut microbiota-derived bacterial consortium with antibacterial activity against fish pathogens. Innov Food Sci Emerg Technol 83:103214. https://doi.org/10.1016/j.ifset.2022.103214
Melo-Bolívar JF, Ruiz Pardo RY, Hume ME, Nisbet DJ, Rodríguez-Villamizar F, Alzate JF, Junca H, Villamil Díaz LM (2019) Establishment and characterization of a competitive exclusion bacterial culture derived from Nile tilapia (*Oreochromis niloticus*) gut microbiomes showing antibacterial activity against pathogenic Streptococcus agalactiae. PLoS ONE 14:e0215375. https://doi.org/10.1371/journal.pone.0215375
Melo-Bolívar JF, Ruiz Pardo RY, Hume ME, Villamil Díaz LM (2021) Multistrain probiotics use in main commercially cultured freshwater fish: a systematic review of evidence. Rev Aquac 13:1758–1780. https://doi.org/10.1111/raq.12543
Melo-Bolívar JF, Ruiz Pardo RY, Quintanilla-Carvajal MX, Díaz LE, Alzate JF, Junca H, Rodríguez Orjuela JA, Villamil Diaz LM (2023b) Evaluation of dietary single probiotic isolates and probiotic multistrain consortia in growth performance, gut histology, gut microbiota, immune regulation, and infection resistance of Nile tilapia, *Oreochromis niloticus*, shows superior monostrain performance. Fish Shellfish Immunol 140:108928. https://doi.org/10.1016/j.fsi.2023.108928
Merrifield DL, Carnevali O (2014) Probiotic Modulation of the Gut Microbiota of Fish. In: Aquaculture Nutrition. John Wiley & Sons, Ltd, Chichester, UK, pp 185–222
Mills S, Ross RP, Coffey A (2011) Lactic Acid Bacteria| *Lactococcus lactis*. In: Encyclopedia of Dairy Sciences. Elsevier, pp 132–137
Mora-Villalobos JA, Montero-Zamora J, Barboza N, Rojas-Garbanzo C, Usaga J, Redondo-Solano M, Schroedter L, Olszewska-Widdrat A, López-Gómez JP (2020) Multi-Product Lactic Acid Bacteria Fermentations: A Review. Fermentation 6:23. https://doi.org/10.3390/fermentation6010023
Mustafa SM, Chua LS, El-Enshasy HA, (2019) Effects of agitation speed and kinetic studies on probiotication of pomegranate juice with lactobacillus casei. Molecules, (2019) Vol 24. Page 2357(24):2357. https://doi.org/10.3390/MOLECULES24132357
Norizan NABM, Halim M, Tan JS, Abbasiliasi S, Mat Sahri M, Othman F, Bin AA (2020) Enhancement of β-Mannanase Production by *Bacillus subtilis* ATCC11774 through Optimization of Medium Composition. Molecules 25:3516. https://doi.org/10.3390/molecules25153516
Padan E, Bibi E, Ito M, Krulwich TA (2005) Alkaline pH homeostasis in bacteria: New insights. Biochimica et Biophysica Acta (BBA) - Biomembranes 1717:67–88. https://doi.org/10.1016/j.bbamem.2005.09.010
Papizadeh M, Rohani M, Hosseini SN, Shojaosadati SA, Nahrevanian H, Talebi M, Pourshafie MR (2020) Screening for efficient nitrogen sources for overproduction of the biomass of the functionally probiotic *L. plantarum* strain RPR42 in a cane molasses-based medium. AMB Express 10. https://doi.org/10.1186/s13568-020-00976-x
Reda RM, Selim KM, El-Sayed HM, El-Hady MA (2018) *In vitro* selection and identification of potential probiotics isolated from the gastrointestinal Tract of Nile Tilapia, *Oreochromis niloticus*. Probiotics Antimicrob Proteins 10:692–703. https://doi.org/10.1007/s12602-017-9314-6
Ringø E, Hoseinifar SH, Ghosh K, Van DH, Beck BR, Song SK (2018) Lactic Acid Bacteria in Finfish—An Update. Front Microbiol 9:1818. https://doi.org/10.3389/fmicb.2018.01818
Sahandi J, Jafaryan H, Soltani M, Ebrahimi P (2019) The use of two *bifidobacterium* strains enhanced growth performance and nutrient utilization of rainbow trout (*Oncorhynchus mykiss*) fry. Probiotics Antimicrob Proteins 11:966–972. https://doi.org/10.1007/s12602-018-9455-2
Singh S, Singh K, Mandjiny S, Holmes L (2015) Modeling the Growth of *Lactococcus lactis* NCIM 2114 under Differently Aerated and Agitated Conditions in Broth Medium. Fermentation 1:86–97. https://doi.org/10.3390/fermentation1010086
Stoyanova LG, Levina NA (2006) Components of fermentation medium regulate bacteriocin synthesis by the recombinant strain *Lactococcus lactis* subsp. *lactis* F-116. Microbiology (n y) 75:286–291. https://doi.org/10.1134/S0026261706030088
Valle Vargas MF, Quintanilla-Carvajal MX, Villamil-Diaz L, Ruiz Pardo RY, Moyano FJ (2024a) Assessment of encapsulated probiotic *Lactococcus lactis* A12 Viability using an *in vitro* digestion model for tilapia. Animals 14:1981. https://doi.org/10.3390/ani14131981
Valle Vargas MF, Villamil Diaz LM, Ruiz Pardo RY, Quintanilla Carvajal MX (2024b) Design of an agro-industrial by-products-based media for the production of probiotic bacteria for fish nutrition. Sci Rep 14:17955. https://doi.org/10.1038/s41598-024-68783-z
Valle-Vargas MF, Ruiz-Pardo RY, Villamil-Díaz L, Quintanilla-Carvajal MX (2023) Production of a potential multistrain probiotic in co-culture conditions using agro-industrial by-products-based medium for fish nutrition. BMC Biotechnol 23:54. https://doi.org/10.1186/s12896-023-00822-5
Verma R, Sharma S, Kundu LM, Maiti SK, Pandey LM (2023) Enhanced production of biosurfactant by *Bacillus subtilis* RSL2 in semicontinuous bioreactor utilizing molasses as a sole substrate. J Biotechnol 362:24–35. https://doi.org/10.1016/J.JBIOTEC.2022.12.007
Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer K-H, Whitman WB (2009) Bergey's manual of systematic bacteriology: volumen 3 the firmicutes. Springer, New York
World Health Organization: Food and Agriculture Organization of the United Nations (2006) Probiotics in food health and nutritional properties and guidelines for evaluation. FAO Food Nutr Pap 85:1–50
Yi Y, Zhang Z, Zhao F, Liu H, Yu L, Zha J, Wang G (2018) Probiotic potential of *Bacillus velezensis* JW: Antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on Carassius auratus. Fish Shellfish Immunol 78:322–330. https://doi.org/10.1016/j.fsi.2018.04.055

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.