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Artículo Original

Isolation, characterization, and scale-up of native bacteria from the cattle ruminal microbiome

Aislamiento, caracterización, e mejoramiento de bacterias nativas a partir del microbioma ruminal del ganado

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Abstract: The Paraguayan meat industry is a key economic pillar and one of the country's main signatures, whereby its productivity depends on strategies that optimize livestock production. Animal supplementation using microorganisms emerges as a sustainable alternative to improve cattle nutrition. In this regard, this study focused on the isolation, characterization, and fermentation using bench-top bioreactors of lactic acid bacteria isolated from the bovine ruminal microbiome. Here, we isolated two strains belonging to the Enterococcaceae family, named here as CR2 and CR3. Their tolerance to different temperature and pH conditions, and growth capacity in alternative media, were evaluated using microplate readers. The isolates showed the highest growth rates in MRS medium, and among alternative media, whey supplemented with glucose (5 g/L) exhibited the best growth parameters. Bench-top fermentation using stir-tank bioreactors showed that, after 72-hour, CR3 reached 25 × 10^{10} CFU/mL, of biomass in MRS, and 24 × 10^{10} CFU/mL in supplemented whey, corresponding to 2.95 and 2.0 g/L of biomass, respectively. Although the results are promising, future optimizations could improve process efficiency and its application in animal supplementation, by their evaluation as probiotics. This study demonstrates the feasibility of scaling these microorganisms and their potential for valorizing agro-industrial byproducts such as whey. Thus, it proposes a sustainable and innovative approach to bacterial biomass production, benefiting both the livestock industry and byproduct utilization.

Key words: *Lactic acid bacteria, agro-industrial by-products, animal supplementation.*

Resumen: La industria cárnica paraguaya constituye un pilar económico fundamental y una de las principales insignias del país, cuya productividad depende de estrategias que optimicen la producción ganadera. La suplementación animal mediante el uso de microorganismos surge como una alternativa sostenible para mejorar la nutrición del ganado. En este contexto, el presente estudio se enfocó en el aislamiento, caracterización y fermentación en biorreactores de mesa de bacterias ácido-lácticas aisladas del microbioma ruminal bovino. Se aislaron dos cepas pertenecientes a la familia Enterococcaceae, denominadas en este trabajo como CR2 y CR3. Se evaluó su tolerancia a diferentes condiciones de temperatura y pH, así como su capacidad de crecimiento en medios alternativos, utilizando lectores de microplacas. Los aislados mostraron las mayores tasas de crecimiento en medio MRS, y entre los medios alternativos, el lactosuero suplementado con glucosa (5 g/L) presentó los mejores parámetros de crecimiento. Las fermentaciones en biorreactores de tanque agitado de mesa demostraron que, tras 72 horas, CR3 alcanzó 25 × 10^{10} UFC/mL en medio MRS y 24 × 10^{10} UFC/mL en lactosuero suplementado, correspondientes a 2.95 y 2.0 g/L de biomasa, respectivamente. Aunque los resultados son prometedores, futuras optimizaciones podrían mejorar la eficiencia del proceso y su aplicación en la suplementación animal, mediante la evaluación de los aislados como probióticos. Este estudio demuestra la viabilidad de escalar estos microorganismos y su potencial para valorizar subproductos agroindustriales como el lactosuero, proponiendo así un enfoque sostenible e innovador para la producción de biomasa bacteriana, en beneficio tanto de la industria ganadera como del aprovechamiento de subproductos.

Palabras clave*: Bacterias ácido-lácticas, residuos agroindustriales, suplementación animal.*

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Introduction

Livestock, especially cattle, is a cornerstone of Paraguay's economy, driving GDP growth, employment, and rural development (Arce, 2012; Laino *et al.*, 2018). Advances in genetics and farm management have propelled Paraguay to become one of the world's leading beef exporters, with 320.000 tons of beef exported in 2024, generating over 1.5 billion USD (SENACSA, 2024). Ruminants, such as cattle, are herbivores that excel at digesting fibrous plant material, thanks to their specialized four-compartment stomach, which facilitates the fermentation of cellulose, hemicellulose, and starch (Briones Ramírez, 2023; Strizler & Rabotnikof, 2019). Here, the microorganisms present in the rumen break down the complex plant polysaccharides into volatile fatty acids (VFAs) and microbial proteins, which are critical for energy and nutrient absorption in ruminants (Kim *et al.*, 2017; Li & Guan, 2017).

Furthermore, as concerns rise over the use of antibiotics in livestock, there is an increasing shift towards alternative strategies, such as by using probiotics, to improve feed efficiency, reduce methane emissions, and promote overall animal welfare (Mansilla *et al.*, 2022). This strategy is gaining importance in regions like the European Union, where the use of antibiotics for growth promotion is banned (Michalak *et al.*, 2021). The challenge lies in the isolation and identification of new natural probiotics, and their subsequent scaling up for production for large-scale use. In bioreactors, the large-scale cultivation of microbes, such as lactic acid bacteria (LAB) and other probiotics, can be achieved by evaluating and maintaining optimal growth conditions for each isolate. These systems are designed for consistent, reproducible production, which is crucial for meeting the demand for probiotic strains in the livestock industry (Serrat Díaz & Méndez Hernández, 2015).

The dominant microbial groups present in the rumen microbiome, such as *Firmicutes* and *Bacteroidetes*, play a key role in fiber degradation, while methanogenic archaea, like *Methanobrevibacter*, are essential for hydrogen homeostasis and in maintaining the rumen's anaerobic conditions (Arias-Islas *et al.*, 2020) (Arias-Islas *et al.*, 2020). The balance of this microbial community is crucial for maintaining the animal's health. For instance, they not only support digestion but also contributes to up to 70% of the animal's energy requirements through VFAs (Li & Guan, 2017). Recent studies on the rumen microbiome aims to enhance livestock productivity and health, by evaluating the use of probiotics, like lactic acid bacteria (LAB), to improve nutrient absorption and growth in livestock (Suharsono *et al.*, 2023). Many of these studies also focus on the bioprospection of microbial isolates from the rumen microbiome to use them as functional probiotics (Kumar *et al.*, 2023; Mamuad *et al.*, 2019; Qadis *et al.*, 2014). Ultimately, the aim is to improve the cattle's gut health and their nutrient efficiency.

Scaling up of microbial production in bioreactors is another challenge. It involves optimizing growth media and ensuring the stability of microbial strains under large-scale fermentation conditions. Given the high cost of defined media, agro-industrial residues, such as whey, are increasingly being evaluated as cost-effective alternatives for large-scale microbial fermentation (Saval, 2012). Whey, a byproduct of dairy processing, is rich in nutrients that can support the growth of lactic acid bacteria (Ramírez Navas, 2012, 2013; Rojas *et al.*, 2016). By utilizing this agro-industrial residue, researchers aim to not only reduce costs but also contribute to sustainable practices in bioprospecting and microbial fermentation, offering an efficient, scalable alternative to traditional media. Thus, the main challenge to leverage microbial biotechnology in the form of natural novel probiotics from the rumen require their isolation, identification, and physiological characterization for effective growth.

Here, we isolated two LAB from the cattle ruminal microbiome, which were biochemically characterized, and evaluated their growth in

alternative whey-based media prior to scaling up at reactor scale.

Materials and Methods

Sample Collection and Bacterial Isolation

Post-mortem rumen content was collected from a 4-year-old, 560-kg bovine (*Bos taurus*) of mixed genetic background (Brahman, Brangus, and Nelore), originally from the Canindeyú department, at the San Lorenzo Municipal Slaughterhouse (San Lorenzo, Central Department, Paraguay). Approximately 400 g of rumen content were collected in a sealed plastic bag and transported on ice to the lab to preserve microbial integrity (Robles *et al.*, 2023). The sample was then stored at –20°C prior to analysis.

To enrich for lactic acid bacteria (LAB), 1 g of rumen content was inoculated into 15 mL of MRS broth (Oxoid CM0359) in sterile 50 mL tubes and incubated at 39°C for 48 hours under microaerophilic conditions, since the aerobic metabolism of LAB results in greater biomass yields—an increase of 25 to 33% compared to anaerobic conditions (Condon, 1983; Cueto-Vigil *et al.*, 2010; Sánchez & Tromps, 2014; Smalley *et al.*, 1968; Yousten *et al.*, 1975). Serial dilutions were performed post-incubation, and 100 µL of the 10^{-6} dilution was plated on MRS agar (1.5% w/v). Plates were then incubated at 39°C under aerobic conditions.

White, round, small colonies were selected and subcultured by streak plate. Gram staining was performed using a modified method by Álvarez Duarte (2020). Gram-positive colonies were selected for catalase testing (Velázquez-López *et al.*, 2018), and were grown in MRS broth at 39°C for 24 hours. Catalase activity was evaluated by adding 3% hydrogen peroxide to 1 mL of culture (Parada *et al.*, 2017; Parra González, 2020). Isolates showing no bubble formation were considered catalase-negative and retained for further study.

Molecular Identification

Isolates were cultured in 30 mL MRS broth at 39°C for 72 hours under static aerobic conditions. After centrifugation (4000 r.p.m., 10 minutes), pellets were resuspended in 0.9% sterile saline. DNA was extracted using the ZymoBIOMICS™ DNA Miniprep Kit, following the manufacturer's protocol (Ojo-Okunola *et al.*, 2020). DNA was quantified with a Nanodrop 8000 spectrophotometer (Thermo Scientific).

Table 1. Sequences of primers 515F and 806R used for the amplification of the 16S rRNA gene of isolates CR2 and CR3 by PCR (Katiraei *et al.*, 2022).

Primer	Sequence
515F	5′-GTGCCAGCMGCCGCGGTAA-3′
806R	5′-GGACTACHVGGGTWTCTAAT-3′

Amplification of the 16S rRNA gene was performed using HiMedia Hi-Proof DNA Polymerase and universal primers 515F and 806R (Table 1). Reactions (20 µL) contained buffer, dNTPs, primers, polymerase, and template DNA. PCR conditions, modified from Katiraei *et al.* (2022), were: Initial denaturation: 94°C for 3 minutes, 30 cycles: 94°C for 45 seconds, 50°C for 1 minute, 72°C for 1 minute, and final extension: 72°C for 5 minutes.

PCR products were verified by electrophoresis on 2% agarose gel (70 V, 90 minutes), stained with Diamond™ Dye, and visualized under UV light. Amplicons were sequenced via Sanger sequencing at Macrogen Inc. (Seoul, South Korea).

Forward and reverse sequences were assembled into consensus sequences using UGENE (v.52.0), with a 70% minimum identity threshold (Lin *et al.*, 2020; Okonechnikov *et al.*, 2012). Taxonomic assignment was performed using BLASTn (megablast algorithm) against the 16S rRNA database (Bacteria and Archaea, type strains only), excluding unannotated sequences (Parra González, 2020; Zhang *et al.*, 2000).

Evaluation of growth conditions: pH and Temperature

Growth kinetics were assessed under conditions similar to the bovine rumen using a microplate

reader (Multiskan FC, Thermo Scientific) measuring optical density at 620 nm, to calculate growth rate (μ) and doubling time (DT).

Four MRS broths pH conditions were evaluated. For this, 100 mL for each condition was sterilized and adjusted to pH 4.5, 5.5, 6.5, and 7.5, using sterile HCl 1 N and NaOH 1 M under sterile conditions. Furthermore, four temperatures were also evaluated, 37°C, 38°C, 39°C, and 40°C (Pérez *et al.*, 2015; Sánchez Ortiz *et al.*, 2015). A factorial combination of pH and temperature yielded 16 treatments. Each condition was performed in octuplicate.

Each well of a 96-well plate received 190 μL of pH-adjusted MRS medium and 10 μL of inoculum (prepared by culturing strains in MRS at 39°C for 24 hours). OD620 was monitored every 30 minutes for 18 hours (Sandoval-Espinola *et al.*, 2015). Growth rates were calculated from the exponential phase slope, converted to natural log (×2.303), and used to determine DT with: $DT = 2\ln/\mu$. Negative controls contained sterile MRS broth. A heatmap of μ values was generated in R using ggplot2, normalized via Z-score (Villanueva & Chen, 2019).

Evaluation of Whey as an Alternative growth medium

Commercial (COPALSA, Capiatá, Paraguay) whey powder was reconstituted at 10% (w/v) (Quille Quille *et al.*, 2021). The solution was clarified by heating at 100°C for 15 minutes, centrifuged (10,000 r.p.m., 5 minutes), and filtered through 0.45 μm nitrocellulose membranes (Muvdi-Nova *et al.*, 2021; Rojas *et al.*, 2016).

Three treatments were evaluated:

- 10% whey
- 10% whey + 5 g/L glucose
- 10% whey + 5 g/L yeast extract

All treatments were sterilized at 121°C for 15 minutes.

Growth was compared between the whey treatments and MRS medium using a microplate reader (39°C, monitoring OD_{620nm} every 30 minutes for 18 hours), as previously mentioned. Each well received 190 μL of medium and 10 μL of inoculum (from 24-hour MRS culture). Each treatment was tested in octuplicate. Negative controls contained sterile MRS.

The treatment with the highest growth rate and subsequent shortest DT was selected for scale-up in a bioreactor.

Bioreactor fermentation

Batch fermentations were performed in a 1-L stirred-tank bioreactor (BioFlo120, Eppendorf) at 39°C, 200 rpm (six-blade Rushton impellers), with continuous pH monitoring for 72 hours, and working volume of 1 L. For inoculum preparation, the selected isolates were cultivated in 20 mL MRS at 39°C for 24 hours. The bioreactor was inoculated at 2% (v/v), containing either MRS or whey medium as described above (Fernández Fernández, 2022; Gutiérrez-Sarmiento *et al.*, 2020).

At the beginning and end of fermentation, 20 mL sample was filtered through 0.22 μm previously dried membranes. Filters were then dried again for 5 hours to determine final dry weight (Padín Gonzáles & Díaz Fernández, 2006). Five aliquots were collected during fermentation, serially diluted in 0.9% saline to $1X10^{-10}$, and plated on MRS agar using the Drigalski loop technique. Plates were incubated aerobically at 39°C for 24 hours.

Statistical Analyses

Data were analyzed using one-way and two-way ANOVA ($\alpha = 0.05$), following García M *et al.* (2013) and Settachaimongkon *et al.* (2016). Assumptions of normality and homogeneity of variances were tested beforehand. Analyses were conducted using InfoStat v.2017 (Di Rienzo *et al.*, 2008; 2017).

Two-Way ANOVA was used to assess interactions between pH and temperature on bacterial growth rate. On the other hand, One-Way ANOVA was applied to detect differences in growth among culture media (MRS and whey treatments).

Results and Discussion

Bacterial Isolation

After enrichment culturing using bovine rumen as initial inoculum in MRS medium, three colonies were obtained. These were subsequently purified through streaking, resulting in colonies CR1, CR2, and CR3. These colonies were selected for biochemical characterization via Gram staining and catalase testing, aiming to confirm that the bacterial isolates were Gram-positive and catalase negative.

These characteristics coincide with lactic acid bacteria, which contain many known probiotics (Hill *et al.*, 2014; Mansilla *et al.*, 2022). Under microscopic observation, the Gram staining revealed violet coloration for both CR2 and CR3, with the cells exhibiting a coccoid morphology, predominantly grouped as diplococci, although forming short chains as well. On the other hand, CR1 exhibited red coloration, indicative of a Gram-negative bacterium (Yupangui Asanza *et al.*, 2024), and a bacillary morphology. The catalase test, which detects the presence and activity of catalase through a reaction with hydrogen peroxide (H_2O_2), was negative for both CR2 and CR3, but positive for CR1 (Table 2). Catalase is an enzyme that breaks down H_2O_2 into oxygen (O_2) and water (H_2O), protecting cells from oxidative damage produced during aerobic metabolism (Moreno & Albarracín, 2016).

Based on the observed characteristics, colonies CR2 and CR3 were selected for further analysis, as they displayed the typical violet coloration of Gram-positive bacteria and were catalase-negative, indicating their potential of being part of the lactic acid bacteria (LAB) group.

Colony	Morphology	Gram Staining	Catalase Test
CR1	Bacilli	-	+
CR2	Cocci	+	-
CR3	Cocci	+	-

Table 2. Characteristics of the isolated colonies based on morphology, Gram staining, and catalase testing.

LAB are Gram-positive, non-motile, non-spore-forming bacteria, typically cocci or bacilli, ranging in size from 0.5 to 0.8 µm (Ringø & Gatesoupe, 1998). These bacteria lack the catalase enzyme (Parra Huertas, 2010); however, LAB are facultative anaerobes, meaning they can grow in the presence or absence of oxygen due to the presence of a hemin group, which allows them to use oxygen as an electron acceptor (Ekinci & Gurel, 2008). They belong to the phylum *Firmicutes* and encompass approximately 20 genera, with *Lactobacillus*, *Lactococcus*, *Enterococcus* and *Streptococcus* being the most notable (Parra Huertas, 2010).

LAB are categorized into homofermentative and heterofermentative species. The former refers to the exclusive production of lactic acid, whereas the latter refers to the production of additional metabolites, such as bacteriocins and ethanol. LAB can be mesophilic or thermophilic, depending on their optimal growth temperature (Parra Huertas, 2010). These properties underscore the significance of LAB in biotechnology, particularly in fermentation processes and as probiotics, not only due to their capacity to produce several compounds, but also because of their potential use for animal microbiome modulation (Hill *et al.*, 2014; Mansilla *et al.*, 2022; Parra Huertas, 2010; Suharsono *et al.*, 2023).

Molecular identification via 16S rRNA Sanger sequencing

DNA extraction and concentration

Total genomic DNA was extracted from CR2 and CR3 as described in Materials and Methods. Their concentration were measured in duplicates using a Nanodrop spectrophotometer at 260 nm and the 260/280 nm absorbance ratio. The average DNA concentration of the CR2 isolate was 49.58 ng/µL with a 260/280 ratio of 2.07. For CR3, the average DNA concentration was 45.61 ng/µL with a 260/280 ratio of 2.185. The absorbance ratio of 260/280 nm indicates high DNA purity,

Table 3. Consensus sequences of CR2 and CR3 isolates after analysis using UGENE software (Unipro v.52.0).

Isolate	Consensus Sequence (5'–3')
CR2	CCACGAGGGGGCAGGCAGTGTCGGATCATTGGGCGTAGCGAGCGCAGGC-GGTTTCTTAACCTGATGTCAAACCCCCGGCTCAACCGGGGAAGGTCCTTG-GAAACTTGGAAACTTCCACTCCGAAAAAGAAAACTGCAATCCATTAC-CAACCGCCCCCGGTTGAGCCGGGGGCATTCAATCAACTTAAAAAACCGCTT-GCTCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTAC-CGCAGCCGCGGGGTACCATCA
CR3	GGGGCCAGCCGTTCCGGGATTTATTGGGGGTAAAGGGAGCCCGGGCG-GTTTTTTAAGTTTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTG-GAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTG-TAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCG-GCTCTCTGGTCTGTAACTGACG

with values above 1,8 suggesting minimal contamination by proteins (Crossley *et al.*, 2020; Osorio *et al.*, 2017).

PCR and Sequence Alignment and Taxonomic Assignment

The identification of the isolates was performed through amplification of the 16S rRNA gene by PCR, using the primers 515F and 806R. The reaction was carried out with the HiMedia Hi-Proof DNA Polymerase kit, following a cycling protocol modified from Katiraei *et al.* (2022). After amplification, the presence of PCR products was confirmed by 2% agarose gel electrophoresis stained with Diamond™ Dye. The amplified products were subsequently sequenced by the Sanger method at Macrogen, Inc. (Seoul, South Korea), using the same primers employed in the PCR reactions.

The consensus sequence for CR2 consisted of 271 base pairs, while for CR3 it was 162 base pairs (Table 3). The consensus sequence of CR3 met the requirement of a 70% similarity between forward and reverse sequences, while CR2 only achieved 60% similarity. This discrepancy might be due to low-quality sequencing. Despite the sequencing issues, taxonomic assignment via BLAST confirmed that CR3 belongs to the *Enterococcus* genus, with *Enterococcus faecium* and *Enterococcus hirae* as the most similar species (99% similarity). For CR2, only a family-level assignment was possible, placing it in the *Enterococcaceae* family. The closest match was *Melisococcus plutonius* (93% similarity), although this result requires further verification due to the sequencing limitations (Al-Shuhaib & Hashim, 2023).

Enterococcus is the third most abundant genus of LAB, after *Lactobacillus* and *Streptococcus*. These Gram-positive cocci are non-spore-forming, catalase-negative, and facultative anaerobes (Anagnostopoulos *et al.*, 2018; Ben Braïek & Smaoui, 2019; Kadri *et al.*, 2015). They are ubiquitous microorganisms found in soil, plants, freshwater, marine environments, and the gastrointestinal tract of animals and humans. Although some strains are opportunistic pathogens, others produce bacteriocins and are used as probiotic supplements in livestock (Beukers *et al.*, 2017; Parra González, 2020). In a study by Parra González (2020), two *Enterococcus faecium* isolates were obtained from milk and Paraguayan cheese. Policani *et al.* (2024) further demonstrated the inhibitory effect of this species against *Listeria*

Table 4. Summary of studies evaluating *Enterococcus faecium* isolation, probiotic potential, and effects on animal health and rumen parameters.

Study	Location/ Subjects	E. faecium use	Key findings
Kumar et al. (2023)	India / 12 Sahiwal cows	Isolation and probiotic potential screening	*E. faecium* showed one of the best probiotic profiles among 43 LAB strains
Šmídková & Čížek (2017)	Newborn calves	*E. faecium* M74 administration for 2 weeks	No significant effect on *E. coli* abundance in the rumen microbiota compared to control.
Azzaz (2023)	*Dairy sheep*	*E. faecium* and *L. rhamnosus* supplementation	Improved digestion and milk yield: +21.7% (*E. faecium*), +20.6% (*L. rhamnosus*). No differences in milk composition.
Qadis et al. (2014)	Holstein calves	LAB probiotic mix (*L. plantarum 220*, *E. faecium 26*, *C. butyricum Mirayi*)	Stable rumen pH (6.6–6.8) in treated group. Significant changes in lactic acid and ammonia nitrogen levels on days 7–14.
Mamuad et al. (2019)	In vitro fermentation	*E. faecium* (0.1–1% concentrations)	Increased VFA, especially propionate, and reduced CH_4 production. No effect on pH or ammonia nitrogen.
Adeniyi et al. (2015)	Nigeria / Cow feces	Isolation and antagonistic activity	*E. hirae*, *E. durans*, *E. faecium*, *E. faecalis* inhibited *E. coli* and *Klebsiella spp.* growth.

monocytogenes.

Enterococcus faecium is the most used *Enterococcus* species in probiotic supplementation for cattle. This lactic acid bacterium is native to the rumen microbiome, though it is also found in dairy and meat products. Its widespread use in animal feed is due to its health and performance benefits in ruminants (Moshokoa *et al.*, 2024).

As summarized in Table 4, several studies worldwide have focused on the isolation of *E. faecium* to evaluate its probiotic potential and its positive impact on animal health.

Growth optimization in terms of pH and Temperature

In the ruminal ecosystem, pH ranges between 5.8 and 7.0 due to fermentation activity. This parameter is heavily influenced by diet, as not all foods impact rumination equally. Rumination, which involves grinding food through strong horizontal jaw movements, mixes the bolus with saliva rich in HCO_3^- and HPO_4^-. Saliva in ruminants has an alkaline pH (8.2-8.4), acting as a buffer to neutralize organic acids produced during fermentation (Ramírez Lozano, 2017). Diets high in concentrated foods reduce rumination and, consequently, saliva secretion, decreasing the buffering capacity and lowering ruminal pH (Ramírez Lozano, 2017).

Growth experiments were performed in 96-well plates in a plate-reader, as described in materials and methods. The evaluation of growth rates for both isolates highlights that CR2 exhibited the best growth at temperatures of 38°C and 39°C, at pH 6.5 and 7.5 (Fig. 1A). In contrast, CR3 showed optimal growth at 40°C and pH 7.5, though it also exhibited good growth across all temperature ranges at this pH (Fig. 1B). The effect of each pH level on the average growth rate of CR2 was dependent on temperature ($F = 4.43$; p-value = 0.0001; Supplementary Table 1). As with CR2, the pH effect on the growth rate of

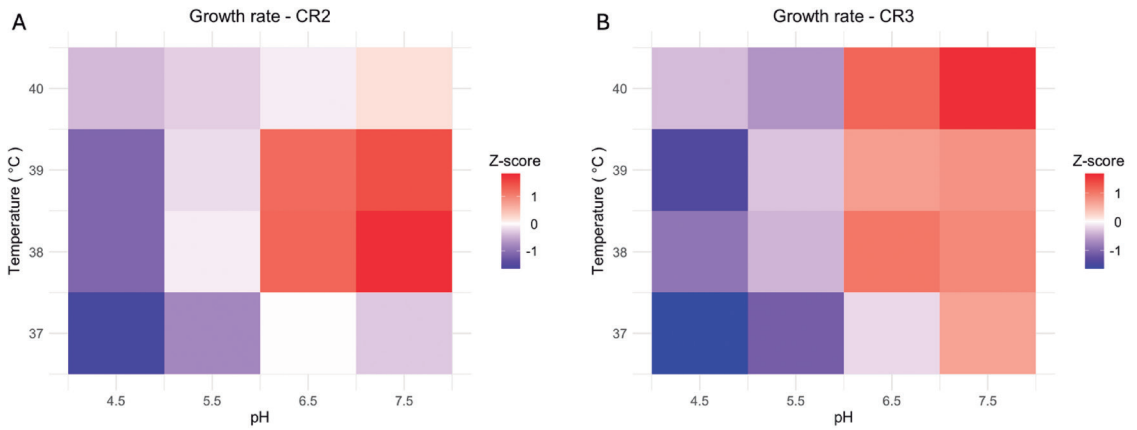

Figure 1. Heatmap of the growth rates of isolates CR2 (A) and CR3 (B) at different pH and temperature values.

CR3 depended on temperature (F = 2.3; p-value = 0.0207; Supplementary Table 1).

The genus *Enterococcus* comprises mesophilic bacteria capable of growing between 10 °C and 45 °C, with an optimal growth temperature range of 30 °C to 35 °C. They can grow within a pH range of 4.4 to 9.6 and tolerate saline environments with up to 6.5% NaCl (Ben Braïek & Smaoui, 2019; García-Solache & Rice, 2019; Murray, 1990).

In the study by El-Sayed *et al.* (2023), it was observed that *Enterococcus faecium* exhibited optimal growth at temperatures between 30 °C and 40 °C and at pH values above 4, with pH 6 being the most favorable for its development. These findings are consistent with the results obtained in the present work, where the isolates CR2 and CR3 showed higher growth rates at pH values between 6 and 7, combined with temperatures ranging from 39 °C to 40 °C.

Evaluation of alternative growth media

In the evaluation of alternative growth media based on whey (10% w/v), it was observed that isolates CR2 and CR3 exhibited the highest growth rates in whey supplemented with glucose (5 g/L). However, the MRS medium continued to support the best growth results for both bacterial isolates (Table 5).

Evaluating the growth kinetics of isolate CR2 using various media, it was observed that under all treatments, CR2 reached the stationary phase around 4 hours after inoculation, with MRS being the medium exhibiting the highest growth rates, followed by whey (10% w/v) supplemented with glucose (5 g/L) (Fig. 2A). Specifically, the MRS medium provided the best results, with a growth rate (μ) of 0.99 h^{-1} and a corresponding doubling time (DT) of 0.70 h. The next best-performing medium was whey (10% w/v) supplemented with

Table 5. Growth rates (h^{-1}) [μ] and doubling time (h) [DT] of isolates CR2 and CR3 cultured in MRS medium, 10% whey, 10% whey supplemented with glucose 5 g/L, and 10% whey supplemented with yeast extract 5 g/L.

	CR2		CR3	
Culturing media	μ (h^{-1})	DT (h)	μ (h^{-1})	DT (h)
MRS	0.99	0.70	1.12	0.62
Whey (10%)	0.65	1.07	0.53	1.32
Whey (10%) + Glucose (5g/L)	0.78	0.90	0.67	1.03
Whey (10%) + Yeast extract (5g/L)	0.48	1.44	0.45	1.55

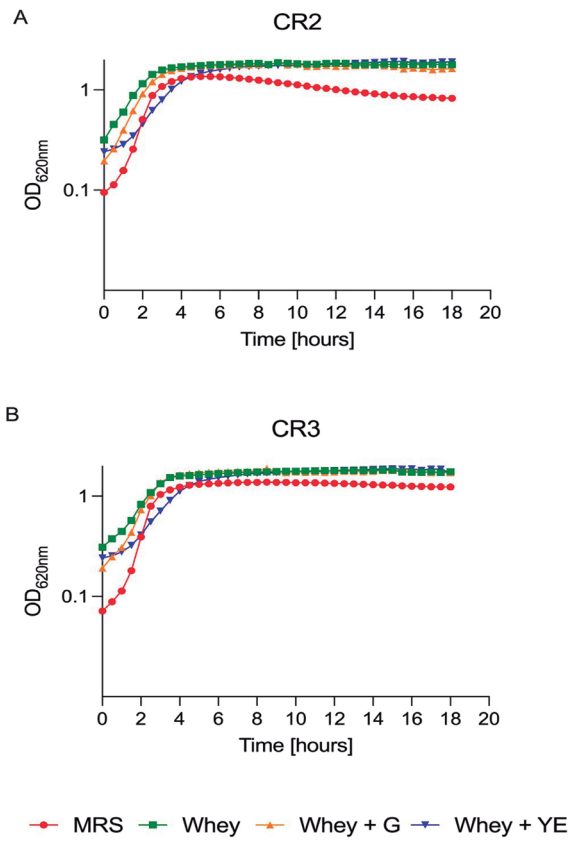

Figure 2. Growth kinetics of isolate CR2 (A) and CR3 (B) in different culture media, measured at 620 nm wavelength. Whey + G: whey supplemented with 5g/L of glucose; Whey + YE: whey supplemented with 5 g/L of yeast extract.

glucose (5 g/L), which resulted in a μ of 0.78 h^{-1} and a DT of 0.90 h, followed by whey (10%) with a μ of 0.65 h^{-1} and DT of 1.07 h. Finally, whey (10% w/v) supplemented with yeast extract (5 g/L) had the lowest results, with a μ of 0.48 h^{-1} and a corresponding DT of 1.44 h (Table 5). Overall, a significant effect of cultivation media on the average growth rate (h^{-1}) was observed (F = 83.91, p-value < 0.0001; Supplementary Table 2).

Similarly, isolate CR3 showed similar results to CR2, with the highest growth rates observed in the MRS medium, with a μ of 1.12 h^{-1} and a corresponding DT of 0.62 h (Table 5). This medium exhibited the highest growth rate, reaching stationary phase after 4 hours cultivation, highlighting the rapid growth of this bacterial isolate under this condition (Fig. 2B). This was followed by whey (10% w/v) supplemented with glucose (5 g/L), which showed a μ of 0.67 h^{-1} and a DT of 1.03 h. Whey (10% w/v) without additives produced a μ of 0.53 h^{-1} and a DT of 1.32 h, while whey (10% w/v) supplemented with yeast extract (5 g/L) displayed the lowest results, with a μ of 0.45 h^{-1} and a DT of 1.55 h (Table 4). For this strain, a significant effect of growth media on the average growth rate (h^{-1}) (F = 10.69, p-value = 0.0001; Supplementary Table 2) was also detected.

Thus, for both, CR2 and CR3 isolates, whey-based media supplemented with glucose (5 g/L) emerged as the best alternative to MRS medium, representing a suitable option for scaling up in a bioreactor. In the context of biomass and lactic acid production using two *Lactobacillus* strains, Botello-Suárez *et al.* (2020) evaluated the use of fresh and deproteinized whey at various concentrations. Their study showed μ values of 0.54 h^{-1} for fresh whey and 0.56 h^{-1} for deproteinized whey at 100% concentration. At 10% whey, μ values were 0.49 h^{-1} for fresh whey and 0.43 h^{-1} for deproteinized whey. Additionally, De La Rosa *et al.* (2023) analyzed the use of various alternative cultivation media, including 15% whey, 10% soy milk, and 15% commercial sugar. In their study, whey supported the highest growth rate, with a μ of 0.47 h^{-1}.

Similarly, Torres Rodelo (2018) examined different supplements for whey, including inulin, yeast extract, and minerals, in combination with MRS as a control. For *Lactobacillus casei*, whey supplemented with all three components achieved a μ of 0.79 h^{-1} and a DT of 0.87 h, while the control MRS medium provided a μ of 0.70 h^{-1} and a DT of 0.82 h. Furthermore, *Lactobacillus rhamnosus* achieved the best growth parameters with whey supplemented with all components, with a μ of 0.80 h^{-1} and a DT of 0.86 h.

Reactor Scale-up

After conducting growth experiments in 96-well plates, the next step involved growth

experiments in bench-top bioreactors, with the purpose of scaling-up. A 72-hour fermentation in a stir-tank bioreactor, with a working volume of 1 L of MRS, the CR3 isolate produced 2.95 g/L of biomass dry weight (DW), corresponding to a final cell concentration of $25x10^{10}$ CFU/mL. The growth rate was 1.57 h^{-1}, with a corresponding doubling time of 0.44 h. The pH decreased from 6.24 to 4.33 during fermentation (Fig. 3A). On the other hand, whey (10% w/v) supplemented with 5 g/L glucose, which was selected for this stage due to its superior growth rate during the microplate assays (Table 5). Here, after 72 hours fermentation, the microbial cell concentration reached $24x10^{10}$ CFU/mL, with a biomass DW of 2 g/L. The growth rate was 1.79 h^{-1}, and the corresponding doubling time was 0.38 h. The pH dropped from 5.70 to 4.50 (Fig. 3B).

Cell viability (CFU/mL) and DW biomass (g/L) were evaluated during both fermentations. Several studies have explored the use of whey as an alternative growth medium for biomass and lactic acid production. Ziadi *et al.* (2020) reported 2.57 g/L DW biomass with whey and 3.16 g/L with M17 medium, producing 32.33 g/L of lactic acid with whey. Gordillo-Andia *et al.* (2024) achieved 13.09 g/L lactic acid with clarified whey, while also observing a drop in pH as observed in this study, likely due to acid production. Studies by Cury R *et al.* (2017) on *L. casei* showed varying cell concentrations in different whey conditions, ranging from $5.28x10^{7}$ to $1.82x10^{8}$ CFU/mL. Escobar *et al.* (2010) demonstrated that whey supplemented with glucose significantly improved biomass production, consistent with the findings in this study, where supplemented media favored bacterial growth more than non-supplemented media.

Besides of the cell viability and biomass yield in fermentations, these results highlight the potential use of whey-based media for culture of LAB in bioreactors, as a less expensive alternative that also contributes to reutilization of agro-industrial residues.

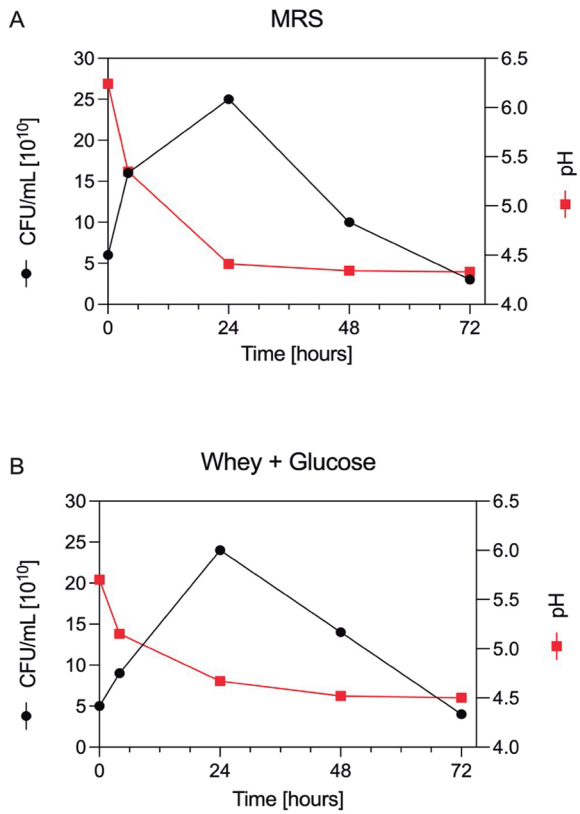

Figure 3. Growth kinetics of CR3 isolate in MRS (A) and Whey (B) medium supplemented with glucose in an stir-tank bioreactor over 72 hours. Black circles represent the CFU/mL, while red squares represent pH values.

Conclusions

Two bacterial strains were successfully isolated from the bovine ruminal microbiome and identified via 16S rRNA gene sequencing as members of the Enterococcaceae family. This group includes native microorganisms commonly found in animal ecosystems, some, such as *Enterococcus faecium,* is widely used as a livestock feed additive.

Both isolates exhibited notable tolerance to pH and temperature conditions simulating the rumen environment, with optimal growth observed at pH values above 7 and temperatures near 40 °C. These findings are essential to ensure the viability and functional activity of the strains under in vivo conditions.

Among the tested media, whey supplemented with glucose emerged as a viable and cost-effective

alternative for bacterial cultivation. This highlights the potential of agro-industrial by-products as sustainable resources for biotechnological applications.

In bioreactor scale-up experiments using stirred-tank bioreactors, glucose-supplemented whey supported efficient biomass production, generating 2 g/L DW after 72 h of cultivation—slightly lower than the 2.95 g/L DW obtained with conventional MRS medium. These results underscore the feasibility of using low-cost substrates for industrial-scale biomass generation.

Author contribution

Mauricio Molinas-Vera and Walter J. Sandoval-Espínola contributed to the conception of the study, data analysis, and manuscript writing. Mauricio Molinas-Vera, Silverio Andrés Quintana, and Yadira Parra carried out the molecular identification of the bacterial isolates. Mauricio Molinas-Vera and Karina Hodara performed the statistical analysis of the results.

Conflicts of interest

The authors declare having no conflicts of interest.

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Appendices

Supplementary Table 1. Analysis of variance (ANOVA) for the effect of pH, temperature, and their interaction on the growth rate (h^{-1}) of bacterial isolates CR2 and CR3.

Bacterial isolate	Growth rate [h-1]					
	F.V.	SS	FD	MS	F	p-value
CR2	Model	11.88	15	0.79	34.14	<0.0001
	pH	7.35	3	2.45	105.62	<0.0001
	Temperature	3.6	3	1.2	51.77	<0.0001
	pH*Temperature	0.93	9	0.1	**4.43**	**0.0001**
	Error	2.6	112	0.02		
	Total	14.48	127			
	F.V.	SS	FD	MS	F	p-value
CR3	Model	6.15	15	0.41	24.6	<0.0001
	pH	4.89	3	1.63	97.66	<0.0001
	Temperature	0.92	3	0.31	18.45	<0.0001
	pH*Temperature	0.35	9	0.04	**2.3**	**0.0207**
	Error	1.87	112	0.02		
	Total	8.02	127			

Supplementary Table 2. Analysis of variance (ANOVA) for the effect of culture medium on the growth rate (h^{-1}) and doubling time (h) of bacterial isolates CR2 and CR3.

Bacterial isolate	Growth rate [h-1]						Dobling time [h]					
	F.V.	SS	FD	MS	F	p-value	F.V.	SS	FD	MS	F	p-value
CR2	Model	1.13	3	0.38	**83.91**	**<0.0001**	Model	2.38	3	0.79	**109.47**	**<0.0001**
	Culture medium	1.13	3	0.38	83.91	<0.0001	Culture medium	2.38	3	0.79	109.47	<0.0001
	Error	0.13	28	0.0045			Error	0.2	28	0.01		
	Total	1.25	31				Total	2.58	31			
	F.V.	SS	FD	MS	F	p-value	F.V.	SS	FD	MS	F	p-value
CR3	Model	1.33	3	0.44	**10.69**	**0.0001**	Model	6.15	15	0.41	**24.6**	**<0.0001**
	Culture medium	1.33	3	0.44	10.69	0.0001	Culture medium	4.89	3	1.63	97.66	<0.0001
	Error	1.16	28	0.04			Error	0.92	3	0.31	18.45	<0.0001
	Total	2.49	31				Total	0.35	9	0.04	2.3	0.0207