Scientific Research Publishing

Advances in Microbiology, 2025, 15(6), 297-312
https://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Presence of *rpoS* Gene and Environmental Stress-Dependent Biofilm Formation in Gram-Negative Bacteria in Mexican Artisanal Dairy Establishments

Ericka Vázquez-Ojeda, Emmanuel Castillo-Quiñones, Bertha Landeros-Sánchez, Lilia Hurtado-Ayala*

Facultad de Ciencias Químicas e Ingeniería, Universidad Autónoma de Baja California, Tijuana, Mexico
Email: vazquez.ericka@uabc.edu.mx, castillo@uabc.edu.mx, bertha.landeros@uabc.edu.mx, *lilyhurtado@uabc.edu.mx

How to cite this paper: Vázquez-Ojeda, E., Castillo-Quiñones, E., Landeros-Sánchez, B. and Hurtado-Ayala, L. (2025) Presence of *rpoS* Gene and Environmental Stress-Dependent Biofilm Formation in Gram-Negative Bacteria in Mexican Artisanal Dairy Establishments. *Advances in Microbiology*, **15**, 297-312.
https://doi.org/10.4236/aim.2025.156021

Received: May 10, 2025
Accepted: June 20, 2025
Published: June 23, 2025

Abstract

Artisanal Mexican cheeses are frequently produced without pasteurization, favoring microbial contamination and potential biofilm formation. **Aim:** To characterize Gram-negative isolates from artisanal cheese outlets in Tijuana, Mexico, evaluate their biofilm-forming ability under different environmental conditions and detect the stress-response gene *rpoS*. **Methods:** Seventeen Gram-negative and 13 Gram-positive bacteria were isolated from cheese, contact surfaces and air. Curves of growth (BHI, 37 ˚C), antimicrobial susceptibility (VITEK 2-AST), biofilm formation in microtiter plates (four media/temperature combinations) and PCR detection of *rpoS* were performed. **Results:** *Escherichia coli* displayed the highest growth rate (μ = 0.28 h^{-1}, g = 149 min), while *Citrobacter freundii* formed the only "strong" biofilm in all conditions. Two *Pantoea agglomerans* isolates were resistant to cefoxitin and one *E. coli* to ampicillin/sulbactam; tetracycline resistance appeared in a single airborne *Staphylococcus epidermidis*. Biofilm biomass (OD_{630}) differed significantly by genus and condition (two-way ANOVA, $p < 0.05$). All Gram-negative isolates were positive for *rpoS*, but gene presence alone did not predict biofilm intensity. **Conclusions:** Gram-negative biofilm-forming bacteria carrying *rpoS* are common in Tijuana artisanal cheeses, yet antimicrobial resistance remains sporadic. Targeted sanitation against mixed-species biofilms and continued AMR surveillance are warranted to enhance food safety.

Keywords

Artisanal Cheese, Biofilm, *rpoS* Gene, Antimicrobial Resistance

1. Introduction

Dairy products are an essential source of high-quality protein, calcium, phosphorus and vitamins, making them a cornerstone of the Mexican diet. According to the Dairy and Products Annual (USDA-FAS), approximately 52% of raw milk produced in Mexico is used to make derivatives such as cheese, with 2023 production estimated at 465,000 t and continuing to rise due to strong domestic demand [1]. The predominance of fresh and artisanal cheeses—many made from unpasteurized milk—increases the risk of microbial contamination and, consequently, foodborne illness (FBI).

Globally, the World Health Organization (WHO) estimates that each year about 600 million people (nearly one in ten) fall ill and 420,000 die from consuming unsafe food, with a disproportionate burden in children under five years of age [2]. In Mexico, bacterial FBIs remain a public health priority: the National Epidemiological Surveillance System (SINAVE) reported over 25,600 cases of bacterial food poisoning (ICD-10 code A05) by the end of epidemiological week 52 of 2023, including 444 new cases in that week alone [3]. Dairy products—especially fresh cheeses—are recurring vehicles for pathogens; a recent study of 111 samples of Cotija and Bola de Ocosingo cheeses (Chiapas, Mexico) detected *Salmonella spp*. DNA in 10.5% and *Staphylococcus aureus* in 13.7% of samples [4].

In artisanal cheeses, Gram-negative pathogens (e.g., *Escherichia coli*, *Citrobacter spp*.) are of particular concern because they combine the tolerance typical of biofilms with an expanding repertoire of antimicrobial-resistance genes [5]. It is estimated that up to 80% of bacteria can adopt a biofilm lifestyle, which protects them from disinfectants, environmental stress and antibiotics [6]. Within the extracellular matrix, horizontal gene transfer and the emergence of persister cells contribute to antimicrobial resistance (AMR), a phenomenon that increases healthcare costs and complicates infection treatment [7].

At the molecular level, the *rpoS* gene—encoding sigma factor S—regulates the transition to stationary phase and stress response in *E. coli* and other Gram-negative bacilli. Its overexpression is associated with enhanced matrix production and more robust biofilms, whereas *rpoS* mutants show drastically reduced adherence to stainless steel, polypropylene and silicone surfaces [8].

Comparative genomic surveys show that an intact *rpoS* locus is present in more than 95% of clinical and food-related isolates of *Salmonella enterica*, *Vibrio cholerae*, *Shigella* spp. and *Cronobacter sakazakii*, underscoring its evolutionary conservation among enteric pathogens. Functional studies demonstrate that in *S. enterica* serovar *typhimurium* a *rpoS* knockout produces ≈ 84% less crystal-violet biomass and suffers a 2-log reduction in survival on stainless steel after desiccation [9]. In enterohaemorrhagic *E. coli* O157:H7, over-expression of *rpoS* doubles acid tolerance and promotes cell aggregation, facilitating colonisation of fresh-produce and cheese matrices [10]. Likewise, in *Pseudomonas fluorescens*—a species frequently recovered from dairy plants—*rpoS* orchestrates extracellular-polysaccharide synthesis and surface attachment through the c-di-GMP network [11]. Col-

lectively, these findings position *rpoS* as a master regulator that links stress adaptation, antimicrobial tolerance and robust biofilm formation in foodborne bacteria. However, the presence of *rpoS* and its relationship to biofilm formation in Gram-negative bacteria isolated from Mexican artisanal cheeses has not been extensively documented.

Therefore, the objective of this study was to evaluate the biofilm-forming capacity of Gram-negative bacterial isolates recovered from artisanal cheese distribution centers in Tijuana (Baja California, Mexico) and to determine the presence of the *rpoS* gene, providing evidence to support more effective control strategies along the dairy chain.

2. Materials and Methods

2.1. Sampling Sites and Study Design

Between January and March 2024, a cross-sectional study was conducted at four artisanal cheese distribution centers in Tijuana, Baja California, Mexico. From each site, three sample types were collected: fresh cheese (Q), food-contact surfaces (S), and ambient air (A). Samples totaled 12 (4 Q + 3 S + 5 A), and all assays were performed in duplicate (Table 1).

Table 1. Sampling sites and main characteristics.

Site code	Sampling sites			
	Environment description	Sample types (n)	Coordinates (WGS-84)	Altitude (m)
M1	Commercial/residential area with local traffic	Q 1, S 1, A 2	32˚31'45.19"N, 116˚58'32.91"W	151
M2	Residential area	Q 1, A 1	32˚29'07.22"N, 116˚51'22.50"W	200
M3	Commercial/residential area	Q 1, S 1, A 1	32˚26'16.62"N, 117˚02'24.34"W	267
M4	Commercial/residential area	Q 1, S 1, A 1	32˚29'18.93"N, 116˚57'04.39"W	70

2.2. Sample Collection and Transport

Fresh cheese samples (100 g) were placed in sterile bags and kept at 4˚C - 8˚C for up to 4 h. Food-contact surfaces (20 - 100 cm^2) were swabbed using pre-moistened swabs in tryptic soy broth, following ISO 18593: 2004 [12]. Ambient air samples were obtained by settle plate method on Plate Count Agar for 10 min at positions determined by $p = 0.15 \sqrt{S}$ (where S is room area in m^2) according to UNE 171330-1: 2008 [13].

2.3. Initial Microbiological Processing

After incubation for 24 h at 37˚C on tryptic soy agar (TSA) and MacConkey agar (BD Difco, USA), each sample was processed in accordance with the relevant Mexican Official Standards (Table 2). From every plate, no more than five colonies that differed in morphology or pigmentation were sub-cultured onto brain–

heart infusion (BHI) agar; duplicate morphotypes were discarded to avoid over-representation. Pure cultures were characterised by Gram stain, oxidase and catalase tests, followed by species-level identification with the VITEK-2 system (bioMérieux).

The analyser was checked every 48 h with *E. coli* ATCC 25922 and *S. aureus* ATCC 29213, and results had to fall within CLSI M100-S34 acceptance limits [14].

Table 2. Mexican official standards applied.

Standard	Purpose	Application in this study
NOM-110-SSA1-1994 [15]	Sample preparation and serial dilution	Homogenization and serial dilution of cheese and swabs
NOM-092-SSA1-1994 [16]	Enumeration of mesophilic aerobic bacteria	Incubation at 35˚C ± 2˚C for 48 ± 2 h
NOM-210-SSA1-2014 [17]	Microbiological sampling of dairy products	Acceptance criteria (absence of pathogens)

2.4. Isolation and Identification of Gram-Negative Bacteria

Presumptive Gram-negative isolates were identified using the VITEK 2 GN system (bioMérieux, Mexico) with a 0.5 McFarland inoculum. Results were available after approximately 18 h. Pure cultures were cryopreserved in TSB with 20% glycerol at −80˚C.

2.5. Antimicrobial Susceptibility Testing

Antibiotic susceptibility was determined by VITEK 2 AST-GN cards and interpreted according to CLSI M100 (2024) breakpoints. **Table 3** lists the antibiotics tested.

Table 3. Antibiotics included in the VITEK 2 AST-GN card.

Class	Antibiotics
Penicillins	Piperacillin, Ticarcillin, Ampicillin, Piperacillin/Tazobactam, Amoxicillin/Clavulanic acid
Cephalosporinas	Cephalothin, Cefuroxime, Cefotaxime, Ceftriaxone, Ceftazidime, Cefepime
Carbapenems	Imipenem, Meropenem, Ertapenem
Monobactams	Aztreonam
Aminoglycosides	Gentamicin, Amikacin, Tobramycin
Fluoroquinolones	Ciprofloxacin, Levofloxacin, Norfloxacin
Polymyxins	Colistin
Other	Trimethoprim/Sulfamethoxazole, Tetracycline, Tigecycline

2.6. Bacterial Growth Curves

To characterize the growth kinetics of five selected Gram-negative genera (*Escherichia coli*, *Serratia marcescens*, *Pantoea agglomerans*, *Raoultella planticola* and *Citrobacter freundii*), each strain was grown for 18 h in BHI at 37˚C and adjusted to 0.5 McFarland (≈1 × 10^8 CFU/mL) with a DensiCHEK Plus (bioMérieux). Only

Gram-negative isolates were included because the study did not plan to analyze the *rpoS* gene in Gram-positive cocci. Erlenmeyer flasks (250 mL) containing 100 mL BHI were inoculated to ~1 × 10^6 CFU/mL (1% v/v) and incubated at 37˚C, 120 rpm. At regular intervals (Table 4), 2 mL samples were withdrawn and the OD_{600} was measured in duplicate on a Genesys 20 spectrophotometer (Thermo Scientific) using sterile BHI as blank. The exponential phase was identified from OD_{600}-versus-time plots, and the specific growth rate (μ) was calculated with Equation (1).

$$\mu = [\ln(OD_2) - \ln(OD_1)]/(t_2 - t_1) \quad (1)$$

and generation time (g) as Equation (2)

$$g = \ln 2/\mu. \quad (2)$$

Data were compared by one-way ANOVA ($p < 0.05$). *E. coli* ATCC 25922 served as quality control, and any curve with $R^2 < 0.95$ was repeated.

Table 4. Schedule of OD_{600} measurements for each genus.

Genus (strain)	No. of readings	Approx. interval	Total duration (h)
E. coli	10	60 min	11
S. marcescens	7	90 min	11
P. agglomerans	8	90 min	12
R. planticola	9	90 min	12.5
C. freundii	7	90 min	11

These kinetic parameters (μ, g) were used to identify the onset of stationary phase for subsequent assays, ensuring all cultures were sampled at comparable physiological states.

2.7. Biofilm Formation Assay

Biofilms were quantified using the microtiter plate method of O'Toole [18] and classified per Stepanović *et al.* [19]. Stationary-phase cultures were adjusted to ≈10^6 CFU/mL (1:100 dilution of 0.5 McFarland cultures). Seventeen Gram-negative isolates were tested in condition C1 and additionally under C2-C4 (Table 5). Briefly, 200 µL of each suspension was inoculated into 96-well polystyrene plates, washed three times with PBS, fixed at 60˚C for 15 min, and stained with 0.1% crystal violet for 15 min. After rinsing and air-drying, dye was solubilized in 200 µL 30% (v/v) glacial acetic acid, and OD_{630} [20] was measured with a Multiskan FC (Thermo Fisher, USA) at the crystal violet absorption maximum in acidic medium. Biofilm production was categorized using ODc = mean blank + 3 SD: non-producers (OD ≤ ODc), weak (ODc < OD ≤ 2 × ODc), moderate (2 × ODc < OD ≤ 4 × ODc) or strong (OD > 4 × ODc). Statistical analysis was performed by one-way ANOVA with Tukey's post-hoc test ($p < 0.05$).

Table 5. Conditions for biofilm assays.

Condition	Medium (200 µL/well)	Temperature	Incubation time
C1	BHI + 2% glucose	37˚C	24 h
C2	Peptone water 0.1%	37˚C	24 h
C3	BHI + 2% glucose	22˚C ± 2˚C	24 h
C4	BHI + 2% glucose	4˚C ± 2˚C	24 h

2.8. Genomic DNA Extraction and Polymerase Chain Reaction (PCR)

Specific primer sets were carefully designed for each targeted genus, as detailed in **Table 6**, resulting in PCR products of varying lengths: 226 bp for *Escherichia coli*, 201 bp for *Serratia marcescens*, 242 bp for *Pantoea agglomerans* 236 bp for *Raoultella planticola*, and 157 bp for *Citrobacter freundii*. As illustrated in **Figure 4**, agarose gel electrophoresis (1.2% TBE) confirmed that all Gram-negative isolates generated bands corresponding to their respective genera. Notably, the positive controls—*E. coli* K-12 MG1655 and *C. freundii* ATCC 8090—produced PCR product sizes aligned with those observed in the field isolates. In contrast, the negative control (PCR-grade water) yielded no amplification. A purification protocol was optimized to isolate high-quality DNA before molecular testing, adapted from Weerakkody *et al.* [21]. This procedure was conducted in a biosafety cabinet under aseptic conditions, using sterilized materials and strict sanitation practices to prevent cross-contamination. Approximately 20 µL of bacterial biomass from fresh cultures (TSA plate scraping) was transferred to a 1.5 mL microtube for cell lysis. 300 µL of lysis buffer (containing NaOH and SDS) was added and mixed for 15 seconds on a vortex mixer. The samples were incubated for 5 minutes at 80˚C in a water bath to denature proteins and disrupt the cell wall. Following incubation, the tubes were briefly cooled, and 1.5 µL of RNase A (10 mg/mL) was added, followed by a 10-minute incubation at 37˚C to degrade residual RNA. Subsequently, 100 µL of precipitation buffer (4M potassium acetate, pH 4.0) was added and mixed gently. The tubes were centrifuged for 3 minutes at 13,000 rpm, discarding the pellet containing precipitated detritus and proteins. The clarified supernatant was transferred to a clean tube, and 300 µL of isopropanol was introduced to precipitate the DNA, which was mixed by inversion. The samples were stored at −20˚C for 24 hours to ensure complete precipitation. Afterward, the samples were centrifuged for 1 minute at 13,000 rpm, with the isopropanol discarded. The resultant pellet was washed with 100 µL of 70% ethanol and centrifuged again. The pellet was air-dried at room temperature until all residual ethanol evaporated. Ultimately, the DNA was resuspended in 100 µL of nuclease-free water and stored at −20˚C until further use [22].

The oligonucleotides were designed from the sequences reported in the National Library of Medicine (https://www.ncbi.nlm.nih.gov/) Gene ID: 947210, (for *Escherichia coli*), Gene ID: 57426935 (*Raoultella planticola*), Gene ID: 66824848 (*Pantoea agglomerans*), Gene ID: 86999769 (*Citrobacter freundii*), Gene ID:

93695333 (*Serratia marcescens*) and using the software Primer3web version 4.1.0. [23].

For the detection of the *rpoS* gene, conventional PCR was performed on the extracted genomic DNA. Each 25 µL reaction mix comprised 12.5 µL of 2 × Taq PCR Master Mix (Bioneer, USA), five µL of a primer mixture (both forward and reverse primers at 10 µM; refer to Table 2), two µL of template DNA, and 5.5 µL of nuclease-free water. Amplification was carried out in a ATC 201 Thermal Cycler (Nyx Technik, Inc. San Diego, CA, USA). following this protocol: initial denaturation at 95˚C for 10 minutes, followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 66.8˚C for 30 seconds, and elongation at 72˚C for 30 seconds, concluding with a final extension at 72˚C for 10 minutes. The PCR products were resolved on a 1.2% agarose gel (in TBE buffer) at 120 V for 25 minutes and visualized under UV light post-staining with ethidium bromide, using a 100 bp ladder (Invitrogen) for size estimation of the amplicons. Positive controls included genomic DNA from *Escherichia coli* K-12 MG1655 and *Citrobacter freundii* ATCC 8090, while PCR-grade water was a negative control. The detection of *rpoS* was confirmed by the presence of a band of the expected size, correlating this finding with the intensity of the biofilm.

3. Results

3.1. Identification of Isolates and Sample Origins

A total of 30 viable isolates were obtained from fresh cheese, food-contact surfaces and ambient air: 17 Gram-negative (56.7%) and 13 Gram-positive (43.3%). Sampling was carried out in duplicate per matrix and each sample was labelled with a unique alphanumeric code (date-site-matrix-replicate). Fresh-cheese wedges (~100 g) were placed directly into sterile Whirl-Pak® bags by the sampling team wearing new nitrile gloves, which were changed between vendors; the outer 1 cm of rind was aseptically trimmed inside the bag with a sterile disposable scalpel to minimise carry-over from retail knives. Bags were stored at 4˚C - 8˚C in insulated coolers equipped with a calibrated thermometer and were processed within 4 h. Surfaces (20 - 100 cm^2) were swabbed with single-use, pre-moistened swabs and transported on ice; ambient air was collected by 10-min settle plates sealed with Parafilm™. A complete set of field blanks (bag, swab, plate) accompanied every round, and none showed growth after incubation. All subsequent handling was performed in a class II biosafety cabinet under aseptic conditions.

Figure 1 summarizes their distribution by sample type. The Gram-negative isolates included *Escherichia coli* (10), *Raoultella planticola* (3), *Pantoea agglomerans* (2), *Serratia marcescens* (1) and *Citrobacter freundii* (1). The Gram-positive isolates comprised *Staphylococcus aureus* (6), *Kocuria kristinae* (3), *Propionibacterium acnes* (1), *Staphylococcus epidermidis* (1), *Staphylococcus auricularis* (1) and *Eggerthia catenaformis* (1). Fresh cheese yielded the greatest diversity (21/30 isolates), followed by surfaces (8/30); only one Gram-positive cocci (*S. epidermidis*) was recovered from air (Figure 1).

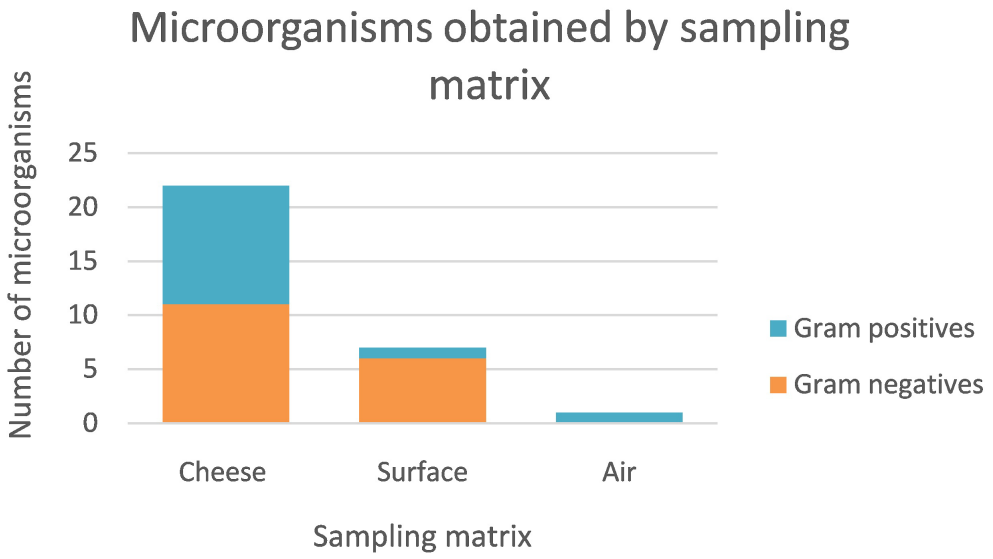

Figure 1. Distribution of gram-negative and gram-positive isolates by sampling matrix (cheese, surface, air).

3.2. Antimicrobial Susceptibility Profile

Using the VITEK 2 AST-GN system, only 3 of 17 Gram-negative isolates (17.6%) exhibited resistance (Table 6): two *P. agglomerans* (cheese and surface) were resistant to cefoxitin (MIC = 16 μg/mL), and one *E. coli* (cheese) was resistant to ampicillin/sulbactam (MIC ≥ 32 μg/mL). All other Gram-negatives—including *R. planticola*, *S. marcescens* and C. *freundii*—were susceptible to the full panel. Among the 13 Gram-positive isolates, only a single *Staphylococcus epidermidis* from air exhibited tetracycline resistance (MIC ≥ 16 μg/mL). All *S. aureus*, *K. kristinae*, *P. acnes*, *S. auricularis* and *E. catenaformis* strains were susceptible to every antibiotic tested.

Table 6. Antimicrobial resistance among gram-negative isolates.

Species	Source	Antibiotic	MIC (μg/mL)	Interpretation
P. agglomerans	Cheese	Cefoxitin	16	R
P. agglomerans	Surface	Cefoxitin	16	R
E. coli	Cheese	Ampicillin/Sulbactam	≥32	R

3.3. Growth Curves of Five Gram-Negative Genera

Figure 2 displays the growth curves at 37˚C for representative strains of *E. coli*, *S. marcescens*, *P. agglomerans*, *R. planticola* and *C. freundii*. All followed a typical sigmoid pattern, with a short lag phase (<45 min) and transition to stationary phase between 6 and 10 h, depending on the genus. Also during the development of the growth curve, the growth rate and generation time were determined (Table 7).

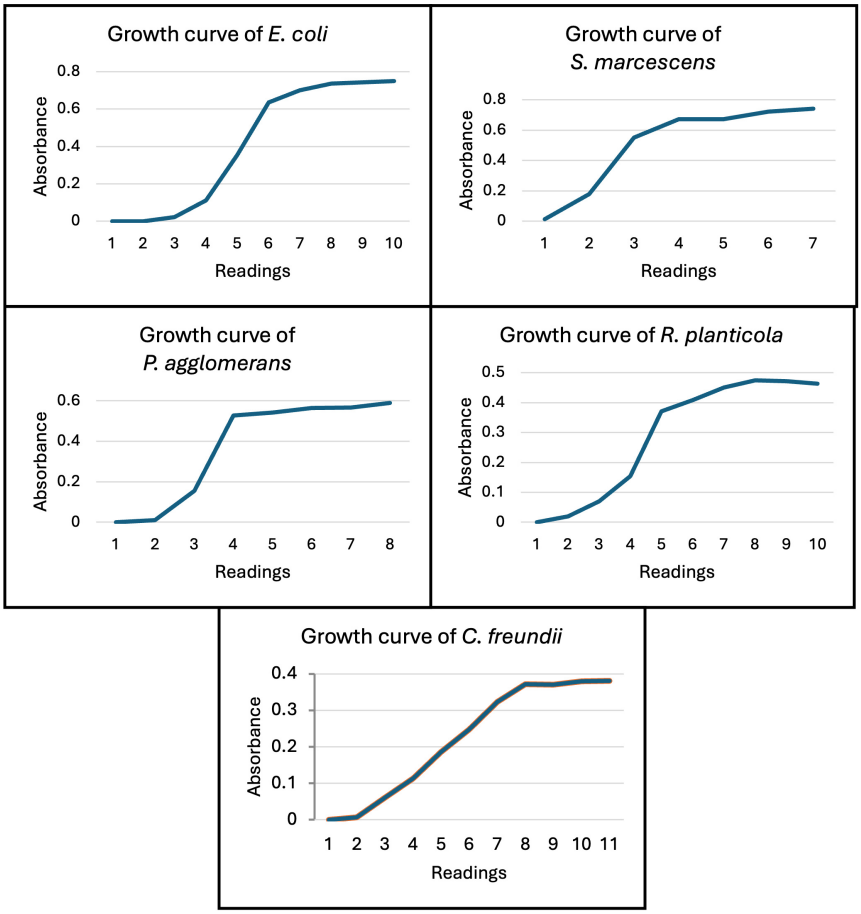

Figure 2. Growth curves (OD_{600} vs. readings) for representative strains of the five Gram-negative genera.

Table 7. Kinetic parameters derived from the exponential phase.

Genus	Specific growth rate (μ, h^{-1})	Generation time (g, min)
E. coli	0.280 ± 0.012	149 ± 6
S. marcescens	0.255 ± 0.010	163 ± 5
P. agglomerans	0.230 ± 0.009	181 ± 6
R. planticola	0.207 ± 0.011	201 ± 7
C. freundii	0.185 ± 0.008	225 ± 8

A one-way ANOVA revealed significant differences in μ among genera ($F = 11.6$; $p < 0.01$). Tukey's post-hoc test showed that *E. coli* grew significantly faster than *P. agglomerans*, *R. planticola* and *C. freundii* ($p < 0.05$), while differences between *S. marcescens* and *P. agglomerans* were not significant ($p = 0.08$). These parameters defined the precise sampling point at $OD_{600} \approx 1.2 \pm 0.1$ for stationary phase prior to biofilm assays, ensuring comparable physiological states.

3.4. Biofilm Formation

All 17 Gram-negative isolates were tested under four conditions: C1 (BHI + 2%

glucose, 37˚C), C2 (peptone water, 37˚C), C3 (BHI + 2% glucose, 22˚C) and C4 (BHI + 2% glucose, 4˚C). Biofilm biomass was measured as OD_{630} after crystal violet solubilization in 30% glacial acetic acid.

3.4.1. Biofilm Biomass by Condition

Figure 3 shows the mean ± SD of OD_{630} for each genus and condition. Overall, C1 produced the highest biomass (mean 0.62 ± 0.14), followed by C3 (0.45 ± 0.12). C2 and C4 reduced biomass to 0.31 ± 0.10 and 0.27 ± 0.09, respectively. A two-way ANOVA (genus × condition) confirmed significant effects of both factors ($p < 0.05$) and their interaction ($p = 0.03$), indicating genus-dependent responses to temperature and nutrient availability.

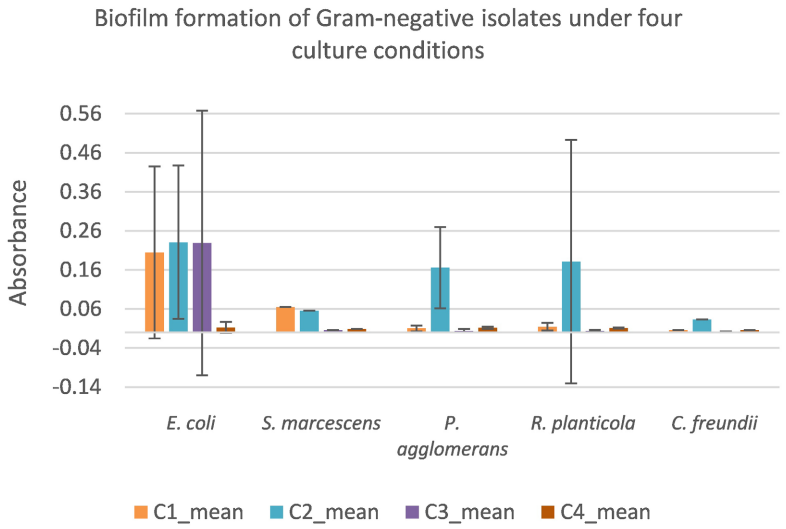

Figure 3. Mean biofilm biomass (OD_{630} ± SD) formed by five Gram-negative bacterial genera under four experimental conditions: C1 (BHI + 2% glucose, 37˚C), C2 (peptone water, 37˚C), C3 (BHI + 2% glucose, 22˚C), and C4 (BHI + 2% glucose, 4˚C). Error bars represent standard deviation (SD).

3.4.2. Biofilm Intensity Classification

Using ODc = mean blank + 3 SD (0.12), isolates were classified as non-producers (OD ≤ ODc), weak (ODc < OD ≤ 2 × ODc), moderate (2 × ODc < OD ≤ 4 × ODc) or strong (OD > 4 × ODc) (Table 8).

Table 8. Biofilm intensity classification by genus.

Genus	Non-producer	Weak	Moderate	Strong	Total
E. coli (n = 10)	2	6	2	0	10
R. planticola (n = 3)	2	1	0	0	3
P. agglomerans (n = 2)	0	2	0	0	2
S. marcescens (n = 1)	0	1	0	0	1
C. freundii (n = 1)	0	0	0	1	1
Total (%)	4 (23.5)	10 (58.8)	2 (11.8)	1 (5.9)	17

A chi-square test of homogeneity (χ^2 = 14.2; df = 6; p = 0.028) showed that intensity distributions differed by genus: *C. freundii* was the only "strong" biofilm former in all conditions, while *R. planticola* was mainly "non-producer" (67%). Most *E. coli* and *S. marcescens* were classified as "weak", although two *E. coli* strains reached "moderate" in C1.

3.4.3. Relationship to Stationary Phase and *rpoS*

All isolates tested positive for the *rpoS* gene confirming the presence of the stationary-phase sigma factor. However, *rpoS* presence alone did not predict biofilm robustness: *R. planticola* carried *rpoS* but produced little biomass, whereas *C. freundii* combined slow growth with dense biofilms under all conditions, suggesting that additional extracellular matrix components and regulatory mechanisms govern biofilm strength.

3.5. Detection of the *rpoS* Gene

Specific primer sets were meticulously designed for each targeted genus, as outlined in Table 9, resulting in PCR products of distinct lengths: 226 bp for *Escherichia coli*, 201 bp for *Serratia marcescens*, 242 bp for *Pantoea agglomerans*, 236 bp for *Raoultella planticola*, and 157 bp for *Citrobacter freundii*.

Table 9. Primers used for amplification of the *rpoS* gene.

Genus	Forward primer (5'→3')	Reverse primer (5'→3')	Expected amplicon size (bp)
Escherichia coli	GCT GAA CGT TTA CCT GCG AA	GGT ATC TTC CGG ACC GTT CG	226
Raoultella planticola	CCC GTA CCA TCC GTT TAC CT	ATC GGC CAG AAT ATC CAG CA	236
Pantoea agglomerans	ATC AAA CCC GTA CCA TCC GT	ATC GGC CAG AAT ATC CAG CA	242
Citrobacter freundii	TAA ACT GGA CCA CGA ACC GA	GGC CAG AAT ATC CAG CAA CG	157
Serratia marcescens	TCG AAC GAG AAT GGA GCT GAG	GCC GCG CAA AAT AGA CTT CT	201

As demonstrated in Figure 4, the agarose gel electrophoresis (1.2% TBE) revealed that all Gram-negative isolates produced bands corresponding to their respective genera. Notably, the positive controls—*E. coli* K-12 MG1655 and *C. freundii* ATCC 8090—exhibited PCR product sizes consistent with those of the field isolates. At the same time, the negative control (PCR-grade water) showed no amplification, effectively ruling out any potential reagent contamination. Gram-positive strains were excluded from this analysis, as the *rpoS* gene is specific to Gram-negative bacilli [19]. The *rpoS* gene is a critical regulator of biofilm formation, enhancing bacterial resilience in challenging environments. Factors influencing *rpoS* activity include oxidative stress, osmotic pressure, and nutrient availability. A thorough understanding of the regulatory networks governed by *rpoS* is pivotal for devising targeted biofilm management strategies, ultimately aimed at augmenting food quality and safety. 100% of Gram-negative isolates carry *rpoS,* although biofilm intensity varied among genera.

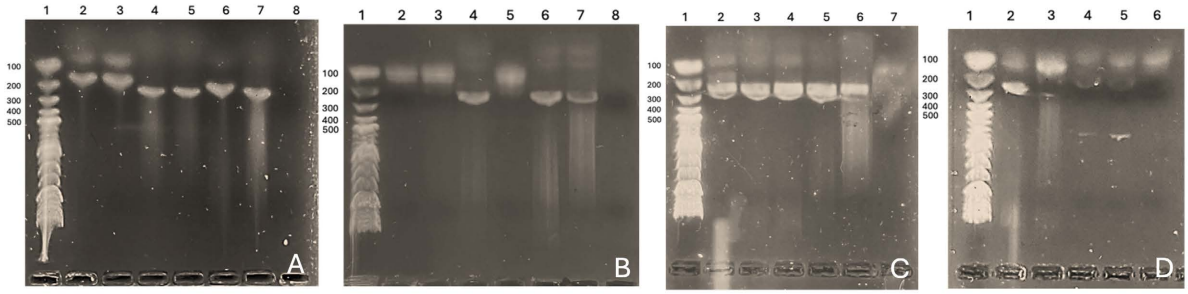

Figure 4. PCR of bacterial strains isolated from cheese samples, characterized by biochemical tests. A. Lane 1: Molecular weight marker, Lane 2: *E. coli*, Lane 3: *E. coli*, Lane 4: *E. coli*, Lane 5: *E. coli*, Lane 6: *S. marcescens*, Lane 7: Positive control (*E. coli* ATCC), Lane 8: Negative control. B. Lane 1: Molecular weight marker, Lane 2: *R. planticola*, Lane 3: *R. planticola*, Lane 4: *E. coli*, Lane 5: *R. planticola*, Lane 6: *E. coli*, Lane 7: Positive control (*E. coli* ATCC), Lane 8: Negative control. C. Lane 1: Molecular weight marker, Lane 2: *E. coli*, Lane 3: *E. coli*, Lane 4: *E. coli*, Lane 5: *E. coli*, Lane 6: Positive control (*E. coli* ATCC), Lane 7: Negative control. D. Lane 1: Molecular weight marker, Lane 2: *P. agglomerans*, Lane 3: *P. agglomerans*, Lane 4: *C. freundii*, Lane 5: Positive control (*C. freundii*), Lane 6: Negative control.

4. Discussion

The recovery of 17 Gram-negative and 13 Gram-positive isolates from fresh cheese and food-contact surfaces underscores the high microbial diversity reported in Mexican artisanal cheeses. That 57% of isolates were Gram-negative bacilli aligns with studies of unpasteurized dairy products in Latin America, where *Escherichia coli* and other Enterobacterales predominate contaminant microbiota [23].

4.1. Antimicrobial Resistance

The detection of only three resistant phenotypes among Gram negatives (17.6%) and one among Gram positives confirms the low prevalence of antimicrobial resistance (AMR) in fresh cheeses observed by Cabrera-Díaz *et al.* [23] in central Mexico. Nevertheless, cefoxitin resistance in *Pantoea agglomerans* and ampicillin/sulbactam resistance in one *E. coli* suggest local selective pressure, possibly due to β-lactam use in dairy herds as documented by the FAO [24]. Although no ESBL or multidrug-resistant profiles were found, the WHO warns that even low percentages can rapidly amplify within biofilm niches [2].

4.2. Growth Kinetics

Growth curves showed that *E. coli* ($\mu \approx 0.28\ h^{-1}$) multiplied significantly faster than *P. agglomerans*, *R. planticola* and *C. freundii*. However, biofilm biomass did not correlate linearly with growth rate: *C. freundii*, despite being the slowest grower ($\mu \approx 0.19\ h^{-1}$), produced the only "strong" biofilms under all conditions. This supports the concept that prolonged stationary phase enhances matrix gene expression and three-dimensional architecture, as described for *Enterobacter* spp. by Pires *et al.* [6].

4.3. Biofilm Formation

Biofilm biomass was the highest in BHI at 37˚C (C1), yet incubation at 22˚C (C3)

retained 73% of the biomass, indicating that typical retail-display temperatures still allow substantial biofilm formation. Cold storage at 4˚C reduced biomass by ≈ 55%, confirming that refrigeration slows but does not eliminate adherence [8]. The significant genus × condition interaction ($p = 0.03$) highlights genus-specific responses: for example, *P. agglomerans* doubled its OD_{630} in peptone water compared to BHI, possibly driven by a "biofilm-by-starvation" mechanism reported for environmental Enterobacterales [6].

4.4. Universality and Relative Impact of *rpoS*

The 100% positivity for *rpoS* confirms a universal stress-response potential among the isolates. However, *rpoS* presence alone did not predict biofilm strength: *R. planticola* carried the gene but was largely a non-producer, while *C. freundii* combined slow growth with dense biofilm formation under all conditions, suggesting that upstream regulatory mutations or interaction with *bolA* modulate matrix synthesis [7]. It is plausible that *R. planticola* has less efficient *rpoS* regulation or produces an EPS with weaker affinity for polystyrene.

Interaction between Biofilm, *rpoS* and Antimicrobial Resistance

No significant correlation was found between biofilm biomass (OD_{630}) and the number of antibiotics to which an isolate was resistant ($\rho = 0.21$, $n = 30$, $p = 0.32$). Similar results were reported by Robbe-Saule *et al.* for *Salmonella* [9] and by Priego-Salado *et al.* for dairy *Citrobacter* isolates [24]. Although oxidative stress can activate the AcrAB efflux pump via *rpoS* and slightly increase multidrug tolerance in *E. coli* [25], recent genomic studies indicate that *rpoS* chiefly aids environmental adaptation and contributes little to clinically relevant AMR [26]. Our data support this view: in artisanal cheeses, *rpoS* enhances persistence through biofilm formation, whereas antimicrobial resistance depends mainly on other factors

4.5. Implications for the Artisanal Dairy Chain

Although AMR prevalence was low and most strains formed weak biofilms, the isolation of *C. freundii*—a strong biofilm former carrying *rpoS*—poses a potential hazard. Pipeline model studies demonstrate that dense *Citrobacter* biofilms protect coliform pathogens and hinder alkaline detergent penetration [27] [28]. Furthermore, tolerance at 22˚C implies that open-market display surfaces facilitate persistence and dissemination.

4.6. Public-Health Implications and Control Strategies

Fresh, raw-milk cheeses sold at room temperature can carry *rpoS*-positive Gram-negative bacteria that form biofilms on knives and display cases. These biofilms may survive routine rinsing for at least two days, seeding the cheese with stress-tolerant cells and occasional β-lactam resistance. Similar strains were traced from artisanal cheeses to patients during recent outbreaks in Europe and Latin America [29]. Even low-level resistance can complicate treatment in vulnerable consumers,

yet Mexico's NOM-243 focuses only on coliform counts and overlooks biofilms. Based on our findings, we recommend: 1) disinfectants validated against biofilms, 2) regular disassembly and scrubbing of utensils, and 3) targeted monitoring of *rpoS* and key AMR genes along the producer-to-retailer chain. These measures would reduce microbial load and the selection pressure for resistance, aligning local practice with the One Health guidance of the FAO and WHO [30].

4.7. Limitations and Future Directions

1) This study was limited to a single municipality and a winter–spring sampling period, so seasonal variation may alter microbial diversity. 2) Biofilm assays were performed on polystyrene, whereas stainless steel predominates in dairy equipment [31] [32]. 3) Biomass was estimated solely by the crystal-violet OD_{630} assay, which stains both living cells and extracellular matrix; differences in matrix composition, washing intensity and plate surface chemistry can over- or under-estimate viable biofilm [33] [34]. Complementary methods such as viable-cell counts or confocal microscopy should be included in future work. 4) Upcoming studies should therefore assess *rpoS* and EPS-gene expression via RT-qPCR. 5) evaluate dual-species biofilms involving Gram-positive cocci. 6) validate cleaning strategies such as enzyme-based disinfectants.

5. Conclusion

Artisanal cheeses marketed in Tijuana (a U.S-Mexico border city), harbour a diverse microbiota in which more than half of the isolates are Gram-negative bacilli that carry the *rpoS* gene and can form biofilms. Although the antimicrobial resistance detected was low and sporadic, the presence of "strong" biofilms in *Citrobacter freundii* and the persistence of several genera at 22˚C and 4˚C indicate a potential food-safety risk. Detection of *rpoS* alone does not explain biofilm intensity; regulatory and environmental factors also contribute. These findings support tightening cleaning protocols aimed at mixed-species biofilms and maintaining continuous monitoring of resistance along the artisanal dairy chain.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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