Research Article

Effect of Synthetic Flavonoids on *Lactobacillus rhamnosus* Aggregation and Exopolysaccharide Production

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Abstract | This study aimed to investigate how synthetic flavonoids influence key probiotic traits of *Lactobacillus rhamnosus*, particularly its exopolysaccharide (EPS) production and cell aggregation. Five methylflavone derivatives were added to the *L. rhamnosus* fermentation medium (200 µg/mL) under optimized conditions. EPS yield and aggregation capacity was subsequently quantified. Results indicated that flavonoid supplementation markedly enhanced EPS production, with the most active compound increased the yield more than threefold compared to the control (371 ± 7.9 mg L^{-1} vs. 114.9 ± 0.3 mg L^{-1}). Auto-aggregation and co-aggregation with beneficial strains was also improved, while adhesion to pathogenic bacteria decreased. Conclusively, these results demonstrated that synthetic flavonoids can considerably strengthen the probiotic functionality of *L. rhamnosus*, offering a promising strategy to overcome its naturally low EPS productivity and improve its potential for industrial and therapeutic applications.

Received | November 14, 2025; **Revised** | December 12, 2025; **Accepted** | December 25, 2025; **Published** | January 14, 2026
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Citation | Khadem, H., B. Boubakeur, M. Adnane, B. Meddah, M. Drabo and A. Tirtouil. 2026. Effect of synthetic flavonoids on *Lactobacillus rhamnosus* aggregation and exopolysaccharide production. *Novel Research in Microbiology Journal,* 10(1): 01-14.
DOI | https://dx.doi.org/10.17582/journal.nrmj/2026/10.1.01.14
Keywords | *Lactobacillus rhamnosus*, Synthetic flavonoids, Exopolysaccharides, Aggregation, Probiotic functionality, Prebiotic effect

Introduction

Exopolysaccharides (EPS) produced by several bacteria, including lactic acid bacteria are highly valued in food industry due to their potential health benefits (Berthold-Pluta *et al.*, 2019; Feng *et al.*, 2025). These polysaccharides have been shown to offer a wide range of positive effects, including cardioprotective, cholesterol-lowering, antioxidant, immunomodulatory, anti-tumor, and prebiotic activities (London *et al.*, 2015; Boubakeur *et al.*, 2018; Copeland *et al.*, 2025). In lactic acid bacteria, EPS are secreted as extracellular polymers that contribute not only to bacterial survival and adhesion but also to the textural and functional quality of fermented foods (Sorensen *et al.*, 2022; Oleksy-Sobczak *et al.*, 2024). This enhances the physicochemical properties of foods such as improving texture, while also providing various health benefits to consumers (Cuevas-Gonzalez *et al.*, 2020; Sorensen *et al.*, 2022; Copeland *et al.*, 2025). Due to their high molecular weight, EPS are resistant to digestion in the upper

gastrointestinal tract, allowing them to reach the colon as intact and selectively stimulate the beneficial gut microorganisms (Caggianiello *et al.*, 2016; Boubakeur *et al.*, 2018; Cichon *et al.*, 2025). These dual nutritional and physiological roles underpin their importance in functional food and probiotic studies.

However, despite of these advantages, one major limitation of many lactic acid bacteria, including *Lactobacillus rhamnosus* (*L. rhamnosus*) is their relatively low EPS productivity (De Vuyst and Degeest, 1999; Sorensen *et al.*, 2022). Reported yields typically range between 0.15 to 0.60 g/L under optimal growth conditions, restricting large-scale application and consistent probiotic performance (Oleksy-Sobczak *et al.*, 2024). Improving EPS yield without compromising bacterial viability has therefore become a major focus in probiotic biotechnology.

In search for natural or synthetic compounds that could enhance EPS biosynthesis, attention has turned to flavonoids, plant-derived polyphenolic compounds with diverse biological impacts (Juca *et al.*, 2020). Beyond their well-documented antioxidant and anti-inflammatory activities, flavonoids have shown the ability to modulate bacterial metabolism and growth (Panche *et al.*, 2016; Cichon *et al.*, 2025; Zhang *et al.*, 2025). Recent studies indicated that polyphenols can act as prebiotic substrates by promoting beneficial microbial populations, including *Lactobacillus* and *Bifidobacterium* spp. (Plamada and Vodnar, 2021; Martin *et al.*, 2023).

Based on this evidence, the present study explored the possibility of using synthetic flavonoid derivatives in enhancing EPS production and aggregation properties of *L. rhamnosus*. This strain was selected due to its recognized probiotic properties, technological robustness, and generally recognized as safe (GRAS) status (Segers and Lebeer, 2014; Aspri *et al.*, 2020). Therefore, by systematically evaluating flavonoids-mediated modulation of key probiotic traits, this work aimed to provide new insights into improving the functional performance and industrial potential of *L. rhamnosus*.

Materials and Methods

Synthetic flavonoids

The synthetic flavonoids used in this study were provided by the Laboratory of Bioconversion, Microbiological Engineering, and Food Safety, Mascara University, Algeria. Two categories of flavonoid derivatives were used, as depicted in Figure 1. These compounds were synthesized by condensing a xylose derivative with 7-hydroxy-8-methylflavone at position 7 through a non-glycosidic linkage, as well as an iTOL derivative. The resulting molecules were obtained either directly (Type A product) or through a space linkage (Type B product). The structural identity of all synthesized flavonoid was confirmed using a combination of analytical techniques, including thin-layer chromatography (TLC) for preliminary screening, liquid column chromatography for purification, and nuclear magnetic resonance spectroscopy (^{1}H-NMR and/or ^{13}C-NMR) for comprehensive structural elucidation. These synthetic flavonoids demonstrated remarkable bioactivity in previous pharmacological assessments, displaying anti-tumor, antiviral, and antibacterial activities. Their influence on lactic acid bacterial growth kinetics has been explored in earlier studies (Boubakeur *et al.*, 2015), which established the rational for the present work to investigate their prebiotic and probiotic-modulating potential.

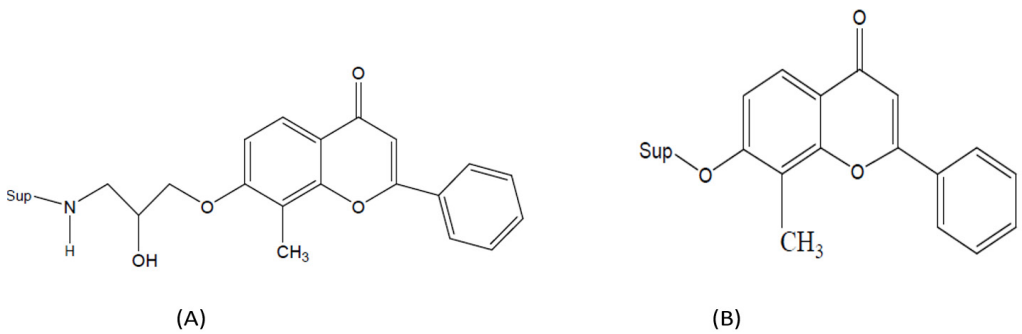

Figure 1: *Chemical structures of the synthetic flavonoids (F1-F5) evaluated in this study. Each compound represents a structural variant of methylflavone designed to assess structure-activity relationships in Lactobacillus rhamnosus. Type A derivatives (F1-F3) carry isopropylidene groups; Type B (F4-F5) contain amino-linked moieties.*

Model microorganisms

The *L. rhamnosus* strain used in this study was obtained from the Laboratory of Bioconversion, Microbiological Engineering, and Food Safety. Strain identification was performed using the API 50 CHL system (Biomerieux, France). For co-aggregation assays, three reference strains were employed; mainly *Streptococcus thermophilus* (*S. thermophilus*) CNRZ 447 (obtained from the INRA microbial collection, Rennes, France), *Staphylococcus aureus (Staph. aureus)* ATCC 43300 (Gram-positive pathogen), and *Escherichia coli (E. coli)* ATCC 10536 (Gram-negative pathogen). These pathogenic strains were provided by the Research Laboratory for the Improvement and Valorization of Local Animal Production, Institute of Veterinary Sciences, University of Tiaret, Algeria.

For inoculum preparation, 100 µL of glycerol-preserved stock culture was streaked onto selective agar plates and incubated for 18-h. *L. rhamnosus* was grown on de Man, Rogosa, and Sharpe (MRS) agar, *S. thermophilus* (CNRZ 447) on M17 agar, *E. coli* on MacConkey agar, and *Staph. aureus* on Chapman agar. After incubation, developing colonies were individually transferred to sterile broth media (*i.e.*, MRS broth and M17 broth for *L. rhamnosus* and *S. thermophilus,* respectively, and Mueller-Hinton broth for the two other pathogenic bacteria) and adjusted to optical density corresponding to 0.5 Mac Farland units.

Characterization of the probiotic potential of L. rhamnosus

The probiotic potential of *L. rhamnosus* was evaluated *via* comprehensive characterization assays. Bacterial strain tolerance to various abiotic stress factors, such as temperature, pH, bile salts, and other factors was assessed following a previously established protocol (Boubakeur *et al.*, 2021).

Optimization of exopolysaccharides production

Production of ESP by a microbial culture is strongly influenced by fermentation conditions and physicochemical parameters. In the present study, the effect of pH (5, 5.5, and 6), incubation temperature (37, 42, and 50 °C), oxygen availability (aerobic and anaerobic), and medium composition, particularly the carbon-to-nitrogen (C/N) ratio were systematically examined following an established procedure (Khadem *et al.*, 2020). The culture medium was modified by supplementing it with sucrose at concentrations ranging from 10 to 90 g/L, while the inoculum density was adjusted between 10^5 and 10^8 CFU/mL to determine the optimal parameters for EPS biosynthesis.

A sequential optimization strategy was employed, in which each variable was independently tested while maintaining the previously optimized conditions for other variables. This approach ensured accurate determination of the most favorable conditions for EPS production. For anaerobic cultivation of *L. rhamnosus,* oxygen-free conditions were established using an anaerobic jar system (AnaeroGen, Oxoid) equipped with palladium catalysts to eliminate residual oxygen. The internal atmosphere was maintained at 85% nitrogen and 15% carbon dioxide, providing ideal conditions for anaerobic growth and EPS production.

Effect of synthetic flavonoids on exopolysaccharides production by L. rhamnosus

The culture medium was supplemented with synthetic flavonoids at a final concentration of 200 µg/mL, identified as the optimal level for maximal bacterial growth in a previous dose-response study (Boubakeur *et al.*, 2015). At this concentration the flavonoids appreciably enhanced the growth kinetics of *L. rhamnosus*, compared to the unsupplemented control. All incubations were conducted under strictly controlled anaerobic conditions, previously optimized for maximal EPS production. The same cultivation parameters established during the optimization phase (*i.e.*, temperature, pH, and sucrose concentration) were maintained to ensure comparability between control and flavonoid-treated cultures.

Estimation of exopolysaccharides yield

Exopolysaccharides produced by *L. rhamnosus* were extracted following the method described by Ricciardi and Clementi (2000). The bacterial cultures were subjected to heat treatment at 80°C for 15 min. and the cells were subsequently harvested by centrifugation at 5,000 x g for 10 min. The resulting supernatant was ultrafiltered, and polysaccharides were precipitated three times with cold ethanol to ensure purity. The precipitation was stored at -20°C for 12 h. Afterward, the samples were centrifuged at 12,000 x g for 20 min. at 4 °C, and the resulting pellet was redissolved in sterile distilled water. The EPS concentration was quantified using the total carbohydrate assay (DuBois *et al.*, 1956).

Effect of synthetic flavonoids on bacterial aggregation

The aggregation behavior of *L. rhamnosus* was evaluated according to a previous literature reported by Kos *et al*. (2003). Cultures were grown in MRS broth supplemented with 200 µg/mL synthetic flavonoids and incubated at 37°C for 18 h. The bacterial cells were harvested by centrifugation at 5,000 x g for 15 min., washed three times with PBS, and resuspended to a final concentration of 10^8 CFU/mL. The suspension was then left to decant at room temperature for 5 h, after which the auto-aggregation percentage was calculated using the following formula reported by Zawistowska-Rojek *et al*. (2022):

$$\text{Auto-aggregation (\%)} = [1 - (A/B)] \times 100$$

Where A is the absorbance after 5 h of decantation and B is the initial absorbance.

For co-aggregation assay, *L. rhamnosus* cells obtained from flavonoid-supplemented cultures were mixed in equal volume (1 mL each) with one of the following bacterial strains: *S. thermophilus*, *Staph. aureus*, and *E. coli*. The co-aggregation percentage was determined as follows:

$$\text{Co-aggregation percentage} = [(Ax + Ay) / 2 - Axy] / [(Ax + Ay) / 2] \times 100$$

Where; Ax = Absorbance of the *L. rhamnosus* suspension, Ay = Absorbance of the other bacterial strains (e.g., *S. thermophilus*, *Staph. aureus*, or *E. coli*), Axy = Absorbance of the mixture of *L. rhamnosus* and the other bacterial strains.

Optical densities for both auto-aggregation and co-aggregation were measured at a wavelength of 620 nm using a spectrophotometer (UV-Vis 2600, Shimadzu, France).

Statistical analysis

All experimental procedures were performed in duplicate to ensure accuracy and reproducibility of the results. Statistical comparisons between experimental groups were performed using one-way analysis of variance (ANOVA), according to Snecdecor and Cochran (1991) with a significance threshold set at $p < 0.05$. When significant differences were detected, Tukey's Honest Significant Difference (HSD) post-hoc test was applied to identify specific group differences. Prior to ANOVA, data normality and variance homogeneity were verified using the Shapiro-Wilk and Levene's tests, respectively, to confirm the suitability of parametric analysis. Significance levels were interpreted as $p < 0.05$: statistically significant; $p < 0.01$: highly significant; $p < 0.001$: very highly significant. All statistical analyses were performed using IBM SPSS 24 (SPSS, 2018) Statistics software (IBM Corp., Armonk, NY, USA).

Results and Discussion

Probiotic properties of L. rhamnosus

Figure 2 illustrates the effect of temperature on growth of *L. rhamnosus*. The strain displayed robust growth across a wide temperature ranges (15-50 °C), reaching its optimal density (8.4 log CFU/mL) at 37 °C after 18 h of incubation. These findings provided valuable insights into the ideal temperature conditions for optimal growth of *L. rhamnosus* and align with the widely accepted optimal growth temperature for this species (Copeland *et al*., 2025). Remarkably, the strain retained viability after exposure to 60-65 °C for up to 2 h, indicating high thermo tolerance possibly linked to the synthesis of heat shock proteins and membrane adaptations (Prasad *et al*., 2003; Zhang *et al*., 2018; Zielińska *et al*., 2025). This thermo resistance offers a considerable advantage for probiotic production, where survival through drying and storage is critical.

In addition to temperature resilience, the strain showed strong tolerance to acid and bile stress (Figure 3). Survival remained above 95% at pH 3.0 and exceeded 96% even at bile concentrations up to 1%. Such resistance ensured passage through the gastrointestinal barriers and efficient intestinal colonization, aligning with prior reports on *L. rhamnosus* acidophilic robustness (Succi *et al*., 2005; Verdenelli *et al*., 2009; Bulut *et al*., 2025).

The bacterial strain also exhibited pronounced antibacterial activity against both Gram-positive (*Staph. aureus*) and Gram-negative (*E. coli*) pathogens, forming inhibition zone diameters between 8-25 mm, in accordance with results reported by previous studies conducted by Riaz Rajoka *et al*. (2017); Sharma *et al*. (2017); Upadhyay *et al*. (2025). This observed antagonistic capacity likely rose from organic acid and bacteriocin secretion (De Vuyst and Degeest, 1999; Plamada and Vodnar, 2021; Sorensen *et al*., 2022; Upadhyay *et al*., 2025), reaffirming the strain's probiotic potential. The strain displayed resistance to

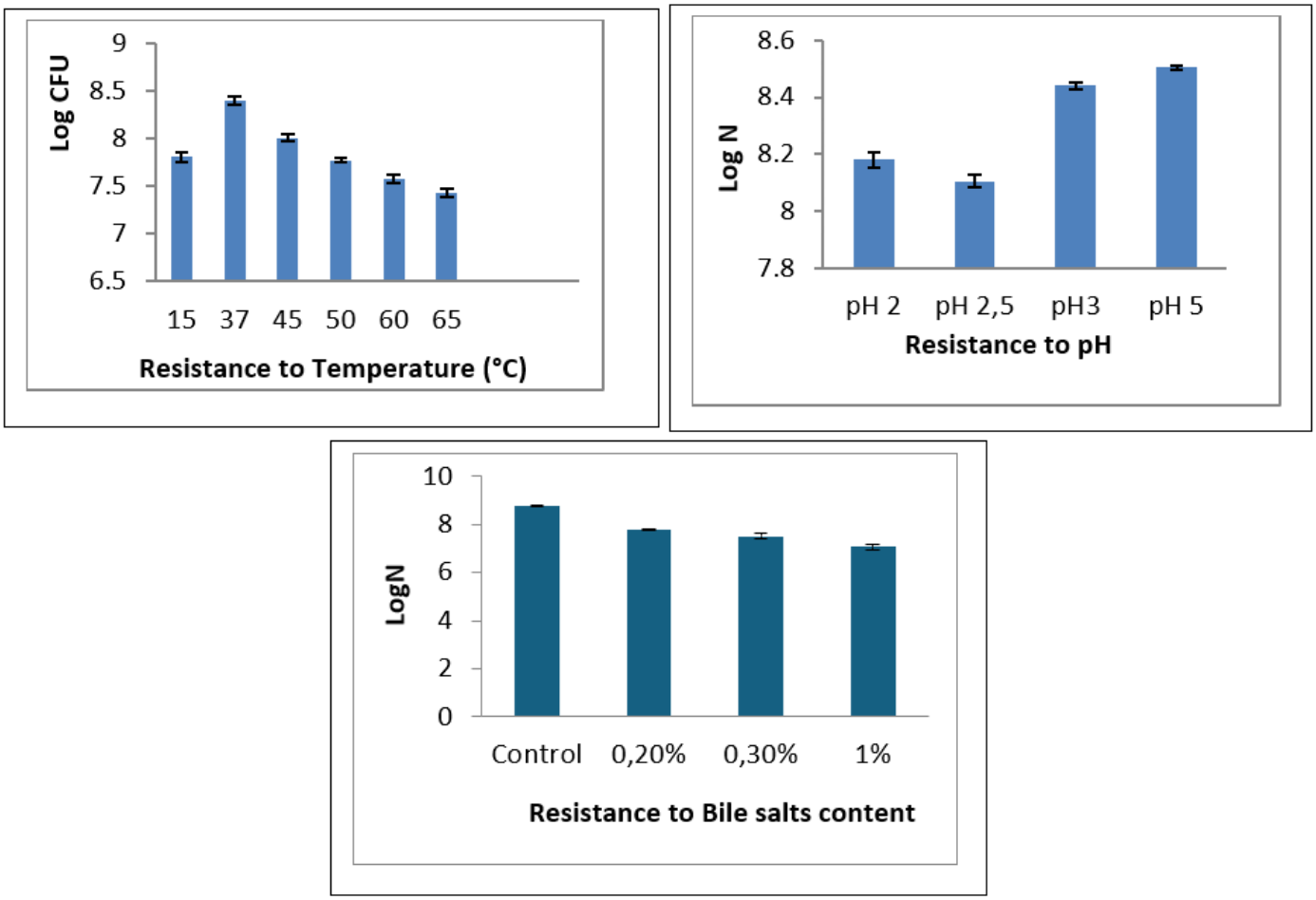

Figure 2: *Effect of temperature, pH, and bile salts on the growth of Lactobacillus rhamnosus. Cells were incubated under variable conditions (temperature 15–50 °C; pH 3.0–8.0; bile 0.3–1 %). The strain showed optimal growth at 37 °C, pH 6.5, and 1 % bile, demonstrating strong tolerance to gastrointestinal-like stress. Results are means of two replicate ±SD.*

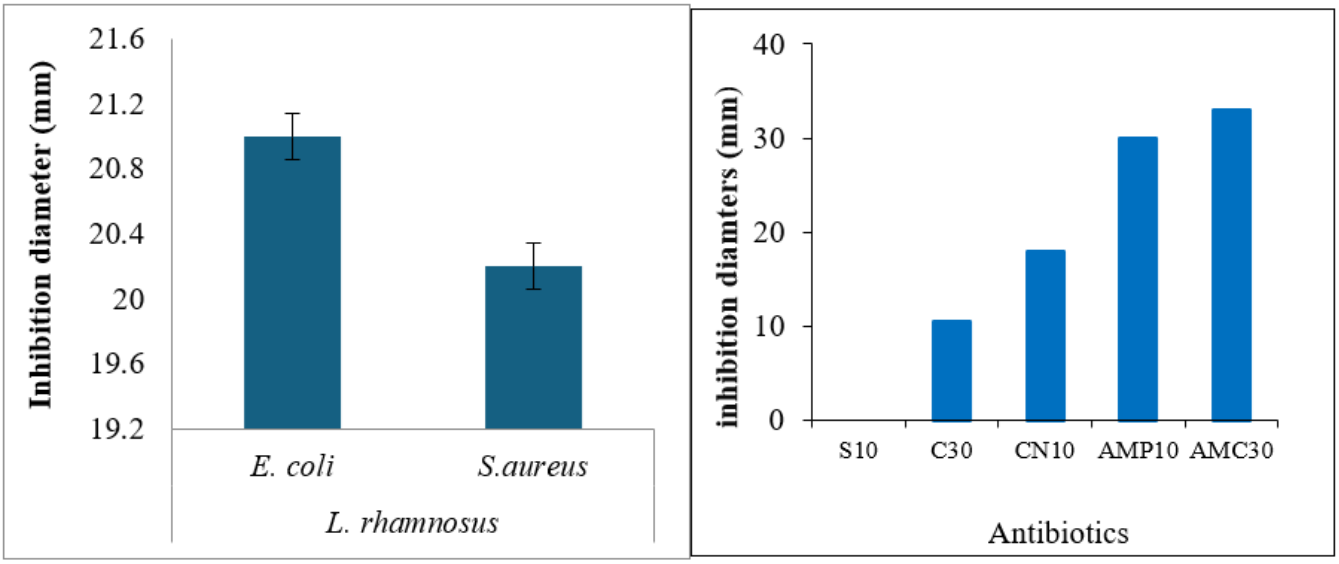

Figure 3: *Antibacterial activity and antibiotic susceptibility of Lactobacillus rhamnosus. Zones of inhibition (mm) were measured against Staphylococcus aureus and Escherichia coli using the agar diffusion method for both tests. The antibiotics utilized were Streptomycin (S10), Chloramphenicol (C30), Gentamicin (CN10), Ampicillin (AMP10), Amoxicillin (AMC30). The results demonstrate a notable variation in the probiotic's antibiotic susceptibility, as well as an antagonistic effect. Results confirm the probiotic's antagonistic effect through secretion of organic acids and bacteriocin-like substances. Results are means of two replicate ±SD.*

streptomycin, while showing variable sensitivity to chloramphenicol, cefalexin, ampicillin, and amoxicillin, in agreement with prior observations reported by Pisano *et al.* (2014); Riaz-Rajoka *et al.* (2017). These results highlighted the importance of strain-specific resistance profiling when considering probiotic co-administration with antibiotics.

Optimization of exopolysaccharides production

Optimization experiments (Figure 4) revealed that EPS biosynthesis was maximized at 35°C, pH 6.5, and 5% sucrose concentration. Production increased with sucrose concentration up to 50g/L, after which further increase let to yield inhibition, suggesting a threshold for carbohydrate metabolism efficiency, likely due to osmotic stress or substrate inhibition (Minari *et al.*, 2024). EPS yield decreased beyond pH 6.5, consistent with earlier findings for mesophilic *Lactobacillus* spp. (Chen *et al.*, 2022). The ideal pH for EPS production is species-specific and controlled pH fermentation in this study improved yield reproducibility, confirming that slightly acidic environment supported optimal enzymatic activity of glycosyltransferases involved in polysaccharide synthesis (Nguyen *et al.*, 2020; Hernández-Figueroa *et al.*, 2025).

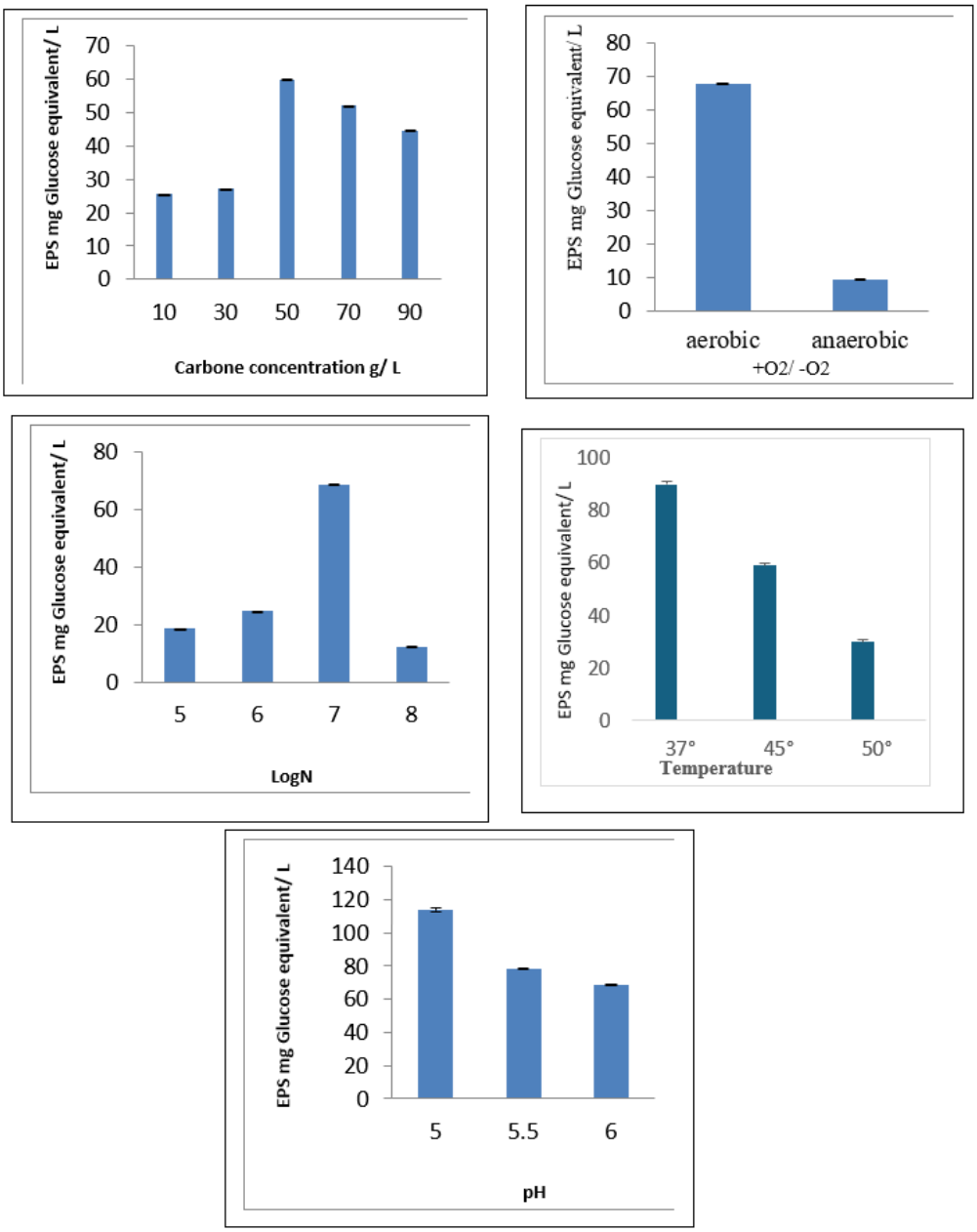

Figure 4: *Optimization of physicochemical parameters for exopolysaccharides (EPS) production by Lactobacillus rhamnosus. EPS yield (mg eq glucose L^{-1}) was determined across gradients of temperature, pH, sucrose concentration, and inoculum density. Maximum production occurred at 35 °C, pH 6.5, and 5 % sucrose, defining the baseline for flavonoid-supplemented experiments. Results are means of two replicate ±SD.*

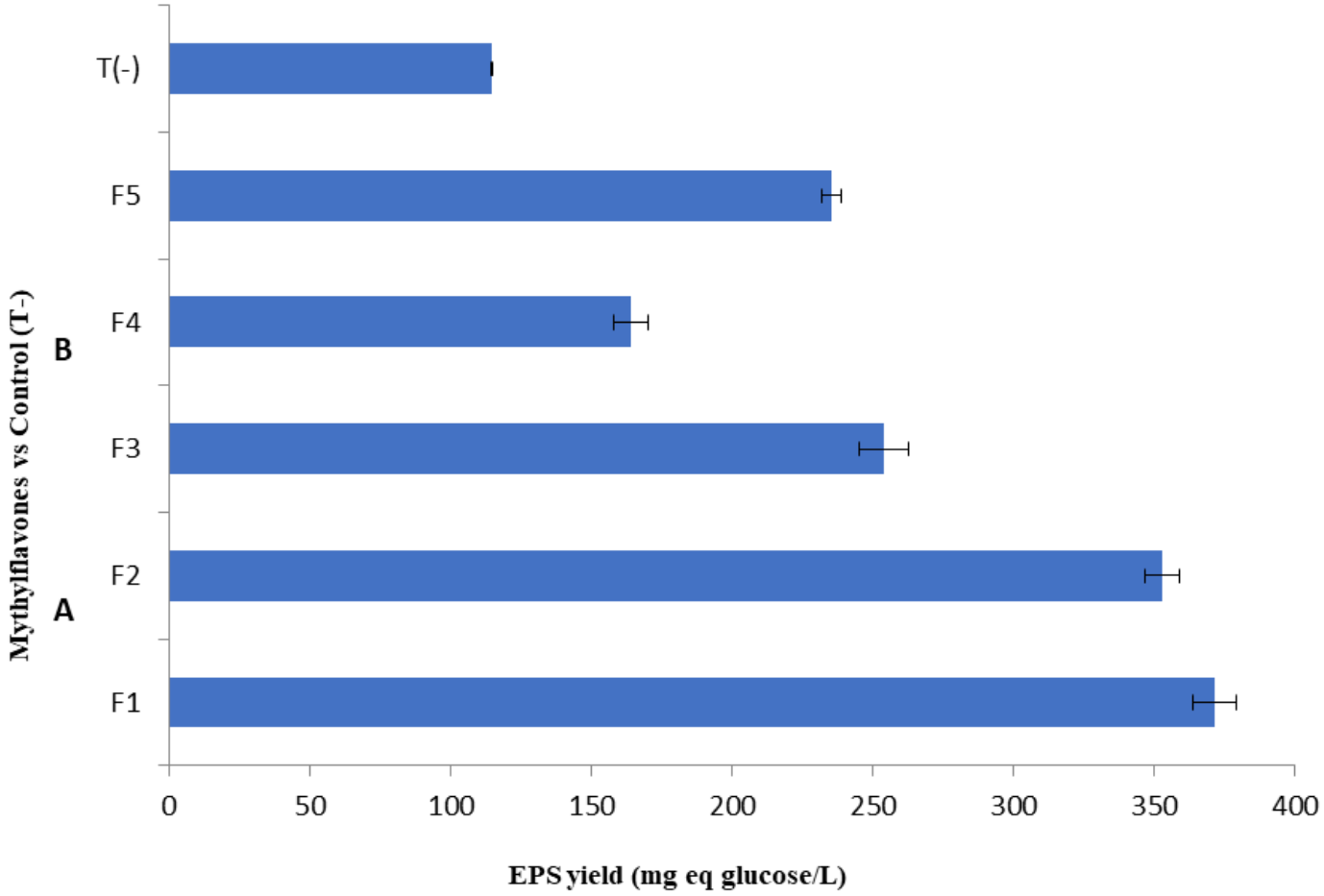

Figure 5: *Effect of synthetic flavonoids (200 µg/ mL) on EPS production by Lactobacillus rhamnosus. Bars represent mean ± SD of triplicate cultures under optimized conditions. All flavonoids enhanced EPS yield relative to the control (114.85 mg/ L), with F1-A giving the highest production (≈3.2-fold increase). Results demonstrate a clear structure–activity relationship favoring Type A derivatives.*

The inoculum density remarkably influenced EPS yield. At 10^7 CFU/mL, EPS production peaked at 68.7 mg eq glucose/L, whereas both lower and higher inoculum densities reduced yield, indicating a balance between metabolic activity and nutrient availability (Zhao and Liang, 2023).

Temperature also played a crucial role. EPS production was highest at 37 °C (90.06 ± 0.226 mg eq glucose/mL), confirming the mesophilic preference of the bacterial strain (Zhang *et al.*, 2023). Production decreased considerably at higher temperatures ($p < 0.001$). This trend supports previous reports showing that thermophilic stress can impair EPS polymerase efficiency (Juraskova *et al.*, 2022; Copeland *et al.*, 2025).

Oxygen availability also affected yield. Aerobic conditions resulted in higher EPS production ($p < 0.05$), compared to anaerobic cultures, likely due to increased energy generation *via* enhanced redox balance (Copeland *et al.*, 2025).

Overall, these optimization steps provided a reliable baseline for evaluating the subsequent impact of synthetic flavonoids. Mesophilic lactic acid bacteria typically produce higher amounts of EPS at lower temperatures (around 25°C), whereas thermophilic bacteria achieve optimal EPS yields at their preferred growth temperatures (Juraskova *et al.*, 2022).

In vitro effect of synthetic flavonoids on exopolysaccharides production

As shown in Figure 5, all five synthetic flavonoids markedly increased EPS yield, compared to the unsupplemented control (114.85 ± 0.28 mg/ L). Among them, the derivative F1-A produced the strongest enhancement, yielding 371.68 ± 7.92 mg/ L, more than a threefold rise over the control. Meanwhile, F2-A and F3-A also displayed substantial stimulatory effects (approximately 2.4-fold and 2.1-fold increases, respectively), while

the type B derivatives (F4-B and F5-B) produced more moderate but still appreciable improvements, yielding 198.2 ± 4.33 mg/ L and 164.1 ± 5.94 mg/L, respectively. These results indicated a clear structure-activity relationship, where type A flavonoids bearing isopropylidene substitutions at positions 2,3 or 4,5 appeared to favor EPS biosynthesis more strongly than type B analogs containing amino-linked glyceryl groups (Konieczna *et al.*, 2018). This trend suggested that molecular configuration influenced how these compounds could interact with the bacterial metabolic pathways involved in carbohydrate polymerization. The markedly higher EPS yield with F1-A implied an enhancement of glycosyltransferases activity or improved precursor availability under anaerobic fermentation. There have been limited published studies on the effect of polyphenols on EPS production by lactic acid bacteria, particularly *Lactobacillus* and *Bifidobacterium* strains. A previous study indicated that polyphenols and flavonoids, which are largely indigestible in the upper gut, can act as prebiotic stimulants, selectively enhancing the growth and metabolic activity of beneficial bacterial genera while modulating carbohydrate metabolism (Plamada and Vodnar, 2021). Flavonoids, due to their indigestibility and breakdown by intestinal flora, may exert a prebiotic effect that promotes the growth, activity, and viability of bifidobacteria and lactobacilli, which are key components of the gut microbiota (Wang *et al.*, 2021; Cichon *et al.*, 2025).

The present results are consistent with earlier findings showing that phenolic compounds can differentially influence EPS synthesis depending on bacterial metabolism (Badra *et al.*, 2022). For instance, *Thymus fontanesii* extract tripled EPS production in *Streptococcus thermophilus* (826 mg/L vs. 219 mg/L control) (Boubakeur *et al.*, 2018), whereas *L. bulgaricus* showed no appreciable change (Khalil, 2010). Such strain-specific responses likely stemmed from variations in carbohydrate-utilization pathways and redox regulation.

In vitro effect of synthetic flavonoids on L. rhamnosus aggregation

Because EPS biosynthesis and aggregation are functionally linked, the relationship between flavonoid treatment and aggregation behavior of *L. rhamnosus* was further evaluated. The tested flavonoid considerably enhanced both auto-aggregation and co-aggregation capabilities of the bacterial strain (Figure 6). Auto-aggregation increased from 80.2% (control) to 90.1% after flavonoid supplementation, while co-aggregation increased substantially from 71.9 ± 1.9%

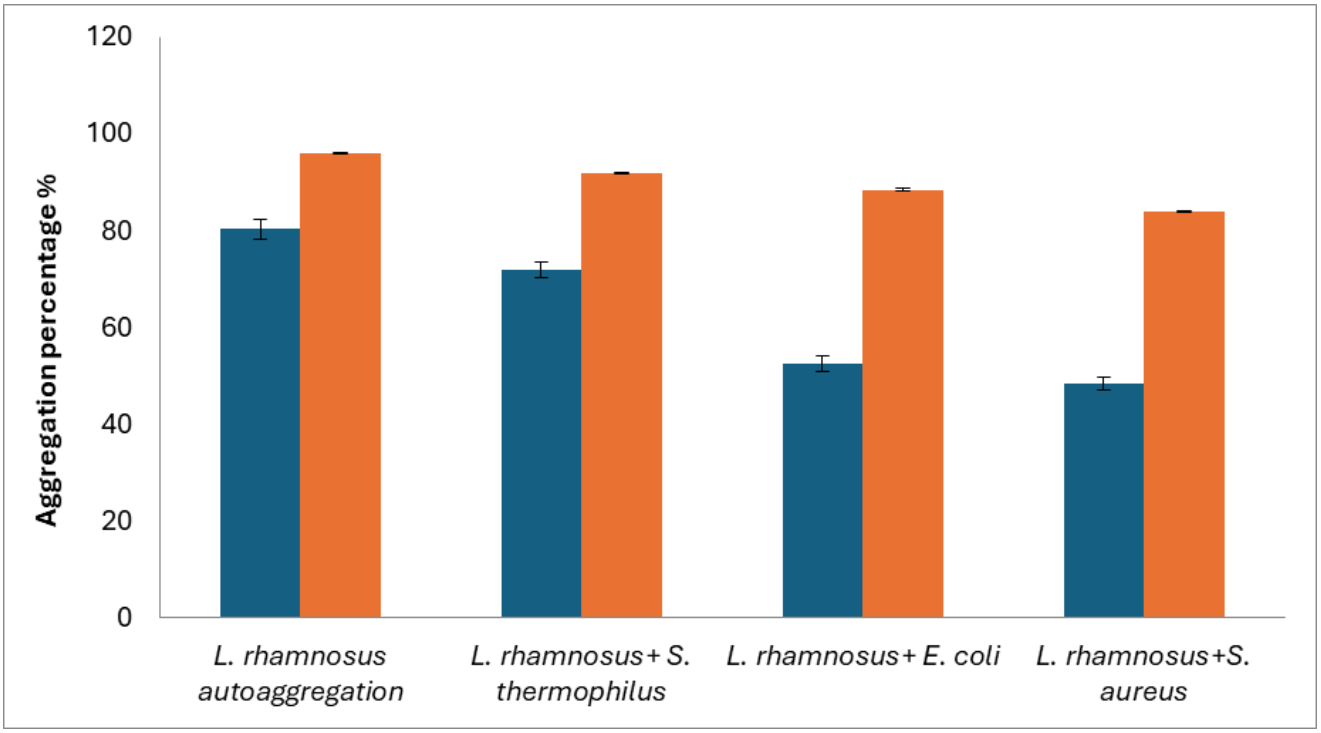

Figure 6: *Effect of flavonoid F1-A on aggregation behavior of Lactobacillus rhamnosus. Auto-aggregation and co-aggregation with Streptococcus thermophilus (S. thermophilus), Staphylococcus aureus (S. aureus), and Escherichia coli (E. coli) were measured after 24 h incubation. F1-A markedly increased both self-adhesion and pathogen coaggregation, suggesting improved probiotic adhesion and colonization potential. Results are means of two replicate ±SD.*

to 91.8 ± 0.07% with *S. thermophilus*, 48.5 ± 1.3% to 83.9 ± 0.1% with *Staph. aureus*, and 52.5 ± 1.6% to 88.5 ± 1.6% with *E, coli*. Among all derivatives, F1-A and F2-A specifically yielded the highest aggregation indices, in parallel with their superior EPS-stimulating effects (Figure 5). These findings demonstrated that flavonoids reinforce the adhesion and surface-interaction properties of *L. rhamnosus*, traits fundamental to probiotic functionality (Polak-Berecka *et al*., 2014). Enhanced aggregation likely facilitates attachment to intestinal epithelial cells, improving colonization and persistence within the gastrointestinal environment (Juraskova *et al*., 2022; Hernández-Figueroa *et al*., 2025). Such effects strengthen the rationale for incorporating flavonoid compounds as prebiotics adjuncts in probiotic formulations aimed at gut health improvement.

Flavonoids found in fruits, including caffeic acid, catechin, epicatechin, coumaric acid, phloridzin, rutin, naringenin, daidzein, genistein, quercetin, and gallic acid have demonstrated inhibitory effects on the growth and adhesion of pathogenic bacteria to human Caco-2 cells (Parkar *et al*., 2008; Wang *et al*., 2025). Additionally, they enhanced the proliferation and adhesion of *L. rhamnosus* (Jafari *et al*., 2022), consistent with the present findings. Similarly, quercetin and resveratrol positively influenced auto-aggregation of *L. plantarum* and *L. fermentum*, as well as their co-aggregation with pathogens such as *L. monocytogenes* INCQS 00266 and *E. coli* INCQS 00219 (Dos Santos *et al*., 2019; Brito Sampaio *et al*., 2022). In prior studies, gallic acid enhanced the aggregation rate (μa) of *Streptococcus thermophilus* without remarkably altering total aggregation (Badra *et al*., 2022; Tsibulskaya *et al*., 2025), highlighting that the flavonoid influence on cell-to-cell interaction is highly strain-specific and depends on structural features of both the bacterial surface and the polyphenol molecule. Altogether, these results reinforced the concept that specific flavonoids can act as molecular cues modulating bacterial adhesion and co-aggregation behavior, potentially through EPS-mediated surface modifications or hydrogen-bonding interactions with cell-wall proteins (Polak-Berecka *et al*., 2014; Wang *et al*., 2025).

Potential applied benefits for animal, poultry, and fish farming

The findings of this study demonstrating that specific synthetic flavonoids considerably enhanced both EPS production and aggregation properties of *L. rhamnosus* have immediate and promising implications for animal husbandry. The current enhanced bacterial strain represents a superior candidate for use as a direct-fed probiotic or as part of a synbiotic (*i.e*., probiotic + prebiotic) formulation in livestock, poultry, and aquaculture operations.

In poultry farming, gut health is paramount for growth performance, feed efficiency, and disease resistance (Atuahene *et al*., 2025). The flavonoid-enhanced *L. rhamnosus* producing strain could be directly applied here. Supplementing this strain into poultry feed could improve intestinal barrier function; a critical defense against pathogens and inflammation. A recent study on broiler chickens demonstrated that dietary supplementation with *L. rhamnosus* GG (LGG) enhanced villus height, increased the expression of intestinal barrier genes (*Mucin2*, *Occludin*), and reduced inflammatory markers following a lipopolysaccharide (LPS) challenge (Zhang *et al*., 2024). By using the current *L. rhamnosus* strain, possessing superior adhesion and colonization potential due to its flavonoid-induced EPS production and aggregation, these beneficial effects could be amplified. Improved colonization would lead to more stable gut microbiota, better nutrient absorption, and enhanced overall performance (Alagawany *et al*., 2021). Furthermore, *L. rhamnosus* strain demonstrated an ability to co-aggregate with several pathogens like *E. coli* and *Staph. aureus*, suggesting that it could actively reduce pathogen load in the gut; a key mechanism for natural disease prevention, as modern industry moves away from the use of antibiotic growth promoters (Alagawany *et al*., 2021).

Aquaculture faces remarkable challenges from bacterial diseases, such as those caused by *Aeromonas hydrophila (A. hydrophila*), which led to high mortality rates (Noshair *et al*., 2023). Probiotics are a critical tool for promoting growth and disease resistance in fish (Noshair *et al*., 2023). The present study provided a new strategy to engineer more effective probiotics for this aquaculture sector. A recent study on Nile tilapia (*Oreochromis niloticus*) showed that dietary *L. rhamnosus* supplementation appreciably improved growth rates, immune parameters, and survival against *A. hydrophila* challenge (Noshair *et al*., 2023). The *L. rhamnosus* strain, with its flavonoid-boosted aggregation and adhesion traits, would be ideally suited for such applications. Enhanced adhesion would

ensure better persistence in the fish's gastrointestinal tract, leading to more consistent probiotic benefits, including improved feed conversion, stress resistance, and survival rates (Sumon *et al*., 2022).

Conclusions and Recommendations

The present study demonstrated that supplementation with synthetic flavonoids positively influenced the functional performance of *L. rhamnosus*. Under optimized conditions, flavonoids treatment enhanced both exopolysaccharides production and aggregation behavior, indicating a coordinated improvement in traits that support probiotic stability and colonization. The obtained results also revealed that the biological effects of flavonoids are structure-dependent, with specific derivatives exerting stronger stimulatory impacts than others. Collectively, these outcomes suggested that synthetic flavonoids can be employed as biofunctional agents to overcome the limited polysaccharide productivity typical of lactic acid bacteria, thereby expanding their potentials for industrial and therapeutic uses. Future investigations are recommended to focus on elucidating the molecular mechanisms underlying these enhancements and characterizing the structural and bioactive features of the resulting polysaccharides.

Acknowledgements

The present work was conducted at the Laboratory of Bioconversion, Microbiological Engineering, and Food Safety. The authors would like to express their gratitude to the laboratory staff for their support.

Novelty Statement

This study presents new insights into the impacts of synthetic flavonoids on the aggregation of *L. rhamnosus* strain and its exopolysaccharide production, contributing to the understanding of their roles in probiotic functionality.

Author's Contribution

Hafidha Khadem: Conceptualization, Resource, Methodology, Validation, Formal analysis, Writing - Original Draft.
Badra Boubakeur: Conceptualization, Methodology, Resource, Writing - Reviewing and Editing.
Mounir Adnane and Boumediene Meddah: Reviewing and Editing.
Moustapha Soungalo Drabo: Investigation, Formal analysis, Reviewing and Editing.
Tir Touil Aicha: Supervision, Reviewing and Editing.

Ethical approval
No ethical approval is required.

Funding source
This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors.

Generative AI and AI-assisted technology statement
We would like to clarify that the article in question did not use artificial intelligence (AI), generative AI or AI-assisted technologies.

Conflict of interests
The authors have declared no conflicts of interest.

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