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Research Article

Keywords: Sinorhizobium meliloti, Medicago sativa, early colonization, desorption kinetics, inoculum density, hydrodynamic shear

Posted Date: November 5th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11311477/v1

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Experimental Characterization of Early Colonization Kinetics of *Rhizobium meliloti* on Alfalfa (*Medicago sativa*) roots

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Author contributions

MEM-R and EDJA performed the experimental work and microbiological assays. EDJA, JGC-M and GM contributed to data processing and interpretation. JGC-M and MAF-R designed and supervised the study. EDJA, MAF-R, JGC-M, MEM-R, EP, and AQ contributed to manuscript preparation and revision.

Abstract

Aims The establishment of nitrogen-fixing symbiosis between *Sinorhizobium meliloti* (syn. *Rhizobium meliloti*) and alfalfa (*Medicago sativa*) depends on early bacterial colonization of root surfaces. This study experimentally evaluated the kinetics of early bacterial attachment, desorption and persistence on axenic alfalfa seedlings using the wild-type strain *S. meliloti* 41.

Methods Colonization dynamics were assessed under different inoculum densities (10^4–10^6 cells mL^{-1}) and sequential washing conditions in phosphate buffer (0.05 M, pH 7.0), including continuous exposure and mechanical agitation (ten cycles), to distinguish weakly retained cells from firmly attached populations. Root-associated and released bacteria were quantified as colony-forming units (CFU) on MR agar, and functional symbiosis was verified by transferring washed seedlings to nitrogen-free Jensen's medium to monitor nodulation.

Results Colonization followed a biphasic pattern, with a rapid association phase (0–2 h) followed by stabilization (3–5 h). Bacterial retention increased with inoculum density (R^2 = 0.8587), the highest density (10^6 cells mL^{-1}) producing the greatest colonization efficiency. Sequential washing and mechanical agitation showed that approximately 93.6% of initially retained bacteria were weakly bound and readily removed, whereas a smaller resistant subpopulation remained stably attached. Longer incubation and higher plant density promoted bacterial persistence and root-surface recolonization.

Conclusions Early root colonization by *S. meliloti* is a dynamic, time- and density-dependent process involving an initial reversible retention phase followed by the establishment of a stable, nodulation-competent bacterial population, providing the biological foundation for subsequent symbiotic development.

Keywords: *Sinorhizobium meliloti; Medicago sativa; early colonization; desorption kinetics; inoculum density; hydrodynamic shear.*

Introduction

The establishment of biological nitrogen fixation through the symbiosis between rhizobial bacteria and leguminous plants is a fundamental process for agricultural sustainability and terrestrial nitrogen cycling. Among these interactions, the association between *Sinorhizobium meliloti* (syn. *Rhizobium meliloti*) and alfalfa (*Medicago sativa*) represents one of the best-characterized legume–rhizobium systems due to its agronomic importance and symbiotic efficiency (Jones et al. 2007; Poole et al. 2018).

Although later stages of symbiosis—such as root hair curling, infection thread formation, and nodule development—have been extensively investigated, the earliest physical interactions between free-living rhizobia and plant roots remain incompletely understood (Rodríguez-Navarro et al. 2007). Successful nodulation depends on a coordinated sequence of early events occurring at the root surface, including bacterial recognition, cell attachment, persistence, and the subsequent colonization of root tissues (Downie 2010). In particular, the transition from weak bacterial retention within the root-associated aqueous environment to stable root-surface attachment constitutes a critical regulatory checkpoint for successful colonization.

Root colonization is influenced by a complex interplay of biotic and abiotic factors, including bacterial population density, root surface physicochemical properties, and environmental conditions affecting bacterial persistence (Lugtenberg and Kamilova 2009; Bogino et al. 2013). Previous studies have demonstrated that rhizobial populations associated with root surfaces may undergo substantial displacement under hydrodynamic or mechanical disturbance, suggesting that only a small fraction of the initial bacterial population establishes stable attachment during the initial stages of interaction (Smit et al. 1992). This highlights the need to understand how environmental shear forces in the rhizosphere impact bacterial anchoring.

Despite advances in understanding rhizosphere colonization, quantitative experimental studies addressing the precise temporal dynamics of bacterial attachment and desorption during the first hours of root interaction remain limited. In particular, few studies have experimentally differentiated weakly retained, transient bacterial populations from firmly attached, resilient cells under controlled washing and shear conditions.

Therefore, the aim of this study was to experimentally evaluate the early interaction kinetics between *Sinorhizobium meliloti* 41 and axenic alfalfa (*Medicago sativa*) seedlings by quantifying bacterial attachment, desorption, and persistence under controlled inoculum densities and sequential washing treatments. The

study further assessed the temporal dynamics of bacterial retention on root surfaces during the initial phases of rhizosphere colonization to define the biophysical boundaries of early symbiotic establishment.

Materials and methods

Bacterial strain and culture conditions

The wild-type strain *Rhizobium meliloti* 41 (Syn. *Sinorhizobium meliloti*), characterized by a prototrophic phenotype, efficient infectivity, and semi-mucoid colony morphology, was used throughout the study. The strain was obtained from the Institut National de la Recherche Agronomique (INRA, France). Stock cultures were maintained under refrigerated conditions in TY liquid medium. For experimental use, bacterial cultures were reactivated on TY solid medium seven days prior to inoculation. Culture purity was verified by Gram staining. Subsequently, bacterial cells were transferred to fresh TY medium and incubated at 30 °C under constant agitation until reaching the logarithmic growth phase (approximately 24 h).

Preparation of bacterial inoculum

Bacterial cells in the exponential growth phase were harvested by centrifugation at 7,000 rpm for 25 min. The resulting pellet was washed and resuspended in sterile distilled water for infectivity assays. For adsorption, desorption, and washing experiments, cells were resuspended in sterile phosphate buffer (0.05 M, pH 7.0). Bacterial suspensions were adjusted to final concentrations ranging from 10^4 to 10^6 cells mL^{-1}, depending on the experimental treatment. These inoculum densities were used to evaluate the effect of bacterial concentration on early root colonization and attachment dynamics.

Plant material and seeding preparation

Axenic seedlings of alfalfa (*Medicago sativa*) were used for all experiments. Two-day-old seedlings were selected to evaluate early-stage bacterial colonization during the initial stages of root development. Prior to inoculation, seedlings were maintained under sterile conditions to minimize microbial interference and ensure controlled plant-bacteria interactions. For experimental assays, seedlings displaying uniform radicle development were selected and transferred aseptically to bacterial suspensions for subsequent colonization and desorption experiments.

Root colonization and sequential washing assay

Alfalfa (*Medicago sativa*) seedlings were immersed in bacterial suspensions containing *Rhizobium meliloti* 41 at inoculum densities ranging from 10^4 to 10^6 cells mL^{-1}, depending on the experimental treatment. Seedlings were exposed to bacterial suspensions for 4–5 h to allow early bacterial colonization processes at the root surface. Following inoculation, seedlings were transferred to sterile washing tubes containing phosphate buffer (0.05 M, pH 7.0) to evaluate bacterial attachment stability and desorption kinetics. Sequential washing experiments were conducted using 17 mL of washing medium during the first washing cycle, followed by transfer to fresh buffer (12 mL) during the second cycle.

To assess bacterial retention under different physical conditions, three experimental washing treatments were applied: (i) immediate removal after transfer (control), (ii) reduced seedling density by sequential removal of seedlings, and (iii) continuous exposure throughout the washing period. Additional mechanical agitation assays were performed to distinguish loosely attached bacterial populations from firmly adhered cells. In agitation treatments, seedling radicles were subjected to ten washing cycles in phosphate buffer before microbiological quantification.

At predefined time intervals, aliquots from the washing medium were collected to evaluate bacterial release kinetics, while retained bacterial populations associated with root surfaces were quantified by colony-forming unit (CFU) counting following incubation on MR agar at 30 °C.

Quantification of root-associated and released bacterial populations

To evaluate bacterial population dynamics during early root colonization, two complementary bacterial fractions were quantified following each washing interval: (i) bacteria firmly associated with the root surface (adhered cells) and (ii) bacteria released into the washing medium (free-living cells). Adhered bacterial cells were quantified by aseptically removing seedling radicles and directly streaking them onto MR agar plates (MR medium) to estimate colony-forming units (CFU) corresponding to bacterial populations retained at the root surface. This fraction represented bacteria capable of resisting removal under washing conditions and

maintaining root attachment.
Free-living bacterial cells were quantified by collecting aliquots from the washing medium at predefined intervals during the desorption process. Samples were serially diluted when required and plated on MR agar medium for CFU determination. Colony counts were used to estimate bacterial populations detached from the root surface and released into the surrounding aqueous phase.
To evaluate the temporal dynamics of bacterial attachment stability, microbiological measurements were performed at different incubation and washing times, allowing assessment of bacterial retention, desorption kinetics, and recolonization behavior during the early stages of *Rhizobium meliloti*–alfalfa interaction.
All microbiological plates were incubated at 30 °C, and bacterial populations were expressed as CFU per root or CFU mL^{-1}, depending on the evaluated compartment.

Desorption kinetics and mechanical stability assays

Desorption kinetics of *Rhizobium meliloti* 41 cells associated with alfalfa (*Medicago sativa*) roots were evaluated to determine the temporal stability of early bacterial attachment under aqueous and mechanical disturbance conditions.
Following bacterial inoculation, seedlings previously exposed to bacterial suspensions (10^5 cells mL^{-1}) for 4–5 h were transferred to sterile phosphate buffer (0.05 M, pH 7.0) and subjected to sequential washing treatments. The desorption process consisted of two consecutive washing stages to distinguish weakly retained bacterial populations from firmly attached cells.
During the first washing cycle, seedlings were transferred to washing tubes containing 17 mL of buffer medium. At predefined intervals, seedling radicles were sequentially removed to monitor bacterial release from the root surface into the surrounding aqueous phase. Remaining seedlings continued incubation under the same washing conditions.
During the second washing cycle, seedlings were transferred to fresh phosphate buffer (12 mL) and the washing procedure was repeated to evaluate the persistence of root-associated bacterial populations following prolonged aqueous exposure.
To discriminate reversible attachment from stable adhesion, mechanical agitation assays were performed as an additional experimental treatment. Seedling radicles were subjected to ten consecutive agitation cycles in phosphate buffer before microbiological quantification. This treatment was designed to selectively remove loosely attached bacterial populations while preserving cells exhibiting stronger attachment to the root surface.
Bacterial populations released into the washing medium and those retained on seedling radicles were quantified by colony-forming unit (CFU) counting at predefined time intervals. Desorption kinetics were evaluated by comparing changes in bacterial abundance under passive washing and mechanical agitation conditions, thereby allowing assessment of attachment stability during early rhizosphere colonization.

Statistical analysis

Experimental data were analyzed to evaluate the temporal dynamics of bacterial attachment, desorption, and persistence on alfalfa (*Medicago sativa*) root surfaces. Bacterial populations were expressed as colony-forming units (CFU) associated with seedling radicles or released into the washing medium, depending on the evaluated compartment.
To assess the relationship between inoculum density and bacterial colonization efficiency, linear regression analyses were performed between bacterial concentration (cells mL^{-1}) and the number of bacteria retained on root surfaces after incubation. The strength of association between variables was evaluated using the coefficient of determination (R^2).
Temporal changes in bacterial populations during sequential washing and mechanical agitation treatments were analyzed descriptively to characterize bacterial retention, desorption kinetics, and recolonization patterns during early rhizosphere colonization. Comparative analyses between passive washing and mechanically disturbed treatments were used to distinguish reversible attachment from stable bacterial adhesion.
All experimental measurements were obtained from independent biological replicates, and data are presented as average values obtained under each experimental condition.

Experimental design and interpretation of colonization dynamics

The experimental design was structured to evaluate the early stages of *Rhizobium meliloti* 41 colonization on alfalfa (*Medicago sativa*) root surfaces under controlled inoculation and washing conditions. Colonization dynamics were assessed as a function of bacterial inoculum density, exposure time, and resistance to aqueous and mechanical disturbance.

Three complementary biological processes were evaluated throughout the experimental period: (i) initial bacterial attachment, corresponding to loosely retained bacterial populations associated with the root aqueous interface; (ii) stable bacterial adhesion, representing bacterial cells resistant to sequential washing and mechanical disturbance; and (iii) bacterial persistence and recolonization, reflected by temporal changes in bacterial populations associated with root tissues after prolonged incubation.

To distinguish transient bacterial association from stable root colonization, sequential washing and mechanical agitation protocols were integrated into the experimental framework. Bacterial populations recovered from washing media were interpreted as weakly retained or reversibly associated cells, whereas bacterial populations remaining attached to root surfaces after prolonged washing represented stable attachment stages associated with early rhizosphere colonization.

Experimental observations were interpreted within the framework of biphasic colonization behavior, allowing discrimination between rapid initial adsorption processes and subsequent stabilization mechanisms associated with bacterial persistence at the root surface.

Effective adhesion and nodulation assay

To evaluate whether bacterial populations remaining associated with alfalfa (*Medicago sativa*) roots after washing were capable of establishing functional symbiosis, an effective adhesion assay based on root nodulation was performed.

Following the sequential washing procedure, seedling radicles previously exposed to *Rhizobium meliloti* 41 suspensions were aseptically transferred to nitrogen-free Jensen's medium and maintained under controlled growth conditions to promote nodulation.

This experimental approach was designed to distinguish bacterial populations that remained physically associated with root tissues from those capable of establishing biologically effective interactions leading to root nodule formation. The appearance of nodules was interpreted as evidence of successful bacterial persistence, stable root attachment, and activation of symbiosis-related signaling pathways.

The nodulation response was used as a biological indicator of effective bacterial colonization following sequential washing and mechanical disturbance treatments, allowing indirect estimation of the fraction of bacteria resistant to hydrodynamic removal and competent for symbiotic establishment.

Results

Colonization kinetics during early root interaction

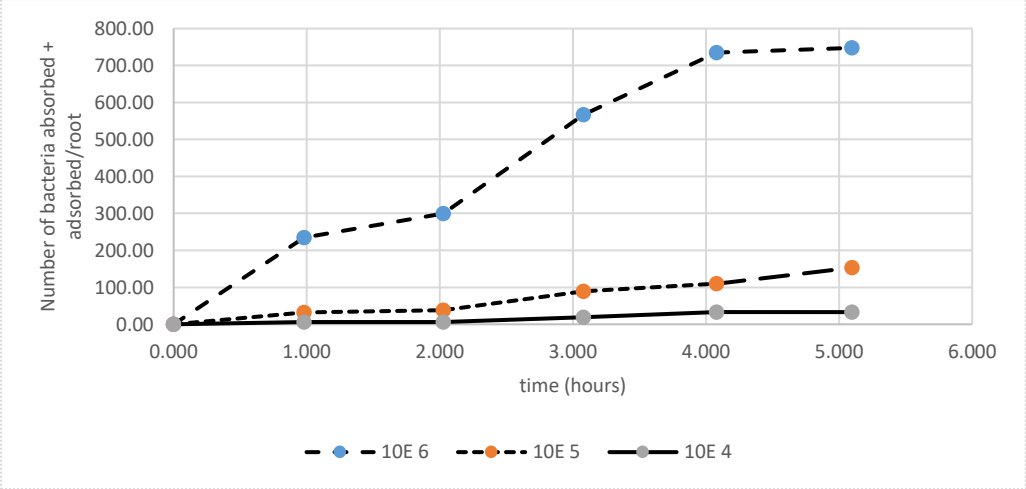


Fig. 1(A) Early Colonization kinetics of *Rhizobium meliloti* on alfalfa (*Medicago sativa*) roots at different inoculum densities.

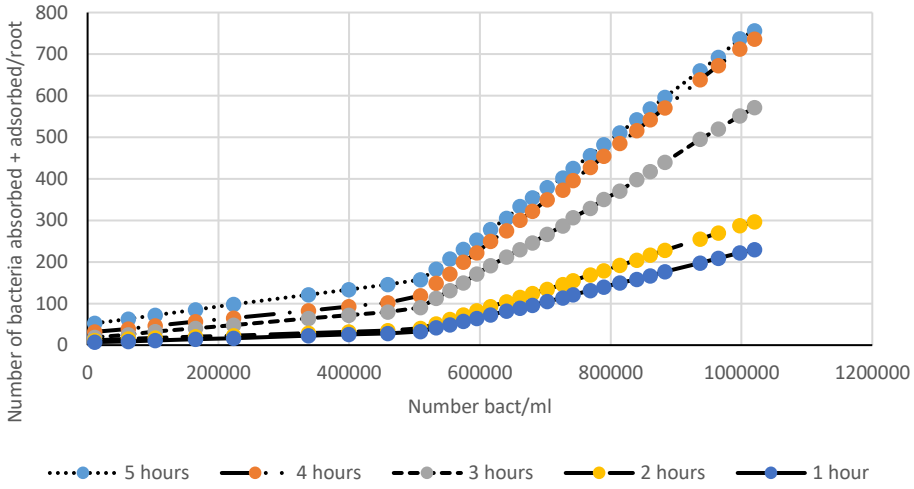


Fig. 1(B) Effect of inoculum density on early root colonization by *Rhizobium meliloti* in alfalfa (*Medicago sativa*).

Figs. 1(A) and 1(B) demonstrate that the early interaction between *Rhizobium meliloti* and alfalfa (*Medicago sativa*) roots follows a clear time- and density-dependent colonization pattern. Root-associated bacterial populations increased progressively during the first hours of incubation, indicating active bacterial attachment and stabilization at the rhizoplane (Drogue et al. 2012; Wheatley et al. 2020).

At the highest inoculum density (10^6 cells mL^{-1}), bacterial retention increased markedly between 2 and 3 h, suggesting a transition from weak, reversible attachment to a more stable colonization phase. During the initial stage (0–2 h), bacterial association appears to be dominated by physicochemical interactions, whereas prolonged incubation (3–5 h) favors stronger bacterial persistence at the root surface (Rodríguez-Navarro et al. 2007; Wheatley and Poole 2018). This behavior is consistent with previous reports describing a biphasic colonization process involving reversible adsorption followed by irreversible adhesion mediated by rhicadhesins, cellulose production, and microcolony formation (De-la-Peña and Loyola-Vargas 2014).

A strong positive relationship was observed between inoculum density and bacterial retention efficiency (Fig. 1B). Increasing bacterial concentration from 10^4 to 10^6 cells mL^{-1} resulted in a substantial increase in root-associated bacterial populations, indicating that higher inoculum densities enhance the probability of bacterial attachment and successful colonization (Beauregard et al. 2013; Weiland-Bräuer 2021).

Linear regression analysis confirmed this relationship, with bacterial retention increasing proportionally as inoculum density increased ($R^2 = 0.8587$). These findings suggest the existence of a minimum bacterial density threshold required to overcome rhizospheric displacement and establish effective early colonization. The observed trend is consistent with previous studies indicating that root exudates, particularly flavonoids such as luteolin in alfalfa, stimulate bacterial signaling pathways involved in motility, attachment, and nodulation responses (Peters et al. 1986; López-Baena et al. 2016).

At prolonged incubation times (~5 h), bacterial accumulation tended to stabilize, particularly at higher inoculum densities, suggesting progressive saturation of available root attachment sites and the establishment of a carrying capacity at the root interface.

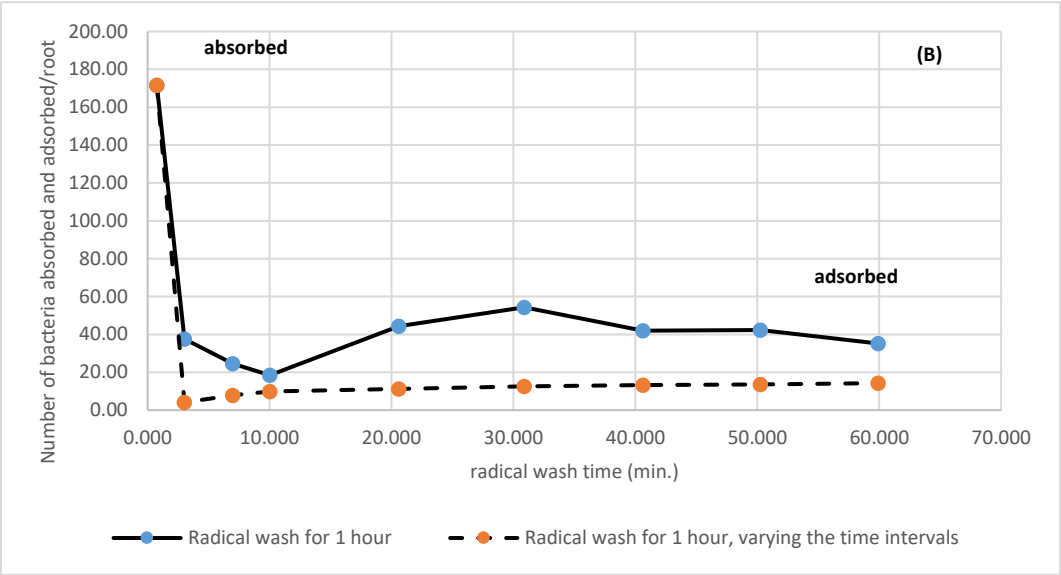


Fig. 2(A) Short-term desorption kinetics of *Rhizobium meliloti* from alfalfa (*Medicago sativa*) root surfaces during the first hour of sequential washing.

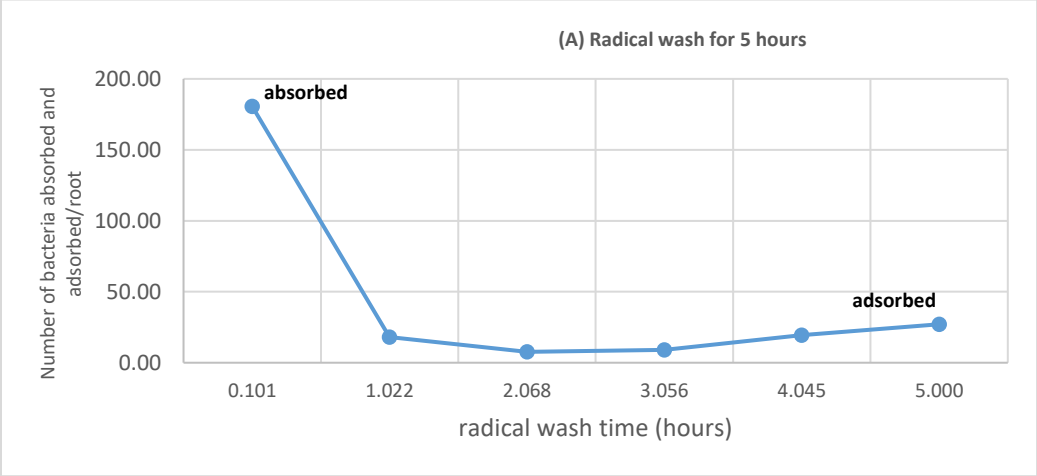


Fig. 2(B) Long-term bacterial retention and desorption dynamics of *Rhizobium meliloti* on alfalfa root surfaces during prolonged washing (5h).

Bacterial desorption and washing dynamics

Short-term bacterial release during sequential washing [Fig. 2(A)]

Fig. 2(A) revealed a rapid decrease in bacterial populations weakly associated with alfalfa root surfaces during the first hour of washing. The number of loosely retained cells sharply declined within the first minutes, indicating that a substantial proportion of bacteria remained associated with the root through weak physicochemical interactions, including van der Waals and electrostatic forces. Similar behavior has been described during early rhizosphere colonization, where an initial reversible adhesion phase precedes stable bacterial attachment (Lugtenberg and Kamilova 2009; De-la-Peña and Loyola-Vargas 2014; López-Baena et al. 2016).

Despite this rapid decline, a residual bacterial fraction remained associated with the root surface throughout the washing intervals, suggesting the existence of early anchoring mechanisms that prevent complete bacterial removal. This retained population likely represents cells transitioning from reversible adsorption toward more stable adhesion states associated with biofilm initiation and localized root colonization (Rinaudi and Giordano 2010; Bogino et al. 2013; Castiblanco and Sundin 2018; Wheatley et al. 2020).

Long-term bacterial persistence under prolonged washing [Fig. 2(B)]

Fig. 2(B) demonstrated a marked reduction in bacterial populations during prolonged washing (5 h), particularly during the initial stages of exposure. However, after the early decline, bacterial numbers

stabilized, indicating that a subset of *Rhizobium meliloti* cells remained firmly attached to the radicle surface despite continued washing.

The results suggest that the aqueous matrix surrounding the root surface functions not merely as a passive compartment, but as an active interface for nutrient exchange and molecular signaling (Hu et al. 2018). Following prolonged washing, approximately 15% of the initially associated bacterial population remained attached to root tissues, indicating the establishment of stable colonization sites.

This persistent bacterial fraction suggests the establishment of stronger adhesion mechanisms, potentially involving exopolysaccharide (EPS) production, microcolony formation, and surface-associated proteins such as rhicadhesins, which collectively enhance resistance to hydrodynamic removal and stabilize bacterial persistence at the rhizoplane (Rinaudi and Giordano 2010; Rodríguez-Navarro et al. 2011; Bogino et al. 2013; Castiblanco and Sundin 2018; Tsyganova et al. 2021).

The persistence of these firmly attached populations supports the concept of biphasic colonization, in which early reversible attachment progressively transitions into stable root colonization, a prerequisite for successful symbiotic establishment (De-la-Peña and Loyola-Vargas 2014; López-Baena et al. 2016; Tsyganova et al. 2021).

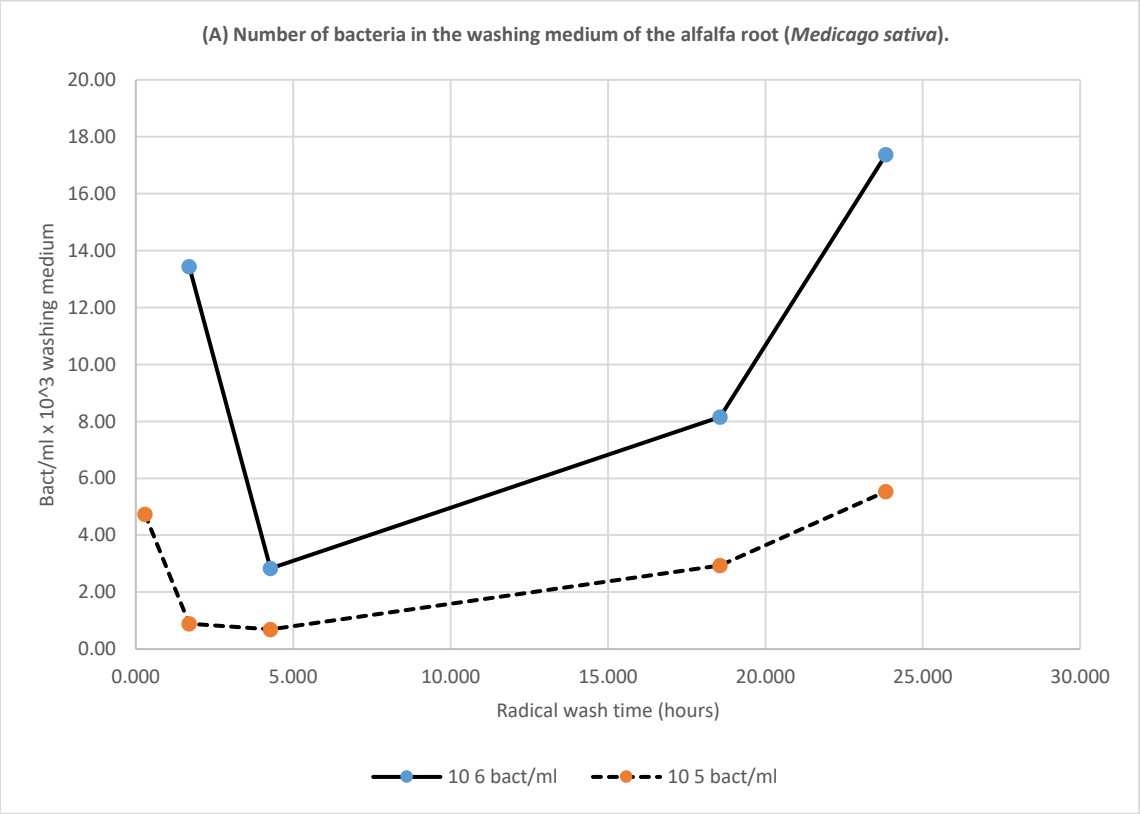


Fig. 3(A) Bacterial release kinetics into the washing medium following root colonization by *Rhizobium meliloti* at different inoculum densities.

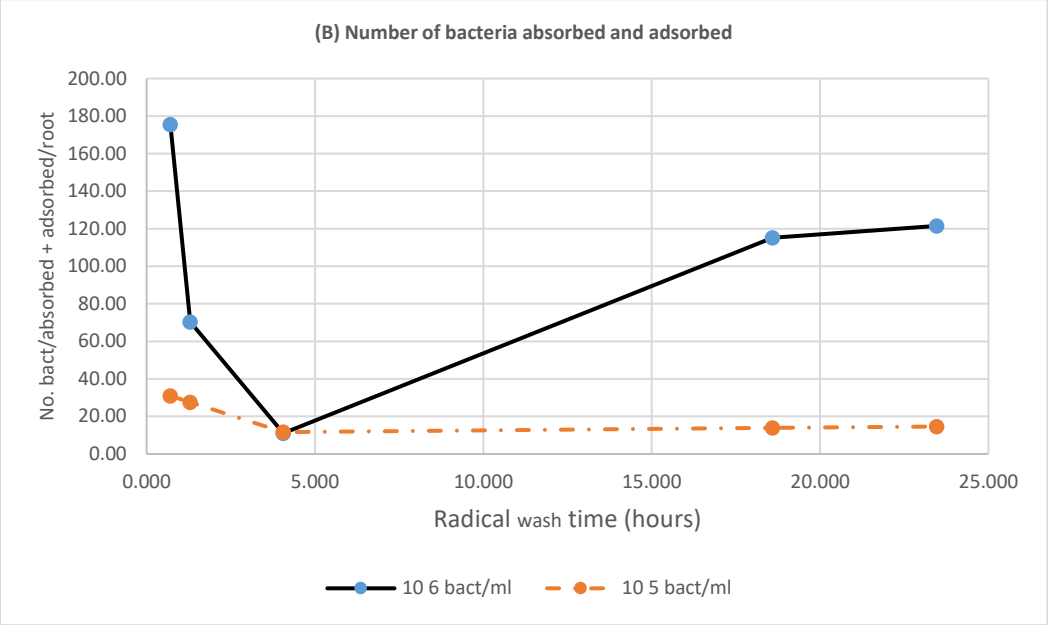


Fig. 3(B) Number of bacteria absorbed and adsorbed. Kinetics of the egress of *Rhizobium meliloti* cells absorbed into the aqueous matrix of the radical surface of the alfalfa that has been incubated with 10^6 and 10^5 cells mL^{-1} for 5 hours.

Bacterial Retention Following Prolonged Incubation and Washing

Bacterial release into the washing medium [Fig. 3(A)]

Fig. 3(A) illustrates the temporal dynamics of bacterial release into the washing medium following alfalfa root colonization by *Rhizobium meliloti* under different inoculum densities (10^5 and 10^6 cells mL^{-1}). During the initial washing intervals, bacterial concentrations in the medium sharply decreased, particularly at the higher inoculum density, indicating the rapid displacement of weakly retained bacterial populations associated with the root surface.

However, after the initial decline, bacterial concentrations progressively increased during later washing periods, particularly at 10^6 cells mL^{-1}, suggesting recolonization and stabilization processes at the root interface. This behavior indicates that higher inoculum densities favor bacterial persistence and recolonization efficiency, likely by increasing the probability of successful attachment and occupation of available root niches (De-la-Peña and Loyola-Vargas 2014; López-Baena et al. 2016).

Effect of inoculum density

A clear density-dependent response was observed between inoculum concentrations (10^5 vs 10^6 cells mL^{-1}), with higher bacterial densities consistently producing greater bacterial retention and recolonization at the root surface. These findings support the existence of a critical threshold density required for stable root colonization, where increased bacterial abundance enhances the likelihood of overcoming hydrodynamic displacement and establishing persistent attachment sites (Rinaudi and Giordano 2010; López-Baena et al. 2016).

At lower inoculum densities, bacterial populations appeared more susceptible to removal during sequential washing, suggesting limited competition for surface occupancy and reduced stabilization potential. Similar density-dependent effects have been reported in rhizosphere colonization studies, where inoculum size directly influences bacterial persistence and microcolony establishment (Castiblanco and Sundin 2018; Tsyganova et al. 2021).

Retention of adsorbed bacterial populations [Fig. 3(B)]

Fig. 3(B) demonstrates the dynamics of bacterial populations weakly retained (absorbed) and firmly attached (adsorbed) to alfalfa root surfaces following prolonged incubation and sequential washing. Although an initial rapid decline in bacterial counts was observed, a persistent fraction remained associated with the root surface throughout the washing period, particularly under higher inoculum densities.

Biological interpretation

The observed persistence of root-associated bacterial populations suggests that the root aqueous matrix functions not merely as a passive compartment but as an active biological interface supporting nutrient exchange, molecular signaling, and bacterial stabilization. The retained bacterial fraction likely reflects the emergence of stronger adhesion mechanisms involving exopolysaccharide (EPS) secretion, surface-associated adhesion proteins, and early microcolony formation, all of which increase bacterial resistance to hydrodynamic removal (Rinaudi and Giordano 2010; Bogino et al. 2013; Castiblanco and Sundin 2018; Tsyganova et al. 2021;).

This transition from weak physicochemical adsorption toward stable attachment supports the concept of biphasic colonization, in which reversible bacterial attachment gradually evolves into persistent root colonization, representing a prerequisite for successful rhizobial symbiosis (De-la-Peña and Loyola-Vargas 2014; López-Baena et al. 2016; Tsyganova et al. 2021).

Table 1. Influence of alfalfa seedling density on bacterial abundance in the washing medium during sequential washing treatments.

Treatment	Washing time (hours)	Root	Number of bacteria medium wash (bact/ml)
Without Plants	3-4 *	8 ± 4.24	471.6 ± 23.69
	1.5		192.8 ± 6.84
	4		120 ± 8.54
	19		46.6 ± 2.33
	24		33.3 ± 2.16
2 Plants	3-4 *	2 ± 0.816	67.80 ± 37.25
	1 ½		186.6 ± 7.71
	4		167.1 ± 10.54
	19		20.0 ± 1.67
	24	6.3 ± 4.28	14.0 ± 0.16
4 Plants	3-4 *		456.6 ± 23.58
	1 ½		313.3 ± 17.7
	4		128.3 ± 11.07
	19		17.5 ± 0.95
	24	3 ± 1.73	15.0 ± 0.5

* 3-4 min interval represents the approximate time required for seedling transfer during the sequential washing procedure.

Effect of plant density on bacterial persistence in the washing medium

Table 1 summarizes the effect of alfalfa (*Medicago sativa*) seedling density on bacterial abundance in the washing medium during sequential washing treatments. In the absence of plants, bacterial concentrations progressively declined over time, decreasing from 471.6 ± 23.69 cells mL^{-1} during the initial washing interval to 33.3 ± 2.16 cells mL^{-1} after 24 h. This reduction suggests gradual bacterial depletion from the aqueous medium in the absence of root-mediated stabilization processes.

In systems containing 2 and 4 seedlings, bacterial dynamics differed from the plant-free condition. Although an initial decrease in bacterial abundance was observed during early washing intervals, subsequent fluctuations indicate active bacterial persistence and recolonization processes associated with root surfaces. Treatments containing 4 seedlings maintained comparatively higher bacterial concentrations during intermediate washing periods, suggesting enhanced bacterial stabilization within the rhizosphere environment.

Effect of plant density

A clear density-dependent effect was observed, in which increasing the number of seedlings modified bacterial persistence in the washing medium. Greater root density likely increased the availability of root exudates, including soluble carbon compounds, amino acids, and signaling molecules that support bacterial metabolism and recolonization efficiency (De-la-Peña and Loyola-Vargas 2014; López-Baena et al. 2016;

Zhalnina et al. 2018).
Furthermore, increasing root surface availability may favor bacterial retention by expanding the number of potential attachment sites, thereby reducing susceptibility to hydrodynamic displacement (Rinaudi and Giordano 2010; Castiblanco and Sundin 2018;). Similar density-dependent responses have been reported in rhizosphere colonization systems, where bacterial persistence is strongly influenced by host root architecture, nutrient gradients, and microbial adaptation to rhizospheric microenvironments (Tsyganova et al. 2021).

Biological interpretation

The observed fluctuations in bacterial abundance suggest that the rhizosphere behaves as a dynamic biological interface rather than a passive aqueous compartment, where hydrated extracellular matrices and structurally organized interfaces may contribute to bacterial persistence and stabilization (Zhalnina et al. 2018; Tsyganova et al. 2021).
Root-associated bacterial populations appear to be continuously regulated through a balance between bacterial detachment, recolonization, and stabilization at the root surface. The presence of multiple seedlings may enhance bacterial persistence by increasing the spatial complexity of the rhizosphere, strengthening plant–microbe interactions, and facilitating the establishment of stable colonization sites during the early stages of symbiosis (López-Baena et al. 2016; Tsyganova et al. 2021).

Table 2. Persistence and release dynamics of root-associated *Rhizobium meliloti* populations during consecutive washing treatments.

	Time	Average number of bacteria Root	Average number of bacteria mL wash medium	No. of plants Tube	Number of bacteria Total/roots	Totals/tube Medium
First radical wash	3 - 4 *	11 ± 8.14	380 ± 132	4	44	6460 ±2254
	2 hours	7 ± 3.5	190 ± 80	4	28	3230 ± 2247
	4 hours	4 ± 2.5	110 ± 32	3	12	1870 ± 544
	26 hours	12 ± 4.3	20 ± 7.5	3	36	340 ± 127.5
Second radical wash	3 - 4 *	1 ± 0.75	8 ± 4.8	4	4	192 ± 43.6
	4 hours	2 ± 1.2	5 ± 2.9	3	6	120 ± 92
	26 hours	4 ± 3.2	3.2 ± 2	2	8	72 ± 32

* The 3-4 min interval represents the approximate time for introducing and removing four alfalfa seedlings during the sequential washing procedure.
The washing medium volume was adjusted to 17 mL during the first desorption cycle and 12 mL during the second cycle.

Sequential washing and bacterial retention dynamics

Table 2 summarizes the persistence and release dynamics of *Rhizobium meliloti* populations associated with alfalfa (*Medicago sativa*) roots during two consecutive washing cycles. Distinct bacterial retention profiles were observed between the first and second washing treatments, reflecting differences in bacterial attachment strength and resistance to hydrodynamic removal.
During the first washing cycle, bacterial abundance in the washing medium progressively decreased over time, declining from 6460 ± 2254 bact.tube^{-1} during the initial interval to 340 ± 127.5 bact.tube^{-1} after 26 h. This reduction suggests the rapid displacement of weakly retained bacterial populations associated with the root surface, corresponding primarily to reversible adsorption mediated by weak physicochemical interactions such as van der Waals and electrostatic forces (Lugtenberg and Kamilova 2009; De-la-Peña and Loyola-Vargas 2014; López-Baena et al. 2016).
In contrast, the second washing cycle showed substantially lower bacterial release into the washing medium, with bacterial abundance decreasing from 192 ± 43.6 bact.tube^{-1} to 72 ± 32 bact.tube^{-1}, indicating that most loosely retained bacterial populations had already been removed during the first desorption phase. The persistence of a residual bacterial fraction following repeated washing suggests the establishment of stronger root-associated adhesion mechanisms resistant to hydrodynamic displacement (Rinaudi and Giordano 2010;

Bogino et al. 2013; Castiblanco and Sundin 2018).

Effect of residence time

In both washing cycles, residence time in the washing medium emerged as a critical factor regulating bacterial persistence and release dynamics. Longer washing periods progressively reduced bacterial abundance in suspension, indicating continuous detachment of weakly retained cells. However, firmly attached (adsorbed) bacterial populations appeared comparatively less sensitive to residence time, maintaining detectable persistence despite prolonged washing conditions.

This behavior suggests the existence of heterogeneous bacterial populations differing in adhesion strength, where early reversible attachment gradually transitions toward more stable bacterial retention states (De-la-Peña and Loyola-Vargas 2014; López-Baena et al. 2016; Tsyganova et al. 2021).

Biological interpretation

The persistence of a reduced but stable bacterial population after consecutive washing cycles supports the hypothesis that root colonization by *Rhizobium meliloti* follows a biphasic colonization model, characterized by an initial phase of weak physicochemical adsorption followed by progressive biological stabilization at the rhizoplane (López-Baena et al. 2016; Tsyganova et al. 2021).

This transition likely involves the production of exopolysaccharides (EPS), formation of microcolonies, and the activity of surface-associated adhesion proteins such as rhicadhesins, which collectively enhance bacterial resistance to hydrodynamic stress and stabilize bacterial attachment to root tissues (Rinaudi and Giordano 2010; Bogino et al. 2013; Castiblanco and Sundin 2018; Tsyganova et al. 2021).

Furthermore, the marked reduction in bacterial release during the second washing cycle suggests that only a specialized bacterial subpopulation remains capable of maintaining stable root association, reinforcing the concept that successful rhizobial colonization depends on both physicochemical compatibility and biological adaptation to the rhizosphere microenvironment (Zhalnina et al. 2018; Tsyganova et al. 2021).

Table 3. Site Occupancy Kinetics of *Rhizobium meliloti* on Alfalfa (*Medicago sativa*) Roots under agitation and washing treatments.

Absorption and Adsorption Time	Treatment of alfalfa roots in the buffer medium prior to radical washing	Radical wash time (hours)	Number of bacteria/root (Δ)
3 – 4 *	Without agitation (a)	0	47 (Ab + Ad)
	Without agitation (a)	4	3 (Ad)
	With agitation (b)	4	0
	With agitation (b)	0	1 (Ad)
30 min	Without agitation (a)	4	7 (Ad)
	With agitation (b)	4	0
2 h	Without agitation (a)	4	9 (Ad)
	With agitation (b)	4	1 (Ad)
4 h	Without agitation (a)	0	111 (Ab + Ad)
	With agitation (b)	0	7 (Ad)
	Without agitation (a)	4	2 (Ad)
	With agitation (b)	4	0

* 3 – 4 min correspond to the time required to place four alfalfa seedlings into the washing tube and remove them.

(a) Roots were introduced into the washing medium and immediately removed.

(b) Each root was agitated 10 times in the washing medium; (Δ) Mean value of four biological replicates.

Site occupancy dynamics and effect of mechanical agitation

Table 3 summarizes the occupancy kinetics of *Rhizobium meliloti* on alfalfa (*Medicago sativa*) roots under different washing and agitation conditions. The results indicate that only a limited fraction of the bacterial population establishes stable interaction sites on the root surface, whereas most bacteria remain weakly associated and are readily displaced by mechanical disturbance.

At the earliest interaction stage (3–4 min), untreated roots exhibited the highest bacterial load, averaging 47

bacteria per root, including both absorbed and adsorbed fractions (Ab + Ad). However, following washing treatment, bacterial retention declined sharply, indicating that most cells were weakly associated with the root surface and susceptible to hydrodynamic displacement. This behavior is consistent with previous reports describing an early reversible adhesion phase dominated by weak physicochemical interactions, including electrostatic forces, van der Waals interactions, and transient rhizosphere contact (Lugtenberg and Kamilova 2009; De-la-Peña and Loyola-Vargas 2014; López-Baena et al. 2016; Tsyganova et al. 2021).

Effect of mechanical agitation

Mechanical agitation revealed that approximately 93.6% of the initially retained bacterial population was removed, demonstrating that the majority of early bacterial attachment is reversible and highly sensitive to hydrodynamic disturbance. Under agitation treatment, bacterial abundance approached negligible levels regardless of incubation time, confirming that loosely attached bacterial populations are readily displaced from the root interface.

Only a reduced bacterial fraction persisted after agitation, suggesting the existence of stronger localized adhesion mechanisms at specific colonization sites. These retained bacterial populations likely correspond to cells undergoing progressive stabilization through exopolysaccharide (EPS) production, microcolony formation, and surface-associated proteins such as rhicadhesins, all of which enhance bacterial resistance to mechanical removal and strengthen root attachment (Rinaudi and Giordano 2010; Bogino et al. 2013; Castiblanco and Sundin 2018; Tsyganova et al. 2021).

Effect of residence time and colonization site occupancy

Residence time strongly influenced bacterial retention and colonization efficiency. After 4 h of incubation, untreated roots exhibited the highest bacterial retention, reaching up to 111 bacteria per root before washing. This increase suggests progressive occupation of available colonization niches and strengthening of bacterial persistence at the root interface, consistent with the establishment of specialized bacterial attachment sites required for successful symbiotic interaction (Rinaudi and González 2009; Knief et al. 2012; Tsyganova et al. 2021).

The observed increase in bacterial retention with incubation time supports the hypothesis that root colonization is not instantaneous but rather develops progressively through repeated bacterial–root contact and localized stabilization mechanisms (De-la-Peña and Loyola-Vargas 2014; López-Baena et al. 2016; Tsyganova et al. 2021). Increased bacterial persistence may also be associated with changes in the rhizospheric microenvironment, including root exudation patterns, hydrated extracellular matrices, and localized physicochemical conditions that contribute to bacterial stabilization and nutrient exchange (Zhalnina et al. 2018; Tsyganova et al. 2021).

Biological interpretation

The results indicate a dynamic equilibrium between adsorption (firm surface adherence) and absorption (retention within the aqueous film surrounding root tissues), where interaction stability progressively increases with prolonged bacterial exposure and repeated root contact. Although most bacterial cells are rapidly displaced during early washing, a small resistant bacterial fraction persists, suggesting the establishment of stable colonization sites required for effective rhizosphere persistence (Rinaudi and Giordano 2010; De-la-Peña and Loyola-Vargas 2014; Tsyganova et al. 2021).

This transition from transient association toward stable bacterial adhesion likely represents a prerequisite for successful rhizobial symbiosis, involving coordinated physicochemical stabilization, microbial adaptation to the rhizosphere, early signaling processes associated with nodulation, colonization site specialization, and symbiotic establishment (Rinaudi and González 2009; Knief et al. 2012; Edwards et al. 2015; López-Baena et al. 2016; Tsyganova et al. 2021).

Conclusions

The early interaction between *Rhizobium meliloti* and alfalfa (*Medicago sativa*) roots follows a highly dynamic and density-dependent colonization process involving progressive stabilization of bacterial populations at the root interface. Colonization kinetics demonstrated a strong positive relationship between inoculum density and bacterial retention efficiency ($R^2 = 0.8587$), indicating that increased bacterial abundance significantly enhances the probability of successful root occupation during the early stages of interaction. These findings

support the existence of a minimum bacterial threshold required for effective colonization and stabilization at the rhizoplane.

Sequential washing experiments demonstrated that bacterial attachment occurs through a biphasic colonization process, beginning with a rapid phase of weak and reversible adsorption followed by the stabilization of a smaller bacterial fraction resistant to hydrodynamic removal. During the initial washing intervals, a pronounced clearing phase was observed, characterized by the rapid displacement of loosely retained bacterial populations associated primarily through transient physicochemical interactions, likely representing the elimination of unstable bacterial populations incapable of maintaining persistent root association.

Following the clearing phase, bacterial populations progressively stabilized, indicating the establishment of stronger attachment mechanisms. Both prolonged washing and mechanical agitation assays showed that approximately 93.6% of initially retained bacterial populations were weakly associated and readily displaced by hydrodynamic disturbance, whereas only a small resistant fraction remained attached following repeated washing and agitation. This persistent subpopulation likely represents bacterial cells undergoing progressive stabilization through localized adhesion, early microcolony establishment, and biological adaptation to root-associated conditions.

The comparison between absorbed and adsorbed bacterial fractions revealed that bacterial persistence is governed by a dynamic equilibrium between bacterial release into the aqueous root-associated matrix and stable retention at the root surface. Recolonization patterns observed during prolonged washing further suggest that bacterial persistence is continuously influenced by local rhizospheric conditions and progressive adaptation to the root-associated microenvironment.

The density of alfalfa seedlings significantly affected bacterial persistence in the washing medium, indicating that increased root density promotes bacterial stabilization through greater surface availability, enhanced root-associated biological activity, and expansion of potential colonization niches. In addition, residence time emerged as a critical factor regulating bacterial persistence, with prolonged exposure periods favoring greater bacterial retention and stabilization at the root interface.

Overall, the findings indicate that successful early colonization of alfalfa roots by *Rhizobium meliloti* depends on the progressive transition from weak and reversible surface association toward stable, root-associated bacterial persistence, establishing the biological foundation for subsequent symbiotic development.

Declaratio|ns

Funding This work was financed by the Authors.

Competing interests The Authors declare no Competing or Conflict of interests.

Ethics approval Not applicable.

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

Author contributions

MEM-R and **EDJA** performed the experimental work and microbiological assays. **EDJA, JGC-M** and **GM** contributed to data processing and interpretation. **JGC-M** and **MAF-R** designed and supervised the study. **EDJA**, **MAF-R**, **JGC-M, MEM-R, EP,** and **AQ** contributed to manuscript preparation and revision.

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