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Colonization and spreading dynamics of *Lactiplantibacillus plantarum* spoilage isolates on wet surfaces

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ABSTRACT

The role of lactic acid bacteria, including *Lactiplantibacillus plantarum,* in food spoilage is well recognized, while the behavior of these non-motile bacteria on wet surfaces, such as those encountered in food processing environments has gained relatively little attention. Here, we observed a fast colony spreading of non-motile *L. plantarum* spoilage isolates on wet surfaces via passive sliding using solid BHI agar media as a model. We investigated the effect of physical properties of agar hydrogel substrate on the surface spreading of six *L. plantarum* food isolates FBR1–6 and a model strain WCFS1, using increasing concentrations of agar from 0.25 up to 1.5% (w/v). Our results revealed that *L. plantarum* strain FBR2 spreads significantly on low agar concentration plates compared to the other strains studied here (with a factor of 50–60 folds higher surface coverage), due to the formation of very soft biofilms with high water content that can float on the surface. The fast-spreading of FBR2 colonies is accompanied by an increased number of cells, elongated cell morphology, and a higher amount of extracellular components. Our finding highlights colonization dynamics and the spreading capacity of non-motile bacteria on surfaces that are relatively wet, thereby revealing an additional hitherto unnoticed parameter for non-motile bacteria that may contribute to contamination of foods by fast surface spreading of these bacteria in food processing environments.

1. Introduction

Traditionally, bacteria were considered individual unicellular organisms primarily found in planktonic environments. However, the significance of biofilm formation on (a)biotic surfaces, where bacteria aggregate into multicellular clusters and create an extracellular matrix, has been well-recognized for many years (Guzmá N-Soto et al., 2021; Schilcher and Horswill, 2020; Verstraeten et al., 2008). Biofilms offer numerous advantages to bacteria, including improved survival, heightened resistance to antimicrobials, and enhanced nutrient intake (Hall--Stoodley et al., 2004; Harshey, 2003). Another crucial aspect of bacterial behavior is motility, which refers to the self-propelled movement of cells. Bacterial cells often have this ability, which allows them to escape unfavorable conditions and explore new resources. Motility helps bacteria find nutrients and occupy specific areas. In this way, motility can also play a role in the interaction between microorganisms and their hosts, especially during colonization or infectious processes (Palma et al., 2022). Biofilm formation and bacterial motility are both important factors influencing bacterial behavior and their ability to colonize diverse surfaces and contamination of devices with applications in medical science, biotechnology and food industry.

Bacterial motility, which enables cells to escape unfavourable conditions and explore new sources, can be classified into two primary types: active motility and passive motility. Active motility often relies on the presence of flagella or pili for self-propulsion (Wadhwa and Berg, 2022). One common active mechanism is swarming, where bacteria move across the surface using rotating flagella (Kearns, 2010). Twitching motility, characterized by the extension and retraction of type IV pili, enables bacterial cells to move in a jerky manner on surfaces (Palma et al., 2022). Gliding motility is a distinct form of surface movement that occurs along the long axis of the cell, without relying on flagella or pili. It often involves the use of focal adhesion complexes for substrate attachment (Wadhwa and Berg, 2022). Additionally, sliding motility is a passive mode of movement driven by the expansion of dividing cells and other substances, allowing bacteria to spread across surfaces (Kearns, 2010; Palma et al., 2022; Wadhwa and Berg, 2022). The sliding

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movement, characterized by the outward expansion of bacterial colonies facilitated by surfactant production and bacterial growth (Pollitt and Diggle, 2017), has been observed in various motile and non-motile bacterial species. For example, double mutants of *Pseudomonas aeruginosa* AO1 expressing neither flagella nor type IV pili, display sliding motility in the presence of specific carbon and nitrogen source (Murray and Kazmierczak, 2008) or a *fleQ* mutant of *Pseudomonas syringae* spread over semi-solid agar substrate despite lacking flagella (Nogales et al., 2015). Spreading of Bacillus *subtilis* colonies on agar substrate is another example that relies on the production of an extracellular matrix. This polymeric matrix, often referred to as extracellular polymeric substances (EPS), can facilitate surface spreading by means of an osmotic pressure difference with the underlying agar substrate (Seminara Agnese et al., 2012) which in turn results in an aqueous nutrient flow towards the colony, causing it to further swell and expand (Yan et al., 2017).

It is known that bacteria can sense and respond to changes in the physical properties of their environment, such as viscosity, stiffness, and mechanical stress induced by flow (Doostmohammadi et al., 2016; Harshey, 2003b; Little et al., 2019a; Patteson et al., 2018; Rashtchi et al., 2022). Thus, colonization dynamics and spreading mechanism of bacteria do not only depend on the motility of the species and/or on the biofilm-induced aqueous nutrient flow by means of the biofilm matrix, but also can be significantly affected by physical properties of the underlying substrate, such as its stiffness and porosity. Often, the porosity and stiffness of soft hydrogels are interdependent; changing one will affect the other. For example, for agar hydrogel substrates, on which biofilm spreading is vastly studied, increasing agar concentration results in increasing the stiffness of the hydrogel and reduction of the pore size (Kalai Chelvam et al., 2014; Little et al., 2019a; Yan et al., 2014, 2017), while the stiffness and pore size can influence colonization dynamics in different ways. It is important to identify which of the above-mentioned effects are important in our case.

Lactiplantibacillus spp. are non-motile gram-positive, non-spore-forming, rod-shaped bacteria that have been extensively studied due to their beneficial role in industrial fermentations and their impact on food quality causing spoilage of a wide range of products including beer, ketchup, salad dressings, and sliced meat (Kubota et al., 2008; Sanders et al., 2015). Although a number of studies addressed biofilm formation of selected species in static and under flow conditions (Akoğlu, 2020; Fernández Ramírez et al., 2015, 2017, 2018; Rashtchi et al., 2022; Van der Veen and Abee, 2011), their surface spreading including sliding motility and the effect of agar plate properties has not been studied. Notably, insight into the possible surface-spreading behavior of *L. plantarum* may contribute to a further understanding of its transmission in food processing environments and recontamination efficacy and spoilage of foods. Therefore, in this study, we investigate the surface spreading behavior of six *L. plantarum* food isolates, FBR1–6 (Sanders et al., 2015), and the extensively studied model strain *L. plantarum* WCFS1, on agar medium with varying agar concentrations. Specifically, in the first step we utilized Brain Heart Infusion (BHI) medium supplemented with 0.005% manganese sulphate and 2% glucose (BHIGMn) with agar concentrations of 0.25%, 0.5%, 0.75%, 1%, and 1.5% (w/v) to examine the effect of substrate properties on colony spreading. Previous studies showed diversity in static and flow biofilm formation of these strains, including CV-stained total biofilm formation, biofilm cell counts and the presence of putative eDNA and proteinaceous matrix components (Rashtchi et al., 2022). We observed that the FBR2 strain exhibited significant spreading capacity on agar plates with lower agar concentrations, while the other strains did not demonstrate any notable colony spreading. Building on these initial findings, our subsequent investigations focused on understanding the mechanism of colony spreading, with a specific emphasis on the FBR2 strain in comparison to the reference strain, WCFS1. To gain further insights into the process of colony spreading, we employed several techniques, including advanced microscopy and rheology measurements, and investigated cell aggregation and production of EPS. Obtained insights on surface-spreading behavior and associated factors influencing colony spreading in non-motile bacteria may provide leads for reducing bacterial contamination via surface colonization in diverse industrial and clinical settings.

2. Material and methods

2.1. Bacterial strains, media, and growth conditions

In this investigation, six food spoilage-causing *L. plantarum* strains (*L. plantarum* FBR1–FBR6) were employed (Sanders et al., 2015) using *L. plantarum* WCFS1 as a reference (Kleerebezem et al., 2013; Fernández Ramírez et al., 2015). An overnight culture (OC) of these strains was prepared by inoculating cells from a −80 °C stock in 10 ml of Man-Rogosa-Sharpe (MRS, Merck) broth and incubated for 18 h at 30 °C. For the spreading assay, brain heart infusion (Becton, Dickinson, France) supplemented with 2% glucose and 0.005% manganese sulfate (BHIGMn) was used (Fernández Ramírez et al., 2015).

2.2. Sliding-motility assay

Autoclaved brain heart infusion (BHI) with different agar concentrations that were supplemented with 2% glucose and 0.005% manganese sulfate (BHIGMn) was poured into plates (diameter, 12 cm). Plates were dried in a safety cabinet for 20 minutes before inoculation with bacteria. In the center of the plates, 5 μl of OD_{600} of *L. plantarum* strains were spotted. After inoculation, the plates were dried in a safety cabinet for 15 minutes and incubated at 30 °C for 24 and 48 hours in a closed container with wet tissue (to avoid evaporation).

To prepare pH indicator on agar plates, a 0.015% phenol red solution was added to the BHIGMn medium, and the plates were prepared as described before.

2.3. Viable cell counting

The number of culturable cells in WCFS1 and FBR2 colonies which were grown on 0.25% and 1.5% agar plates after 24 and 48 hours was measured using plate counting. The entire colony on the plate was carefully removed and suspended in 20 ml of phosphate-buffered saline (PBS; pH 7.4 (Merck), NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 1.44 g/l, KH_2PO_4 0.24 g/l). The serial dilution of cells was prepared in Peptone Physiological Salt Solution (PPS) and plated on MRS agar and CFUs were determined after 48 hours incubated at 30 °C.

2.4. Cells aggregation assay

L. plantarum WCFS1 and FBR2 were inoculated in BHIGMn broth from −80 °C stock and incubated for 18 h at 30 °C. For the experiment bacterial cells were collected by centrifuging at 8000 g for 5 min (Eppendorf Centrifuge, 5804 R), and resuspended in 10 ml PBS tubes with an OD_{600} of 0.3–0.4. The tubes were placed at 30 °C. The optical density at 600 nm (OD_{600}) (OD final) was measured after 24 and 48 hours. The formula used to get the auto-aggregation was: $100 \times [1 - (OD_{final}/OD_{initial})]$.

In this assay, the final optical density (OD) measurement is affected by the sedimentation of cells, particularly when they form aggregates. Cells with a higher degree of aggregation tend to experience more rapid and extensive sedimentation, leading to a significant decrease in the final OD measurement.

2.5. Cell surface hydrophobicity

Bacterial hydrophobicity was evaluated by measuring microbial cells' attachment to n-hexadecane (MATH) as a solvent, based on Mattos et al. (Luiza Mattos-Guaraldi et al., 1999), with slight modification. An overnight culture (OC) of both strains was prepared by inoculating cells from a −80 °C stock in 10 ml of BHIMnG broth and incubated for 18 h at

30 °C. Bacterial cells were obtained by centrifuging at 8000 g for 5 min (Eppendorf Centrifuge, 5804 R), resuspended in PBS at a final OD_{600} of 0.3–0.4, and then combined with 1 ml of n-hexadecane in a volume of 4 ml. This mixture was then vortexed for 1 min. The phase separation took place when the mixture was left at room temperature for 30 minutes. The optical density of the aqueous phase was measured at 600 nm. Distribution in water versus solvent is given in percentage based on the following equation: [OD_{600}(initial bacterial suspension) - OD_{600}(aqueous phase) /OD_{600}(original bacterial suspension)]*100. In this procedure, the more hydrophobic cells will move to the hexadecane phase which causes a lowering of OD in the water phase.

2.6. Live/dead cells fluorescent imaging

Live/Dead cell tests were performed using two DNA-binding fluorophores, membrane-permeant SYTO9 and non-membrane permeant propidium iodide (PI). The latter nucleic acid stain emits red fluorescence after binding to DNA so only dead cells with ruptured membranes are stained, while SYTO9 enters both live and dead bacterial cells, emitting green fluorescence following binding to DNA. Undiluted samples from the center and edge of colonies that formed on 0.25% and 1.5% agar plates after 48 hours were combined with 3 μl SYTO9 (0.0334 M) and 3 μl PI (0.2 M) to prepare a sample for microscopy analysis. The excitation/emission maxima of these dyes are ~480/500 nm and ~490/635 nm for SYTO 9 and PI, respectively. Fluorescent microscopic images were made using an axioskope epifluorescence microscope equipped with a 50 W mercury lamp, carboy fluorescein (cF), and diacetate (cFDA) filter set (excitation wavelength 450–490 nm, emission wavelength >500 nm), an x100 Plan-Neofluar objective lens, and a camera (Carl Zeiss, Germany).

2.7. Scanning electron microscopy (SEM)

For the Scanning Electron Microscopy (SEM) images, the bacterial colonies were grown as described above in Section 2.2. After growing the colonies carefully sampled biofilm material was placed on poly-L-lysine coated coverslips (Corning^{R}) for 1 hour to immobilize the samples on the surface. The glass slides with the attached cells were put in 2.5% glutaraldehyde in phosphate buffer pH 7.2 at room temperature for one hour, rinsed 3 times in the same buffer and postfixed for 1 hour in 1% OsO4 (w/v) in the same buffer. Hereafter they were washed with water twice, dehydrated in a graded ethanol series (10, 30, 50, 70, 90, 100, 100%) and were critical point dried with carbon dioxide (Leica EM CPD 300). The glasses were attached on a sample holder by carbon adhesive tabs (EMS Washington USA), sputter coated with tungsten (Leica EM SCD 500) and imaged with a field emission scanning electron microscope (FEI Magellan 400).

2.8. Extraction and characterization of EPS

L. plantarum WCSF1 and FBR2 were inoculated on agar plates with 0.25% (w/v) and 1.5% (w/v) agar concentrations, as described in Section 2.2. The colonies grown on the 0.25% (w/v) agar plate displayed a very soft and liquid-like texture, making it difficult to completely scrape them off the agar surface, therefore, they were first dissolved in PBS. The PBS was gently added onto the agar plate, ensuring that it covered the bacterial colony and it was left to interact with the colony for 5 minutes, promoting dissolution. Next, using an inoculation loop, the colony completely was dissolved in PBS without damaging the agar. Finally, the dissolved colony was collected by pipetting the suspension from the agar plate into the 50 ml tube. The collected colony was then centrifuged at 8000 g for 20 minutes, and the supernatant was separated for subsequent dialysis and quantification of loosely bound EPS, following the method reported by Felz et al. (Felz et al., 2019). The resulting pellet was used for extracting the EPS using the cation exchange resin method. To measure biofilms' mineral and organic content total (TS) and volatile solids (VS) were measured according to APHA standard method Nr. 2540E. To extract EPS from the biofilm pellet, a slightly modified method of Frolund et al. (1996) was followed (Frolund et al., 1996). The pellet was mixed with 30 ml of PBS and 20 g of cation exchange resin (DOWEX 50×8, 20–50 mesh, FLuka) in an Erlenmeyer flask. The mixture was stirred at 250 rpm for 2 hours at 4 °C and then centrifuged at 8000 g for 20 minutes at 4 °C. The resulting liquid phase was collected as the extracted EPS solution. The extracted EPS solution and loosely bound EPS which was in the supernatant from the first step were dialyzed against demi water for 24 hours at room temperature using a SnakeSkin™ with a 3.5 K MWCO. To quantify mineral and organic fraction of the extracted EPS, approximately 1/3 of the dialyzed sample was used for VS/TS measurements. To correct for any soluble organics originating from the agar plates, the clean agar plates were treated in the same way as colony samples.

Sugar and protein content of EPS were characterized with colorimetric methods. The protein content was estimated by measuring the total nitrogen (TN) content of EPS with LCK314, LCK514 and LCK1414 kits (HACH GMBH, Germany) and the TN values were multiplied by a factor of 6.25. The phenol–sulfuric acid method was used to quantify polysaccharides as glucose equivalents (Mohr and Wolfbeis, 1994).

2.9. Confocal microscopy for visualization of EPS

Confocal microscopy was employed to visualize biofilm formed by *L. plantarum* WCSF1 and FBR2 on 0.25% and 1.5% (w/v) agar medium. Protein and polysaccharide distribution within the biofilm formed by WCFS1 and FBR2 were assessed using two fluorescent dyes, FITC (Fluorescein isothiocyanate) and Calcofluor White respectively. Undiluted samples were taken from the formed biofilm on 0.25% (w/v) and 1.5% (w/v) agar plates for 48 hours and were combined with a 5 μl mix of Calcofluor White (0.1% (w/v)) and FITC (0.01% (w/v)) to prepare a sample for confocal microscopy, using 408 nm and 490 nm filters for visualizing Calcofluor White and FITC fluorescence, respectively.

2.10. Rheology measurements

Because of the nonuniform thickness of the colonies and to prevent "slip" of the biofilm on the agar surface, we chose to conduct the rheology measurements by using plate-plate geometry with biofilms that were carefully collected by gently scraping them off the agar plates. During this process, minimal pressure was applied to the agar surface to prevent damage to the agar that could contaminate the biofilm samples. The samples were placed on rheometer's plate as described previously (Jana et al., 2020). Rheology measurements were performed on an MCR 502 Rheometer (Anton Paar, Graz, Austria) equipped with 25 mm sand-blasted plates. Standard frequency and amplitude sweep tests were conducted on the samples to comprehensively analyze the material characteristics and determine the linear viscoelastic regime. The rheological properties of agar substrates with different concentrations of agar were also measured using the same method.

3. Results

3.1. Colony spreading on the agar plate

To assess the spreading capacity of *L. plantarum* WCFS1 and the FBR1–6 food spoilage strains, 5 μl containing ≈ 7 $\log_{10}$CFUs of overnight culture were applied onto BHIGMn media with varying agar concentrations (0.25%, 0.5%, 0.7%, 1%, 1.5% (w/v)) and incubated at 30 °C for 24 and 48 hours. The colony area which was measured by ImageJ software after 24 and 48 hours of incubation is presented in S.1 (Supplementary Information, SI) and Fig. 1, respectively. The data indicates that the colony area increased over time for all strains, with FBR2 exhibiting the most significant increase. Furthermore, colony size was influenced by the agar concentration in most strains, with larger

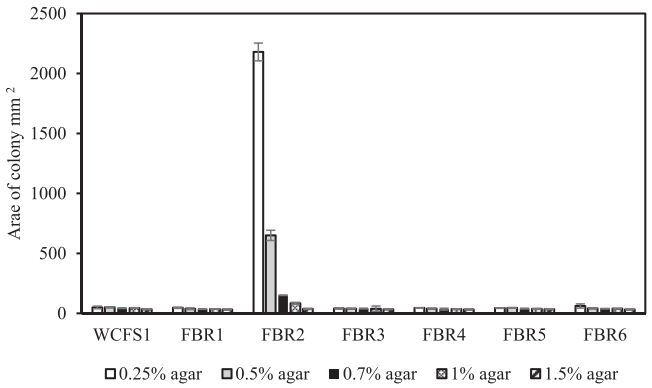

Fig. 1. The area of colony formed by *L. plantarum* WCFS1 and FBR1-FBR6 on varying agar concentrations (0.25%, 0.5%, 0.7%, 1%, 1.5%(w/v)) after 48 h incubation at 30 °C.

colonies forming on lower agar concentrations. Notably, this dependency was most pronounced in FBR2. Overall, FBR2 displayed the largest colony size compared to other strains across all agar concentrations. On 0.25% (w/v) agar concentration FBR2 formed colonies with colony surface areas of 300 and 2180 mm^2 after 24 and 48 h incubation time respectively. The latter was approximately 60-fold higher than that on 1.5% (w/v) agar plates after 48 hours.

Based on the above results, FBR2 shows a unique behaviour in spreading over agar plate surfaces with respect to the other strains. Therefore, in the rest of the paper, we focus on FBR2. In addition, to further investigate the colony formation of FBR2, we make a comparison with the reference strain WCFS1 on agar plates with extreme concentrations (0.25% and 1.5% (w/v) agar medium). Fig. 2 shows the colonies formed by both strains on plates containing 0.25% and 1.5% agar. As previously mentioned, it is evident that FBR2 produced a much larger colony on the 0.25% agar plate compared to the reference strain WCFS1. Moreover, the colony size of FBR2 exhibited noticeable expansion throughout the duration of the incubation period (Shown in S. 2, SI). To qualitatively monitor the pH change in the medium and the growth of the bacteria, we used phenol red as a pH indicator which gives a yellow colour in acidic pH. The bottom row of Fig. 2 shows that by growing *L. plantarum* in BHIGMn, the environment becomes more acidic due to bacterial metabolism, which results in yellow-coloured regions around the colonies.

3.2. Number of culturable cells in a colony

To investigate the composition of the formed biofilms on different substrates we first measured the number of culturable cells. Fig. 3 illustrates the relation between the agar concentration, incubation time,

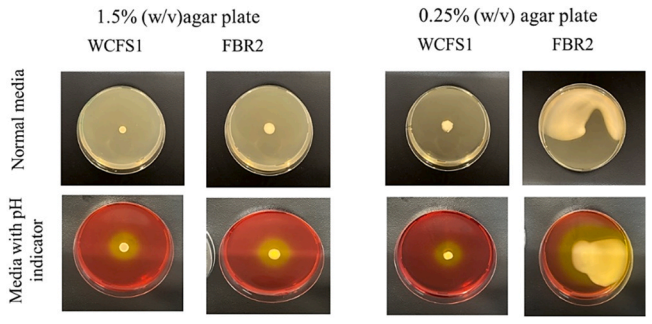

Fig. 2. Colony formation by *L. plantarum* WCFS1 and FBR2 on 1.5% (left) and 0.25% (W/V) agar plate (right) after 48 h incubation at 30 °C. The first and second rows show images of colonies on BHIGMn with phenol red, added as pH indicator.

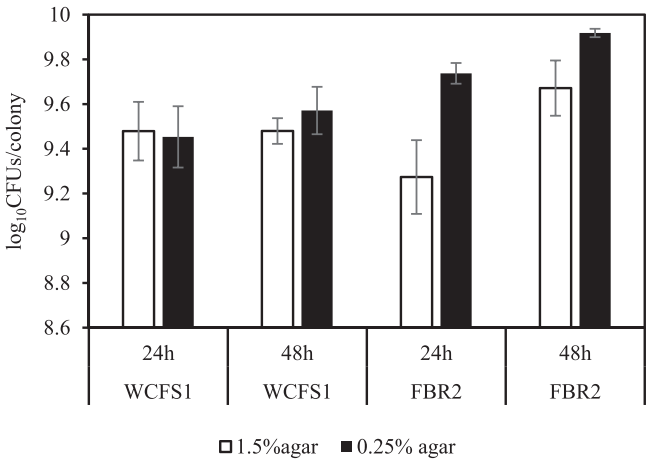

Fig. 3. Number of culturable cells in the formed colonies by *L. plantarum* WCFS1 and FBR2 on 0.25% (w/v) and 1.5% (w/v) agar plates after 24 and 48 h incubation time at 30 °C.

and the number of culturable cells per colony for *L. plantarum* WCFS1 and FBR2. Initially, both strains had approximately 7.3 $\log_{10}$ CFU after spotting. The data presented for WCFS1 indicates no significant difference in the number of cells per colony between the two types of agar plates and incubation times. However, in the case of FBR2, the number of culturable cells per colony was considerably higher on the plate with 0.25% (w/v) agar, and this number further increased with longer incubation periods. The colony formed by FBR2 on the low concentration (0.25% (w/v)) agar plate displayed an approximately 1.7-fold higher count of culturable cells compared to the FBR2 colony on the high concentration (1.5% (w/v)) agar plate after 48 hours incubation time.

3.3. Fluorescent images of biofilms

Besides measuring the culturable cells, fluorescence microscopy was employed to visualize live/dead cells in the colonies of WCFS1 and FBR2 on agar plates with concentrations of 0.25% (w/v) and 1.5% (w/v). The biofilm was stained with Syto9 to identify living cells and propidium iodide (PI), which binds to intracellular DNA in cells with compromised membranes (dead cells). Samples were collected from both the edge and centre of the colonies. As it is evident from Fig. 4, there were no considerable variations in the ratio of dead to alive cells among different WCFS1 samples, indicating a consistent cell distribution. In contrast, FBR2 exhibited a significantly higher ratio of dead cells to alive cells compared to WCFS1. Based on our selected samples of FBR2, the edge of the colonies displayed a higher dead-to-alive cell ratio compared to the centre. To this statement, there is one caveat: due to the limited sampling area, the selected samples may not provide a comprehensive representation of the overall number of living cells.

3.4. Hydrophobicity and auto-aggregation of cells

The hydrophobicity and auto-aggregation capacity of cells can affect spreading dynamics on the surface. WCFS1 exhibited a significantly higher drop in OD at both 24 and 48 h, compared to FBR2, which results in higher values of cell auto aggregation (Table 1). WCFS1 cells also showed higher hydrophobicity compared to FBR2.

3.5. Scanning electron microscopy (SEM)

The morphology of the cells in the WCFS1 and FBR2 colonies formed on 0.25% (w/v) and 1.5% (w/v) agar plates was also examined using SEM microscopy to see how the cell morphology may change under different conditions. Samples were collected from both the edge and

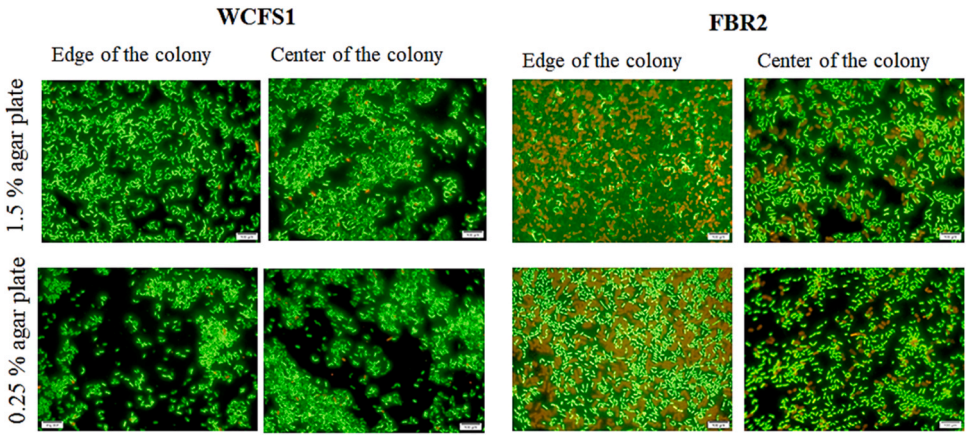

Fig. 4. Fluorescent images of *L. plantarum* WCFS1 and FBR2 samples of biofilms on 0.25% (w/v) and 1.5% (w/v) agar plate after 48 h incubation time at 30 °C. Cells were stained with LIVE/DEAD BacLight viability kit. Green and red colors represent alive and dead cells respectively. The scale bar is 100 μm.

Table 1
Cell hydrophobicity and auto-aggregation of *L. plantarum* WCFS1 and FBR2 in BHIGMn broth media at 30 °C incubation.

Strain	Cell auto-aggregation		Cell hydrophobicity
	24 h	48 h	
WCFS1	31±1%	57±1%	29±8%
FBR2	8±3%	27±3%	8±3%

centre of the colonies. Additionally, the cells in the broth culture media were visualized for comparison. In Fig. 5, SEM images of different samples were shown which indicates that the number of cells per area in WCFS1 samples was higher compared to FBR2. In addition, the morphology of cells can be compared. Based on the SEM images in Fig. 5 (A) size of WCFS1 cells was consistent across all samples; therefore, the strain's morphology remained almost unchanged when transferred from broth media to solid media. Moreover, different agar concentrations had no discernible effect on the WCFS1 cell size. Interestingly, the samples taken from the centre of the colony exhibited a greater amount of matrix surrounding the cells compared to the samples from the edges. In contrast, Fig. 5(B) demonstrates that FBR2 cells could experience a change in their length upon transitioning from broth media to solid media. When grown on a 1.5% (w/v) agar plate, both at the centre and the edge of the colony the cells showed a slight elongation with respect to the cells in the broth medium. However, on a 0.25% (w/v) agar plate, only the cells at the edge of the colony were slightly longer, and the ones in the centre did not exhibit any considerable change in their morphology with respect to that of the broth cells. We again note, as a word of caution, that due to the limited sampling, the selected samples may not provide a comprehensive representation of the overall system. In addition, it is noted that an increase in the agar concentration from 0.25% (w/v) to 1.5% (w/v) resulted in a larger population of cells in general.

3.6. EPS Extraction and composition

To investigate the chemical composition of the formed biofilms under different conditions, we first measured the water content and then extracted and characterized the EPS. The data presented in Table 2 illustrates the water content of WCFS1 and FBR2 biofilms formed on 0.25% (w/v) and 1.5% (w/v) agar. It is evident that the biofilms of both strains exhibit higher water content on the lower agar concentration as expected. Specifically, FBR2 on the 0.25% (w/v) agar plate by 93% shows the highest water content, while WCFS1 on the 1.5% (w/v) agar plate by 78% exhibits the lowest water content.

The amount of EPS in the colonies created by WCFS1 and FBR2 on 0.25% (w/v) and 1.5% (w/v) agar was measured for further analysis. Fig. 6(A), shows the amount of dry matter extracted from each colony. These data indicate that the amount of EPS extracted from colonies was higher for colonies formed on 0.25% (w/v) agar plate in both strains. Moreover, colonies formed by FBR2 on 0.25% (w/v) agar plate, produced much more EPS compared to WCFS1. This is consistent with a higher amount of colony formed by FBR2 on 0.25% (w/v) agar. However, the total amount of EPS extracted from the colony formed on a 1.5% (w/v) agar plate was comparable in both strains. A comprehensive analysis of the extracted materials revealed a higher proportion of protein content compared to polysaccharides in all samples. The polysaccharide extracted from the WCSF1 colony on both agar plates and FBR2 on the 1.5% (w/v) agar plate was close to zero. It is worth mentioning that the data presented in Fig. 6 is the amount of material produced in one colony after incubation time. Although the total EPS production is significantly higher in FBR2 colonies on low agar concentration substrates, due to their very high water content the concentration of EPS is not necessarily higher than the other biofilms. Fraction of total EPS extracted from dried colonies is also calculated and presented in S. 3 (Supplemental data). In addition, confocal images of the colony composition for biofilms formed by WCFS1 and FBR2 on both agar plates are shown in S. 4. in the supplemental data.

3.6.1. Rheological properties of L. plantarum WCFS1 and FBR2 Colonies on 0.25 and 1.5% (w/v) Agar Plates

To characterize the mechanical properties of the biofilm formed by *L. plantarum* WCFS1 and FBR2 on 0.25% and 1.5% agar concentration plates, we measured the storage (shear) modulus (G′), which quantifies the resistance to shear deformations, and the viscous loss (shear) modulus (G″) of the biofilms. The former relates to elastic energy stored upon shear deformation, while the second relates to energy lost during shear. The shear sweep tests were carried out at a deformation oscillation frequency of 1 Hz, for strain amplitudes ranging from 0.1% to 100%. Fig. 7 shows the G' and G'' of the colonies as a function of shear amplitude for both strains on different agar plates. It illustrates that the linear viscoelastic regime, indicated by the plateau part of G′ data in the amplitude sweep plot, varies between the two strains. WCFS1 forms significantly stiffer biofilms on both substrates with respect to FBR2, by approximately one order of magnitude difference. Besides that, comparing 0.25% agar with 1.5% (w/v) agar, the stiffness of the grown biofilms differs by two orders of magnitudes, for both the WCFS1 and FBR2 strains. The biofilm formed by FBR2 on 0.25% (w/v) agar substrates has very low elastic and loss moduli of a few Pa, which is a signature of a very soft viscoelastic layer capable of flowing under its own weight. Beyond the plateau part of the G' plot (i.e. the so-called linear viscoelastic regime), the elastic moduli of all biofilms

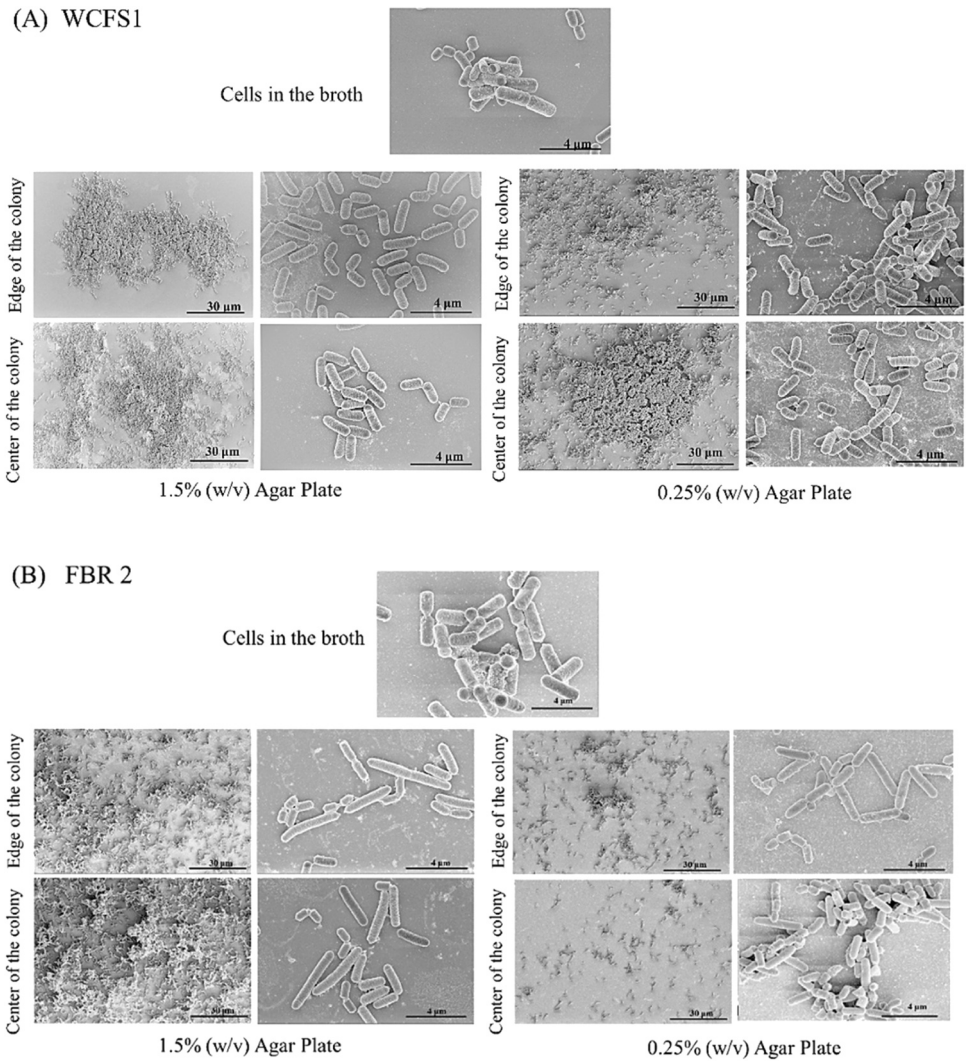

Fig. 5. SEM images of *L. plantarum* WCFS1(A) and FBR2 (B). Images of planktonic cells in the broth media (top) and cells in the formed colony on 0.25% (w/v) and 1.5% (w/v) agar plate after 48 hours incubation time at 30 °C. Images of samples from the centre and edges of the colony are shown separately.

Table 2
Water content (weight percentage) *of L. plantarum* WCFS1 and FBR2 formed colonies on 0.25% (w/v) and 1.5% (w/v) agar plate after 48 h incubation time at 30 °C.

Strain	Agar concentration of plate (w/v)	Water content of biofilm (%)
WCFS1	1.5%	78± 3
WCFS1	0.25%	86 ±2
FBR2	1.5%	86 ±3
FBR2	0.25%	93 ±1

continuously decreased by increasing strain amplitudes, indicating a strain-softening behaviour as we expect from typical hydrogels. In summary, the results indicate that both strains formed softer biofilms on low agar concentrations compared to high agar concentrations, with WCFS1 forming a stiffer biofilm than FBR2. More rheological characterization of both biofilms, including frequency sweep data is shown in the S. 5 (SI).

Rheological data of agar substrates indicate that the stiffness of the agar increases considerably (G' increases from about 100 Pa to 10000 Pa) by increasing the agar concentration from 0.25% (w/v) to 1.5% (w/v) (S. 6, SI).

4. Discussion

Recent studies proved that bacteria are able to sense the mechanical properties of their environment by translating mechanical signals of the environment into biochemical signals via mechanosensitive signalling pathways (Inclan et al., 2016; Persat et al., 2015; Song et al., 2018). Such signals enable bacteria to modulate gene expression and cellular differentiation (Persat et al., 2015) in response to changes in the physical properties of the surroundings, for example, by enhancing EPS production. This means that characterizing the physical properties of the surfaces can help with understanding colonization on surfaces and may provide opportunities to avoid contamination with diverse applications in the food industry and medical science. Agar hydrogel has emerged as the main model substrate for investigating colonization on surfaces due to its biological compatibility, affordability, accessibility, ease of preparation, and resistance to bacterial decomposition. One key property of agar gels is their high water-binding capacity, resulting in a limited presence of free water on the gel surface (Harshey, 2003a; Manuscript, 2013). Typical agar concentration as a substrate is in the range of 0.5%–2%. In our study, we employed varied agar concentrations, (from 0.25% (w/v) to 1.5% (w/v)) to investigate the effect of substrate physical properties on the colony-spreading dynamics of different *L. plantarum* strains. Among these strains, FBR2 consistently exhibited the largest colony size across all agar concentrations studied, with a substantially

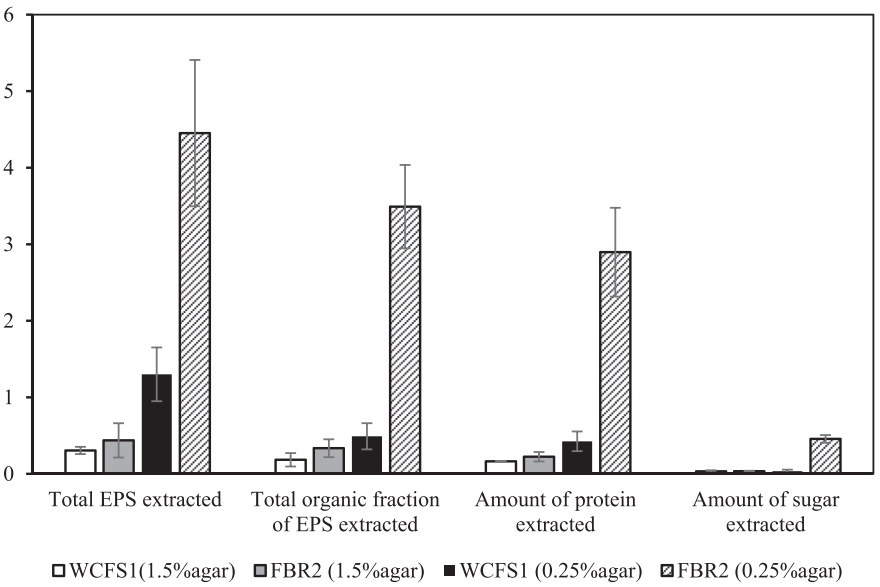

Fig. 6. The amount of EPS, protein and polysaccharide extracted from the *L. plantarum* WCFS1 and FBR2 formed colony on 0.25% (w/v) and 1.5% (w/v) agar plates after 48 hours incubation time at 30 °C.

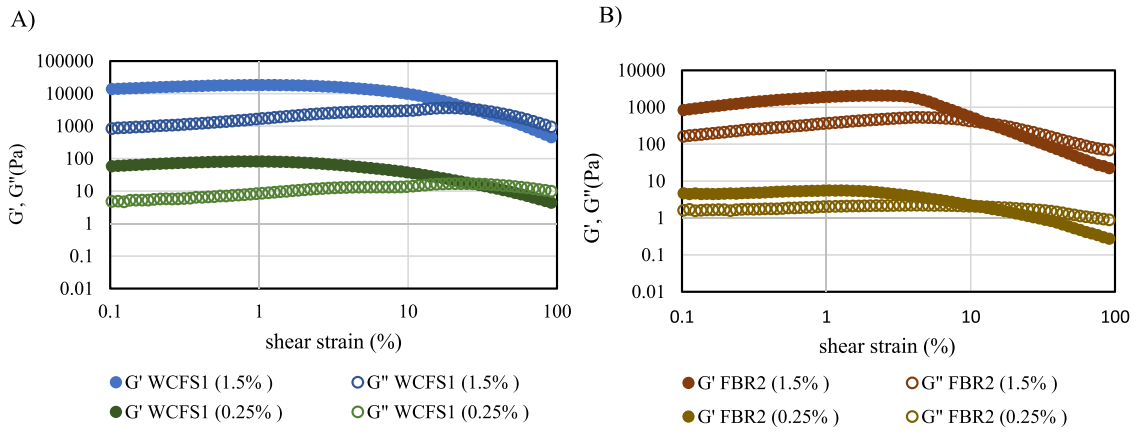

Fig. 7. Log-log plot of the elastic (G′, denoted by a close circle) and loss (G″, denoted by an open circle) moduli as a function of applied strain (at a frequency of 1 Hz using a plate-plate geometry) for colonies formed by *L. plantarum* WCFS1(A) and FBR2 (B) on 1.5% (w/v) and 0.25% (w/v) agar plates.

rapid spreading rate on the lowest agar concentration (0.25% (w/v)).

The surface spreading of non-motile bacteria is governed by a mechanism in which the bacteria themselves take only a passive role. Based on SEM microscopy, and previous studies, due to the absence of flagella or pili, spreading behaviour of FBR2 and other *L. plantarum* strains could be described by sliding motility, as originally presented by Henrichsen (Henrichsen, 1972). All studied strains of *L. plantarum* exhibited a reduction in spreading rate by increasing the agar concentration, this effect was much more pronounced in FBR2 than in WCFS1. Although the reduction of the spreading rate by an increase in the agar concentration could be correlated to an increase in the stiffness of the agar substrate, it was recently shown that this is not the case (Asp et al., 2022). Increasing the agar concentration also changes the pore size of the hydrogel, and this may contribute to the colony spreading differently. Lowering the agar concentration increases the pore size. The water uptake by the biofilm will be largely governed by the osmotic pressure difference between the substate and the biofilm (Asp et al., 2022). At lower agar concentration the osmotic pressure of the agar will become lower. This would facilitate more water transport from the agar substrate to the biofilm. On the other hand, the osmotic pressure difference will be governed mostly by EPS production in the biofilm, which will increase its osmotic pressure and the osmotic pressure difference and will promote water uptake. Such an increase of water uptake was observed in *Bacillus subtilis* colony biofilms, resulting in swelling of the biofilm (van Gestel et al., 2015). Water and nutrient uptake creates a nutrient-rich environment that enhances cell growth, and even enhances the EPS production, leading to more water uptake and consequently to flooding and spreading of the colony (van Gestel et al., 2015; Yan et al., 2017). Data from Fig. 7, clearly shows a significantly higher EPS production in the colonies formed by *Lactiplantibacillus* FBR2 on low agar concentration media during the incubation time compared to WCSF1. It is conceivable that the higher EPS production facilitates water uptake by osmotic pressure, resulting in elevated water content in the biofilm supporting colony spreading.

We have also compared the mechanical properties of the biofilm colonies (S5, SI) and showed that the FBR2 strain displayed an elastic shear modulus of about one order of magnitude lower than the WCFS1 strain, suggesting a softer biofilm structure for the FBR2 strain. In fact, the FBR2 biofilm exhibited lower elastic and viscous properties at all tested agar concentrations, indicating an overall lower stiffness and viscosity in the FBR2 biofilm. Furthermore, rheological data of FBR2 on 0.25% (w/v) agar prove that this biofilm is a very weak gel that can

easily be destroyed under its own weight, and flows like a viscous fluid on the agar surface, as was observed by tilting the plate, enhancing spreading on the surface.

The SEM microscopy images reveal that the morphology of WCFS cells in broth media and on low and high agar concentration plates is similar, while the FBR2 strain displayed a slight elongation of the cells when grown on all agar substrates. Cell elongation was reported for motile bacteria such as *Salmonella*, *Bacillus*, *Serratia*, and *Proteus mirabilis* (Kim and Surette, 2005; Little et al., 2019b; Manuscript, 2013; Senesi et al., 2002). For *P. mirabilis*, cell elongation was observed for an increase in agar concentration and concomitant enhanced population migration (Little et al., 2019). The elongation of motile bacteria allows for more area to accommodate lateral flagella and enables adequate cell-to-cell contact, thereby increasing swarming efficiency (Turner et al., 2010). However, *L. plantarum* is non-motile and cell elongation in this case may serve to align the cells, resulting in more efficient spreading. This should be investigated more thoroughly.

The number of cells, specifically culturable cells, plays an important role in surface contamination by colony spreading. As mentioned, FBR2 exhibited a higher number of culturable cells on the 0.25% (w/v) agar plate compared to the 1.5% (w/v) agar plate, and the number of cells increased with longer incubation times. This is in agreement with the higher uptake of water and availability of nutrients for FBR2 colonies on low agar substrates. In contrast, the number of culturable cells in WCFS1 colonies on both agar plates was similar. Despite the same initial cell count during the spotting stage for both strains, FBR2 exhibited a higher overall cell count and higher culturable cells, increasing the risk of contamination. Furthermore, the data in Table 1 demonstrates that FBR2 cells exhibited lower hydrophobicity and auto-aggregation compared to WCFS1 cells (confirmed by confocal images) which illustrates that WCFS1 cells formed clusters, indicating higher levels of auto-aggregation. The low hydrophobicity of FBR2 cells, coupled with their low auto-aggregation, facilitate their dispersion and sliding over the wet low agar concentration surface. This is again an important feature of FBR2, which increases their potential for surface contamination.

These findings highlight the significant potential for colony spreading by the FBR2 strain of *L. plantarum*, which could contribute to contamination and spreading on wet surfaces in food processing environments. However, FBR2 exhibits significant differences in colony phenotype compared to the five other tested *L. plantarum* isolates. To investigate the wider distribution and efficacy of the colonization dynamics of *L. plantarum*, a larger collection of isolates obtained from diverse food processing environments and spoiled products should be included in future studies. In addition, more detailed mechanistic understanding can be obtained via proteomics analysis providing information about proteins involved in the spreading process, while comparative genome sequence analysis may identify genetic factors contributing to colony-spreading behaviour, including the capacity of EPS production. Such investigations will contribute to a better understanding of the relationships between types of bacteria, bacterial surface spreading capacity, biofilm formation, and thus provide additional measures to prevent microbial contamination in food production environments.

5. Conclusion

In conclusion, we investigated the colony-spreading dynamics of different non-motile strains of *L. plantarum* on agar substrates of different concentrations. We observed a significant colony formation capability and a high rate of biofilm growth by the FBR2 strain, particularly on low agar concentrations substrates. Furthermore, we investigated the biofilm rheological properties, cell viability, cell morphology, cell hydrophobicity, and EPS production on different substrates, and showed that FBR2 biofilms displayed a considerably softer structure (lower elasticity and viscosity) than WCFS1 biofilms with higher water content. Additionally, FBR2 cells exhibited lower hydrophobicity and auto-aggregation, which may contribute to their enhanced spreading capability on low agar concentrations. These findings enhance our understanding of colonization dynamics of non-motile bacteria on surfaces, which may have practical relevance that warrants further investigations in food processing and in clinical settings.

CRediT authorship contribution statement

Mehdi Habibi: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Conceptualization. **T. Abee:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **E. van der Linden:** Writing – review & editing, Supervision, Project administration, Conceptualization. **D. Sudmalis:** Writing – review & editing, Methodology, Formal analysis. **P. Rashtchi:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mehdi Habibi reports financial support and article publishing charges were provided by Wageningen University. Mehdi Habibi is the editor-in-chief of the Journal of Applied Rheology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.127674.

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