International Journal of Food Microbiology 271 (2018) 15–23

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Role of cell surface composition and lysis in static biofilm formation by *Lactobacillus plantarum* WCFS1

Mónica D. Fernández Ramírez[a,b], Masja N. Nierop Groot[a,c], Eddy J. Smid[a,b], Pascal Hols[d], Michiel Kleerebezem[a,e], Tjakko Abee[a,b,*]

[a] Top Institute Food and Nutrition (TIFN), Wageningen, The Netherlands
[b] Laboratory of Food Microbiology, Wageningen University, Wageningen, The Netherlands
[c] Wageningen Food and Biobased Research, Wageningen University and Research, Wageningen, The Netherlands
[d] Institut des Sciences de la Vie, Université Catholique de Louvain, Louvain-la-Neuve, Belgium
[e] Host-Microbe Interactomics Group, Wageningen University, Wageningen, The Netherlands

ARTICLE INFO

Keywords:
Autolysis
Extracellular DNA
Capsular polysaccharides
Sortase A

ABSTRACT

Next to applications in fermentations, *Lactobacillus plantarum* is recognized as a food spoilage organism, and its dispersal from biofilms in food processing environments might be implicated in contamination or re-contamination of food products. This study provides new insights into biofilm development by *L. plantarum* WCFS1 through comparative analysis of wild type and mutants affected in cell surface composition, including mutants deficient in the production of Sortase A involved in the covalent attachment of 27 predicted surface proteins to the cell wall peptidoglycan (Δ*srtA*) and mutants deficient in the production of capsular polysaccharides (CPS1–4, Δ*cps1–4*). Surface adhesion and biofilm formation studies revealed none of the imposed cell surface modifications to affect the initial attachment of cells to polystyrene while biofilm formation based on Crystal Violet (CV) staining was severely reduced in the Δ*srtA* mutant and significantly increased in mutants lacking the *cps1* cluster, compared to the wild-type strain. Fluorescence microscopy analysis of biofilm samples pointed to a higher presence of extracellular DNA (eDNA) in *cps1* mutants and this corresponded with increased autolysis activity. Subsequent studies using Δ*acm2* and Δ*lytA* derivatives affected in lytic behaviour revealed reduced biofilm formation measured by CV staining, confirming the relevance of lysis for the build-up of the biofilm matrix with eDNA.

1. Introduction

Lactobacilli are Gram positive, generally non-motile bacteria which can be found in a diverse range of habitats. They are widely used in the food industry as probiotics (Boesten and de Vos 2008; Turroni et al. 2014) and starter cultures for the production of fermented food products (Caplice and Fitzgerald 1999; Leroy and De Vuyst 2004). However, besides their desired properties, they are also associated with food spoilage (Bartowsky and Henschke 2008; Bjorkroth and Korkeala 1996; Lyhs et al. 2001; Samelis et al. 2000). One contamination route with lactic acid bacteria is via the presence of biofilms in the food production environment (Somers et al. 2001). Biofilms are defined as microorganisms attached to a surface embedded in a matrix of extracellular polymeric substances (O'Toole et al. 2000). *L. plantarum* has been shown to form submerged biofilms, both as single species but also in multispecies biofilms (Fernández Ramírez et al. 2015; Kubota et al. 2008; Kubota et al. 2009; Metselaar et al. 2015; van der Veen and Abee 2011). Several stages in biofilm development can be recognized including surface adhesion, microcolony formation, biofilm growth, matrix formation, and biofilm dispersion as the final stage (Abee et al. 2011; Watnick and Kolter 2000).

Surface components of the cell envelope of bacteria have been shown important for interaction with the environment (Kleerebezem et al. 2010; O'Toole et al. 2000; Pratt and Kolter 1998). The main constituents of the Gram positive cell envelope are peptidoglycan, teichoic acids, proteins and polysaccharides (Kleerebezem et al. 2010; Silhavy et al. 2010). Especially, polysaccharides were found to play a role in biofilm formation contributing to the formation of the biofilm matrix (Branda et al. 2005; Stewart and Franklin 2008). Four different gene clusters encoding capsular polysaccharide biosynthesis are located on the *L. plantarum* WCFS1 genome and the role of these cell surface polysaccharides in probiotic functionality was studied in deletion mutants lacking either individual or multiple *cps* gene clusters (Remus et al. 2012). In addition, some of the cell surface proteins present in *L.*

* Corresponding author at: Laboratory of Food Microbiology, Wageningen University, Wageningen, The Netherlands.
E-mail address: tjakko.abee@wur.nl (T. Abee).

https://doi.org/10.1016/j.ijfoodmicro.2018.02.013
Received 13 September 2017; Received in revised form 26 January 2018; Accepted 11 February 2018
Available online 21 February 2018

Table 1
Strains used in the present study.

Strain	Description	Notation	Reference
WCFS1	*L. plantarum* NCIMB8826		(Kleerebezem et al. 2003; Siezen et al. 2012)
NZ3548Cm	Cm^R; WCFS1 derivative; chromosomal replacement of the Δ*cps1A-I* gene cluster	Δ*cps1*	(Remus et al. 2012)
NZ3533ACm	Cm^R; WCFS1 derivative; chromosomal replacement of the Δ*cps2A-J* gene cluster	Δ*cps2*	
NZ3549Cm	Cm^R; WCFS1 derivative; chromosomal replacement of the Δ*cps3A-J* gene cluster	Δ*cps3*	
NZ3534Cm	Cm^R; WCFS1 derivative; chromosomal replacement of the Δ*cps4A-J* gene cluster	Δ*cps4*	(Andre et al. 2011)
NZ3550Cm	Cm^R; WCFS1 derivative; chromosomal replacement of the Δ*cps1A-3J* cluster	Δ*cps1–3*	
NZ3680Cm	Cm^R; WCFS1 derivative; chromosomal replacement of the Δ*cps1A-3J*, Δ*cps4A-J* cluster	Δ*cps1–4*	
NZ3513Cm	Δ*srtA*	Δ*srtA*	(Remus et al. 2013)
NZ3557	Cm^R; WCFS1 derivative; *acm2::cat*	Δ*acm2*	(Fredriksen et al. 2012)
TR0011	NZ7100 derivative; *lp_3093* (*lys2*)::lox72	Δ*lys2*	(Rolain et al. 2012)
TR006	NZ7100 derivative; lp_*3421* (*lytA*)::lox66-P32-cat-lox71	Δ*lytA*	

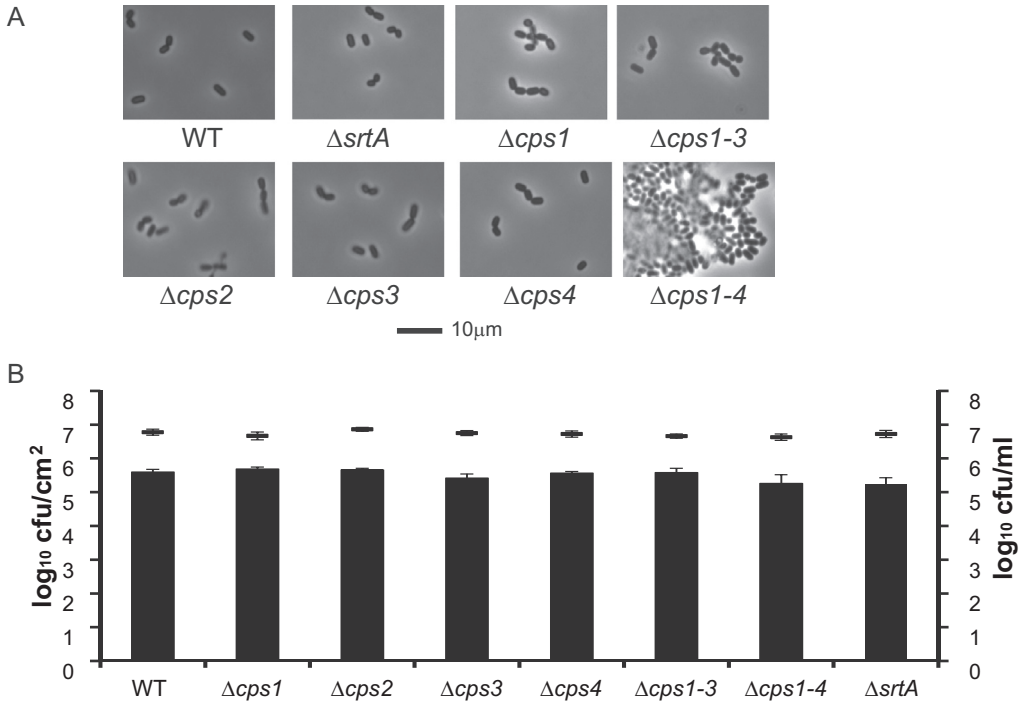

Fig. 1. Morphology and initial attachment of *Lactobacillus plantarum* WCFS1 and its mutants affected in cell surface properties (A) Morphology of *L. plantarum* WCFS1 (WT) and mutants affected in cell surface properties grown in BHIMnG at 30 °C for 18 h. (B) The number of cells attached to polystyrene after 1 h incubation at 30 °C in BHIMnG was determined and represented by the columns with the lines on top of each columns marking the initial inoculum (log_{10} cfu/ml). The error bars display the standard deviation of the experiment. The experiment was performed in triplicate and with three biological replicates. No significant difference from the WT was found (ANOVA, $P < 0.001$).

plantarum, such as mannose-specific adhesins, have been reported to play a role in attachment to biotic surfaces including host epithelial cells (Pretzer et al. 2005). Notably, the major sortase SrtA mediates covalent binding of 27 predicted cell-surface proteins encoded in the *L. plantarum* WCFS1 genome, including mucus-binding proteins, a hydrolase, as well as mannose-specific and collagen-binding adhesins (Boekhorst et al. 2005; Kleerebezem et al. 2010). Among these, the function of three sortase dependent proteins (SDPs) in microbe-host interactions has been experimentally validated (Du et al. 2015; Pretzer et al. 2005; Sturme et al. 2005). The deletion of sortase A in *Enterococcus faecalis* resulted in a lower initial attachment and to defective biofilm formation under static and dynamic conditions, showing its relevance in initial attachment and biofilm formation (Guiton et al. 2009). Additionally, Malik et al. (2013) reported that the highly autoaggregative, adhesive and biofilm forming phenotype of the vaginal *Lactobacillus plantarum* Strain CMPG5300 is sortase-dependent. Although the exact matrix composition of *L. plantarum* biofilms remains to be determined, enzyme treatments have shown proteins and eDNA to be part of the biofilm matrix (Fernández Ramírez et al. 2015). DNA can be released into the biofilm matrix either by active secretion or cell lysis (Jakubovics et al. 2013). The latter has been reported for different species including enterococci (Guiton et al. 2009; Thomas et al. 2008) and staphylococci (Qin et al. 2007; Rice et al. 2007) conceivably caused by autolysins that play a role in cell wall degradation (Bayles 2007; Frese et al. 2013).

The current study focuses on the role of capsular polysaccharides and cell-wall associated proteins in biofilm formation of *L. plantarum* WCFS1. Comparative analysis of *L. plantarum* WCFS1 (wild type) and selected mutants affected in cell surface composition (Δ*srtA*, Δ*cps1–4*) and cell lysis activity (Δ*acm2*, Δ*lytA*) provided evidence that cell wall autolysis and release of DNA are major determinants of *L. plantarum* WCFS1 static biofilm formation.

2. Materials and methods

2.1. Strains and media

The bacterial strains used in the present study are listed in Table 1. The strains were streaked from a −80 °C glycerol stock on De Man-Rogosa-Sharpe (MRS) agar (Merck) plates and incubated for 48 h at 30 °C. A single colony was inoculated in 10 ml of MRS at 30 °C for 18 h to prepare a starting culture. The media were supplemented with 10 μg/ml chloramphenicol when appropriate. Biofilms were grown in brain heart infusion (BHI; Becton Dickinson) supplemented with 0.005% manganese sulphate and 2% glucose (Merck) (BHIMnG) as it has been shown previously that this medium favours biofilm formation of *L.*

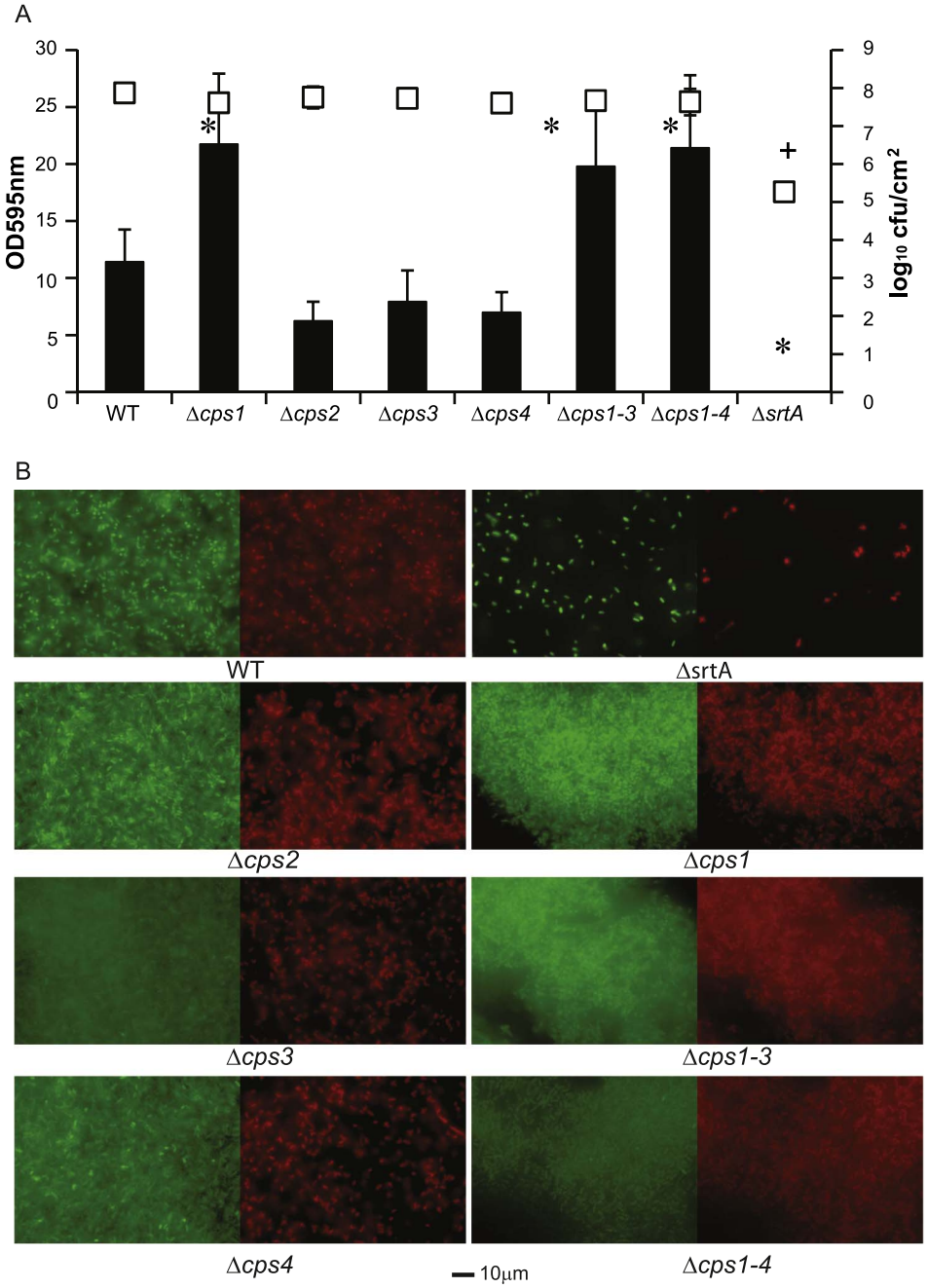

Fig. 2. Biofilm formation of WCFS1 and its mutants affected in cell surface properties. Biofilms were grown in BHIMnG for 48 h at 30 °C on polystyrene. (A) The data represent the average of three biological replicates in triplicate and the standard deviation of biofilm formation measured by CV assay (columns) and number of cells in the biofilm (squares). Statistical difference (ANOVA, Dunett's test $P < 0.005$) is marked * for CV and + for number of cells in the biofilm compared to the WT. (B) Representative fluorescent images of biofilms formed by the different strains stained with LIVE/DEAD BacLight bacterial viability kit: Syto9 (left) and propidium iodide (right).

plantarum (Fernández Ramírez et al. 2015; van der Veen and Abee 2011). A concentration of 0.01 mg/ml of chicken egg lysozyme (Sigma) was added to BHIMnG when indicated.

2.2. Biofilm formation

Biofilms were formed under static conditions as described previously (Merritt et al. 2005). The biofilms were grown in polystyrene (PS) 12 well plates (Greiner Bio-One). Each well was filled with 1.5 ml of BHIMnG inoculated with 1% (v/v) of an overnight grown culture (18 h at 30 °C) containing approximately 9.2 $\log_{10}$ colony forming units (cfu) per ml. The biofilms were grown at 30 °C for either 1 h (for determination of initial attachment) or 48 h and the plates were sealed with parafilm to avoid evaporation. The initial attachment of strain WCFS1 and its Δ*acm2* derivative was also evaluated in the presence of lysozyme (0.01 mg/ml) in BHIMnG. From the overnight culture, 1 ml was taken and centrifuged for 10 min 5000 x *g* and washed once with phosphate buffered saline (PBS, NaCl 8 g/l; KCl 0.2 g/l; Na_2HPO_4 1.44 g/l; KH_2PO_4 0.24 g/l; pH 7.4) (Merck). Resulting cells were resuspended in 1 ml of PBS with 0.01 mg/ml lysozyme and incubated for 1 h at 30 °C. From this suspension, 0.01% (v/v) was inoculated as described above in BHIMnG containing 0.01 mg/ml lysozyme. The control sample was treated in the same way but without 0.01 mg/ml lysozyme in PBS or BHIMnG.

The biofilm formation was measured both by the Crystal Violet (CV) assay and plate counting to determine the total biofilm formation and

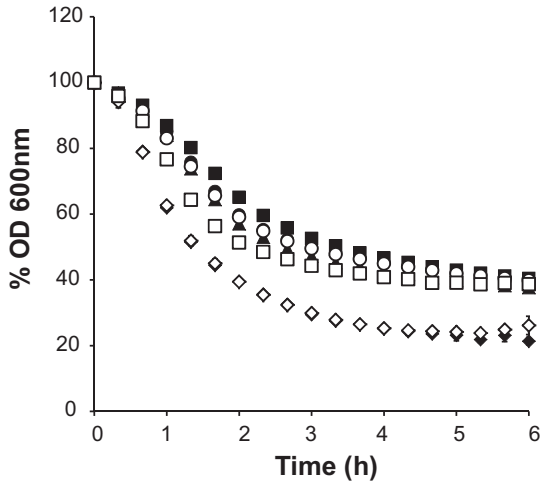

Fig. 3. Triton X-100 (0.05%)-induced autolysis of WCFS1 and its mutants affected in cell surface properties. The data represent the average and the standard deviation of three biological replicates of residual OD 600 (%) after treating exponentially growing cells of WT (filled circle), Δ*cps1* (filled diamonds), Δ*cps2* (filled squares), Δ*cps3* (filled triangles), Δ*cps4* (open circles), Δ*cps1–3* (open diamonds) and Δ*srtA* (open squares) with 0.05% Triton X-100. %OD 600 nm for Δ*cps1* and Δ*cps1–3* is significantly different from the WT after 40 min; Δ*srtA* is significantly different from the WT only between 1 and 2.6 h; Δ*cps2* is significantly different between 1.3 and 2.3 h (ANOVA, Dunett's test $P < 0.005$).

number of culturable cells in the biofilm, respectively. For the initial attachment only the number of culturable cells attached was determined by plate counting. For cell enumeration, the biofilms were washed three times with 2 ml of PBS to remove unattached cells. Next, the remaining biofilm was resuspended in 2 ml of PBS by scraping the surface with the tip of the pipette and vigorous pipetting. Additionally, the absence of clumps or cell aggregates was analysed by phase contrast microscopy (Fernández Ramírez et al. 2015). Serial dilutions were prepared in PBS and plated on MRS agar and incubated at 30 °C for 48 h.

For the CV assay, the medium was carefully removed with a pipette and the biofilm was washed as described above. The attached biofilm was stained for 30 min with 1.5 ml of 0.1% (w/v) of CV (Merck). The excess of CV was removed by washing three times with 2 ml of PBS as described above. The dye attached to the biofilm was solubilized in 70% ethanol for 30 min at room temperature and the optical density (OD) was measured at 595 nm (SpectraMax, Molecular Devices). When OD_{595} values exceeded a value of 1, the samples were diluted in 70% ethanol and the resulting OD measurements were corrected for the dilution factor and by subtracting the average of the blank. The resulting measurement was defined as the total biofilm formed. Each plate was prepared in triplicate and three independent biological replicates were analysed.

The LIVE/DEAD® Bacterial Viability Kit (BacLight™) was used to visualise the biofilms following the supplier's protocol. The biofilms were grown in six well plates in 3 ml of inoculated BHIMnG as described above. For microscopy experiments, the fluorescent filters UMNBV (SYTO9) and U-MWIG (PI) were used at a magnification of 1000 times with a BX41 microscope (Olympus).

2.3. Triton X-100 induced autolysis in buffer solution

This assay was carried out as previously described (Rolain et al. 2012) with some modifications. Briefly, *L. plantarum* strains were grown to mid exponential phase in BHIMnG (OD_{600} = 0.8) without (all strains) and with lysozyme (0.01 mg/ml) in the case of WCFS1 (WT) and its Δ*acm2* derivative. Bacteria were harvested by centrifugation (5000 × *g*, 10 min, 4 °C) and washed once with PBS. Washed cells were resuspended at a final OD_{600} of 1 in PBS containing 0.05% Triton X-100 (Sigma-Aldrich). Aliquots of 200 µl were placed in 96-well polystyrene microtiter plates (Greiner Bio-One) and incubated at 30 °C, and the OD_{600} was determined every 20 min (SpectraMax, Molecular Devices).

3. Results

3.1. Cell surface composition does not affect initial attachment

Initial attachment of cells to the surface is important in the early stages of biofilm formation. Cell surface characteristics involved in initial attachment are expected to affect biofilm development. Mutants affected in cell-surface protein composition (Δ*srtA*) and capsular polysaccharide production (Δ*cps*) were examined for their capacity to interact with polystyrene (PS) surfaces. First, cell morphology of *L. plantarum* WCFS1 (WT), Δ*srtA* and Δ*cps* mutants was analysed using phase contrast microscopy following incubation of the respective cultures for 18 h in BHIMnG at 30 °C. Mutants with a deletion of the *cps1* cluster (either alone or in combination with other *cps*) were found to form chains of cells, with Δ*cps1–4* forming cell aggregates (Fig. 1A). Free cells were obtained from all mutants by vigorous pipetting and samples were processed within 1 h for cell enumeration, a time frame in which no aggregation was observed (data not shown). Subsequent analysis of the initial attachment of WT, Δ*srtA* and the Δ*cps* mutants revealed no significant differences in adhesion capacity after 1 h of incubation at 30 °C (ANOVA, $P < 0.001$; Fig. 1B).

3.2. Biofilm formation is affected by cps1 and srtA deletions

The biofilm forming capacity of WT, Δ*srtA*, Δ*cps1*, Δ*cps2*, Δ*cps3*, Δ*cps4*, Δ*cps1–3*, and Δ*cps1–4* was tested to determine the influence of the surface modifications on biofilm formation following initial adhesion. The biofilm forming capacity was analysed after 48 h of incubation at 30 °C by both CV staining and enumeration of culturable cells in the biofilm (Fig. 2A). The number of culturable cells in the biofilms formed by the mutants was comparable to the number obtained with the WT strain, except for the Δ*srtA* mutant. The Δ*srtA* mutant biofilm contained an approximately 100-fold reduced level of culturable cells. Notably, CV staining for this mutant was also below the detection limit, suggesting that this mutant strain is severely hampered in biofilm formation. For strain *L. plantarum* WCFS1, targeted mutants for three putative SDPs have been previously constructed: lp_1229 (*msa*, mannose surface adhesin) and lp_0373 (Pretzer et al. 2005), and lp_2940 (Bron et al. 2007). Determination of their biofilm formation capacity in BHIMnG medium at 30 °C measured as CV-stainable material and the number of culturable cells in the biofilm, showed similar performance for WCFS1 wild type and the three mutants (Supplementary fig. 1).

For the *cps* mutants, CV staining showed a remarkable increase in total biofilm formation for the strains lacking the *cps1* gene cluster (i.e., Δ*cps1*, Δ*cps1–3* and Δ*cps1–4*) relative to the WT strain (Fig. 2A). The biofilms were stained with propidium iodide (PI) which binds to intracellular DNA of cells with damaged membranes but also to eDNA released into the matrix, conceivably following lysis of cells. The images obtained by fluorescence microscopy of propidium iodide (PI)-stained biofilms, suggest denser agglomerates in biofilms formed by strains lacking the Δ*cps1* gene cluster, suggesting higher eDNA levels in the biofilm matrix (Fig. 2B).

3.3. Sensitivity of Δcps1 mutants to chemically-induced autolysis differs from the WT strain

To analyse the autolysis capacity, exponentially growing cells of WT and mutant strains were exposed to triton X-100 and the degree of cell lysis was determined (Fig. 3). Autolysis under these conditions was comparable to the WT for all tested strains with the exception of the Δ*cps1* and Δ*cps1–3* mutant strains, which showed a higher degree of lysis. The increased lysis activity of these two mutants corroborates the

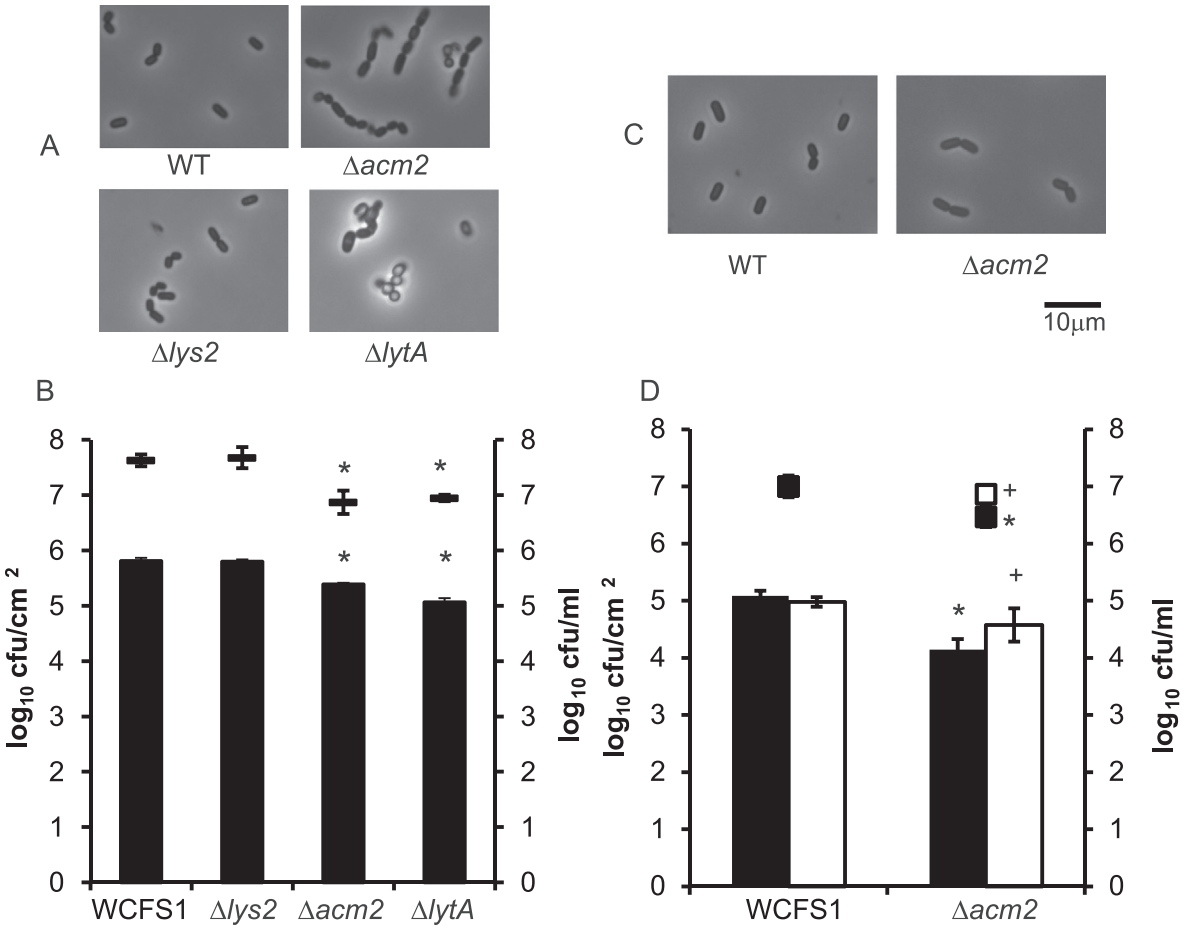

Fig. 4. Morphology and initial attachment of *L. plantarum* WCFS1 and mutants affected in peptidoglycan hydrolases. (A) The morphology of the strains grown in BHIMnG at 30 °C for 18 is shown. (B) Shows the number of cells attached to polystyrene (log_{10} cfu/cm^2) after 1 h incubation at 30 °C in BHIMnG (black bars) and on top of each bar the initial inoculum (log_{10} cfu/ml) is indicated. (C) Shows the morphology of WT and Δ*acm2* strains grown in BHIMnG at for 18 h at 30 °C with 0.01 mg/ml lysozyme (D) Shows adhesion capacity, with the initial inoculum pre-incubated for 1 h with PBS (black squares) or PBS with 0.01 mg/ lysozyme (open squares) in log cfu/ml, and initial attachment after 1 h incubation at 30 °C in BHIMnG without (black bars) or with 0.01 mg/ml lysozyme (open bars). The error bars display the standard deviation of the experiment. The experiment was performed in triplicate and with three biological replicates. Statistical differences were determined by comparing the results to the WT (ANOVA, Dunett's test $P < 0.005$) and are marked (*). Significant difference (*t*-test $P < 0.05$) resulting from addition of lysozyme are marked (+).

increased CV and PI staining of the biofilms of these strains relative to the WT (see above; Fig. 2B), which suggests that more eDNA is present in the matrix of these mutants. The lysis behaviour of the Δ*cps1–4* mutant could not be determined by this method as a consequence of cell aggregation at later stages in the assay (Fig. 2).

3.4. Cell lysis is important for biofilm development

To further explore how cell lysis affects biofilm formation, we characterised *L. plantarum* mutants deficient for specific peptidoglycan hydrolases (Δ*acm2*, Δ*lys2*, Δ*lytA*) that are potentially affected in cell lysis during biofilm formation. Microscopy analysis of BHIMnG grown cultures showed that the Δ*acm2* mutant formed chains of cells and cells of the Δ*lytA* mutant displayed slight aggregation and loss of rod shaped morphology, confirming previously reported phenotypes for these mutants after growth in MRS medium (Rolain et al. 2012) (Fig. 4A). The initial attachment of the WT and mutant strains to PS was evaluated after 1 h of incubation in BHIMnG and significant difference in adhesion capacity was observed for Δ*lytA* and Δ*acm2* (ANOVA, Dunett's test $P < 0.005$; Fig. 4B). The Δ*lytA* mutant strain showed a slightly reduced inoculum size possibly caused by increased cell death in the stationary phase, which is in agreement with the previously reported reduced viability of this mutant (Rolain et al. 2012). The slightly lower colony counts observed in the initial inoculum of the Δ*acm2* mutant is likely explained by the formation of cell-chains that lead to an underestimation of the viable cells using colony forming unit enumeration. It was not possible to separate these chains into single cells by vigorous pipetting. However, the average chain length of the Δ*acm2* mutant has been reported to be approximately 4 cells (Rolain et al. 2012) and correction of the obtained colony counts for this factor results in similar number of attached and initial inoculum cell numbers for this mutant as compared to WT (Fig. 4B and D). Notably, it has previously been shown that inactivation of the major autolysin AcmA of *Lactococcus lactis* also resulted in chain formation and it was suggested that a reduced number of peptidoglycan breaks affected adhesion capacity to surfaces (Mercier et al. 2002). Therefore, adhesion was also studied with *L. plantarum* Δ*acm2* cells grown in the presence of 0.01 mg/ml of lysozyme using WT as a control. This concentration of lysozyme did not damage the cells as reflected in the similar numbers of culturable cells for both strains (Fig. 4D). Notably, lysozyme treatment of the Δ*acm2* mutant seemed to disintegrate chains with a cell morphology that appeared comparable to that observed for the WT (Fig. 4C). The WT strain showed similar PS adhesion efficiency when grown in absence or presence of lysozyme, whereas lysozyme treatment of the Δ*acm2* mutant led to higher cell numbers in both the initial inoculum and the PS-attached fraction (Fig. 4D), suggesting that indeed the enzymes disintegrated the chain leading to a higher number of single cells and this may have affected initial attachment of the Δ*acm2* mutant.

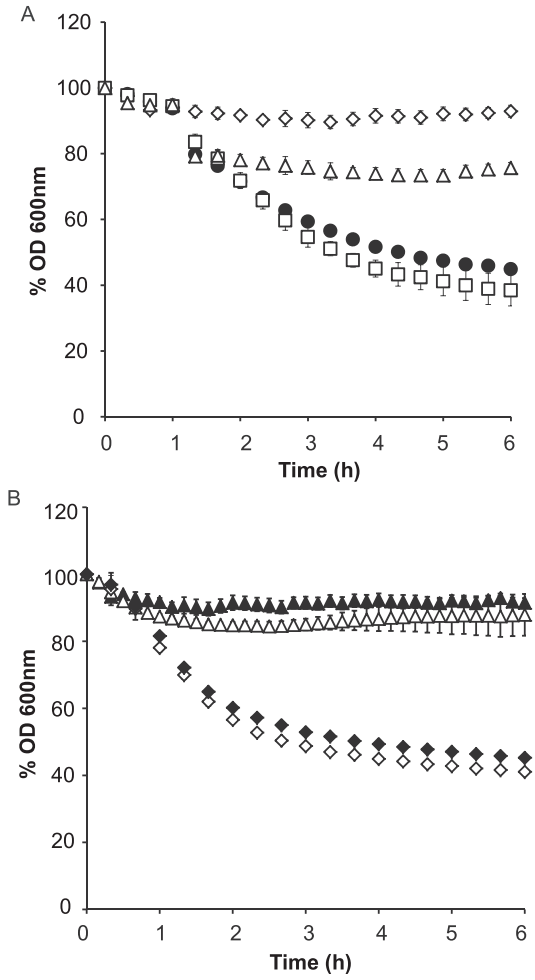

Fig. 5. Triton X-100 (0.05%)-induced autolysis of WCFS1 and mutants affected in peptidoglycan hydrolases. (A) Shows OD 600 (%) for WT (filled circle), Δ*acm2* (diamonds), Δ*lys2* (squares), and Δ*lytA* (triangles). %OD 600 nm for Δ*acm2* and Δ*lytA* are significantly different from the WT after 1.3 h and 2 h respectively (ANOVA, Dunett's test $P < 0.005$). (B) Shows additional data for strains WCFS1 (diamonds) and Δ*acm2* (triangles) grown in the absence (black) or presence (open) of 0.01 mg/ml lysozyme. %OD 600 nm for lysozyme treated strains was not significantly different from untreated strains (Student's *t*-test $P < 0.005$). The data represent the average and the standard deviation of three biological replicates.

Next, induced autolysis behaviour was analysed in BHI supplemented with Mn(II) and glucose (BHIMnG) that was used in adhesion and biofilm formation experiments for the mutants (Fig. 5A), as well as for WT and Δ*acm2* mutant cells grown in the presence of lysozyme (Fig. 5B). In BHIMnG, Δ*lytA* and Δ*acm2* mutants showed a lower degree of lysis as compared to the WT, whereas the Δ*lys2* mutation did not significantly affect lysis behaviour (ANOVA, $P < 0.001$), these results are in concordance with previously reported results obtained in MRS medium (Rolain et al. 2012). In addition, the lysis behaviour of the Δ*acm2* mutant was not affected by lysozyme treatment (Fig. 5B). This indicates that although the chaining is disrupted, the degree of lysis is not influenced.

The biofilm forming capacity of WT and Δ*lytA*, Δ*lys2*, Δ*acm2* mutants was further characterised by enumeration of the culturable biofilm cells and CV staining using BHIMnG (Fig. 6A) and for the WT and Δ*acm2* mutant also using BHIMnG containing 0.01 mg/ml lysozyme (Fig. 6C). Although WT and the Δ*lytA* mutant had a comparable number of culturable cells in the biofilm, the biofilm produced by the Δ*lytA* mutant showed a lower level of CV binding as compared to the WT. Without lysozyme, the Δ*acm2* mutant did not form measurable biofilms based on CV staining, but appeared to develop a submerged pellicle that was not attached to the PS surface (data not shown). However, the biofilm formation capacity of the Δ*acm2* mutant significantly increased in the presence of lysozyme (Fig 6C), containing similar numbers of culturable cells and an approximate 50% reduced CV staining compared to biofilms formed by WT. These observations were supported by fluorescence microscopy using Live/Dead staining (Fig. 6D). Images of the Syto9-stained samples showed that very few cells of the Δ*acm2* mutant were attached when incubated in BHIMnG without lysozyme, while the images obtained for the Δ*acm2* mutant in BHIMnG with lysozyme were similar to those obtained for the WT (without and with lysozyme) and the Δ*lytA* and Δ*lys2* mutants. PI-staining of biofilms of the Δ*acm2* and Δ*lytA* mutant strains show only dead cells and reduced presence of eDNA compared to the WT and Δ*lys2* mutant biofilms (Fig. 6B). This observation is in agreement with the reduced CV staining indicative of lower total biofilm formation for the Δ*acm2* and Δ*lytA* mutants (Fig. 6A).

4. Discussion

This study has provided new insights into biofilm development by *L. plantarum* WCFS1 through comparative analysis of wild type and selected mutants affected in cell surface composition and cell lysis, establishing a role of eDNA in the biofilm matrix.

The initial attachment of *L. plantarum* to PS surfaces was not affected in mutant strains lacking genes in either one or multiple combinations of the four clusters encoding CPS production or the sortase gene (*srtA* gene). Previously, it was shown that the effect of *cps* deletion in *L. plantarum* strains was strain dependent and no clear relation could be established between hydrophobicity and adhesion (Lee et al. 2016). Notably, in our study the deletion of *srtA* resulted in a severe impairment of subsequent biofilm development by *L. plantarum*. The importance of Sortase A in biofilm formation was previously described for *Enterococcus faecalis* and both initial attachment and biofilm development were affected in this species (Guiton et al. 2009). Additionally, the *srtA* mutant derivative of *L. plantarum* CMPG5300 showed reduced attachment to epithelial cells as well as reduced biofilm formation in polystyrene surfaces (Malik et al. 2013). SrtA covalently anchors proteins with a LPTXG motif, collectively called sortase dependent proteins (SDPs), to the cell wall peptidoglycan (Fischetti et al. 1990). Previously, it was shown that *srtA* was significantly upregulated during biofilm formation in *L. plantarum* DB200, supporting a possible role for one or more SDP's in biofilm formation in this strain as well (De Angelis et al. 2015). For strain *L. plantarum* WCFS1, 27 SDPs are predicted (Boekhorst et al. 2005; Kleerebezem et al. 2010) and targeted mutants for three putative SDPs have been previously constructed: lp_1229 (*msa*, mannose surface adhesin) and lp_0373 (Pretzer et al. 2005), and lp_2940 (Bron et al. 2007), the latter two being cell surface protein precursors. Determination of their biofilm formation capacity in BHIMnG medium at 30 °C measured as CV-stainable material and the number of culturable cells in the biofilm, showed similar performance for WCFS1 wild type and mutants (Supplementary fig. 1). A role for these SDPs in biofilm formation in *L. plantarum* WCFS1 in other conditions cannot be excluded, especially since a recent study by Malik et al. (2016) using *L. plantarum* CMPG5300, a highly aggregative vaginal isolate, showed loss of the aggregative phenotype and a (two-fold) reduction in biofilm formation to polystyrene in MRS medium at 37 °C for a mannose-specific lectin (Msl) deletion mutant. Our results indicate that one or more of the *L. plantarum* WCFS1 SDPs are involved in biofilm development and their specific role(s) remain to be elucidated.

The impact of capsular polysaccharides on *L. plantarum* WCFS1 surface adhesion and biofilm formation was studied using strains lacking the *cps1* cluster, either individually or in combination. Notably, the Δ*cps1* mutant displayed a chaining phenotype, whereas the Δ*cps1–4* mutant formed cell aggregates (cell clumping) that could be dissociated by vigorous pipetting. Chaining and clumping phenotypes are indicators of cell envelope modification, which could cause the observed

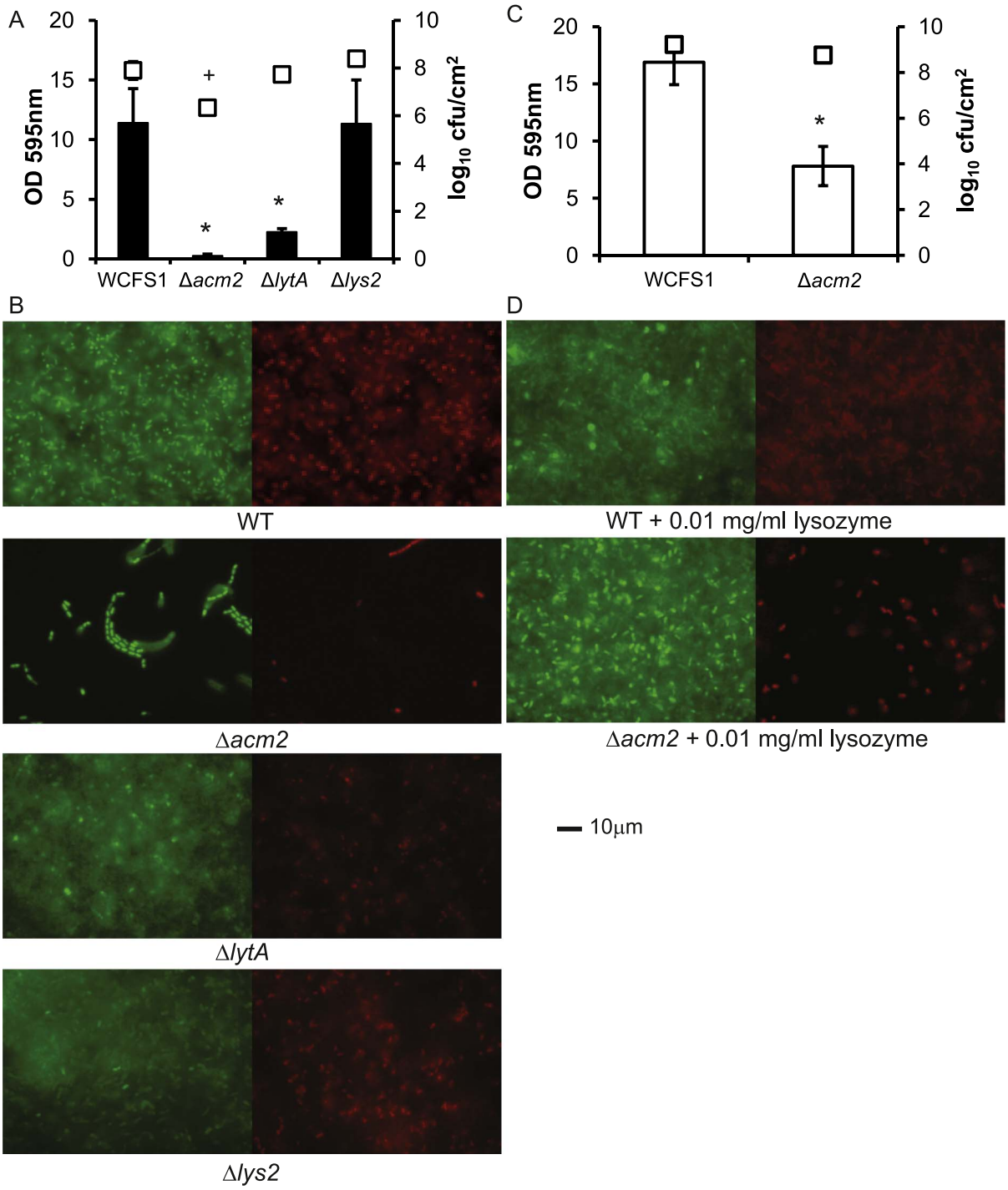

Fig. 6. Biofilm formation of WCFS1 and mutant derivatives. Biofilms were grown in BHIMnG for 48 h at 30 °C on polystyrene. The data represent the average of three biological replicates in triplicate and the standard deviation of biofilm formation CV assay (columns) and number of cells in the biofilm (squares) without (A) or with lysozyme (C) for WT and Δ*acm2*. (B and D) Representative fluorescent images of biofilms stained with LIVE/DEAD BacLight bacterial viability kit: Syto9 (left) and propidium iodide (right). Statistical difference (ANOVA, Dunett's test $P < 0.005$) is marked * for CV and + for number of cells in the biofilm when compared to the WT.

differences in biofilm formation. Biofilm formation analysis revealed that strains lacking the *cps1* gene cluster showed increased CV staining, whereas the number of culturable cells was comparable to the WT. Previous studies showed that deletion of *cps1* resulted in a significant decrease of the amount of galactose and rhamnose (> 99 and 95%, respectively) and reduced chain length of the capsule polysaccharides (Remus et al. 2012). Deletion of *cps3* or *cps4* in *L. plantarum* WCFS1 did not have an impact on the monosaccharide composition of the capsular polysaccharides whereas for mutants lacking *cps2*, the relative amount of galactose and rhamnose in capsular polysaccharides was also found to be significantly decreased by approximately 50% (Remus et al. 2012). The initial attachment of none of the *cps* mutants was affected, and similar numbers of culturable cells were reached in the biofilm as compared to the WT. The numbers of culturable cells in the Δ*cps1* mutant biofilm were only slightly lower (approximately 20%) than the WT but given the fact that CV measurement is expressed on a linear scale, this slight reduction resulted in double the amount of total biofilm as quantified by CV suggesting that the increase in CV staining is

best explained by the enhanced lysis capacity of single and multiple *cps1* deletion mutants resulting in higher amounts of eDNA in the biofilm matrix. The mechanism underlying enhanced cell lysis of these mutants remains to be elucidated. Notably, several studies have reported that the lack of capsular polysaccharides in *Streptococcus pneumoniae* increased the total biofilm amount (Moscoso et al. 2006; Muñoz-Elías et al. 2008), but did not provide mechanistic insight into how the loss of capsule affects biofilm formation. Extracellular polysaccharides have also been studied in probiotic L. *rhamnosus* GG and the production of a galactose containing polysaccharide (Francius et al. 2008; Landersjö et al. 2002) decreased attachment to polystyrene surfaces and subsequent biofilm forming capacity, conceivably due to shielding of other cell surface structures (Lebeer et al. 2009).

Current data obtained with *cps* mutants support our previous study that showed *L. plantarum* biofilms to contain eDNA as matrix component (Fernández Ramírez et al. 2015). A role for eDNA in *L. plantarum* biofilm formation was further confirmed by biofilm formation analysis of mutants deleted in the major cell wall autolysin Acm2, or the redundant peptidoglycan hydrolase Lys 2, and the D, L-endopeptidase LytA. None of these enzymes are predicted to be sortase dependent (Boekhorst et al. 2005; Kleerebezem et al. 2010) and therefore do not play a plausible role in the impaired biofilm formation of the *srtA* mutant. Reduced lytic behaviour of the autolysin mutants correlated with decreased biofilm formation of the mutants as measured by CV staining. The deletion of *lys2* did not have an impact on biofilm formation which is in line with the observation that its lytic behaviour is similar to the WT. The Δ*lytA* mutant displayed a lower degree of lysis compared to the WT. Although the number of culturable cells in the biofilm of the Δ*lytA* was comparable to the WT, the total biofilm amount as measured by CV staining was 5-fold lower compared to the WT. It is conceivable that reduced levels of eDNA in the Δ*lytA* biofilm as observed using fluorescence microscopy match the lower biofilm formation as measured by CV staining.

Notably, the Δ*acm2* mutant strain displayed extensive cell chaining, typically observed for mutants lacking major cell wall hydrolase activity since these enzymes have been found to be responsible for cell separation and the introduction of peptidoglycan breaks (Mercier et al. 2002; Rolain et al. 2012). Therefore the biofilm performance of the Δ*acm2* mutant was analysed also in the presence of low levels of lysozyme. This condition was shown to prevent cell-chain formation by complementing the cell separation defect by exogenously introducing peptidoglycan breaks. Notably, in presence of lysozyme, still the biofilm formation of Δ*acm2* mutant differed significantly from the WT. In addition, the Δ*acm2* mutant showed virtual absence of autolysis induced by Triton X-100 even when treated with lysozyme. This lack of autolysis behaviour coincided with reduced biofilm formation capacity compared to WT as measured by CV staining. These observations are in line with the previously suggested role for autolysins in cell lysis and DNA release to the biofilm matrix reported for *E. faecalis* and *Staphylococcus epidermidis* (Bayles 2007; Frese et al. 2013; Qin et al. 2007; Thomas et al. 2008).

Taken together, results obtained with single and multiple Δ*cps1* mutants (enhanced lysis), and Δ*lytA* and Δ*acm2* mutants (reduced lysis) indicate that there is a correlation between the lytic behaviour of *L. plantarum* and total biofilm formation measured by CV staining with only slightly lower numbers of culturable biofilm cells. The absence of peptidoglycan hydrolase LytA and the major autolysin Acm2 proved to affect biofilm formation as the lack of cell lysis resulted in a lower contribution of eDNA to the matrix. This suggests that, in addition to proteinase treatment (Fernández Ramírez et al., 2015), implementation of DNAse treatments in surface cleaning strategies could support *L. plantarum* biofilm eradication in food processing environments. Such a strategy may not be economically feasible in a daily cleaning regime but could be useful in eradication of persistent biofilm problems in process environments. Moreover, a search for natural or chemical alternatives which disintegrate eDNA-supported biofilm cell interactions may increase opportunities for implementation of such an eDNA targeting strategy. In addition, a role for sortase A dependent proteins in biofilm formation was identified conceivably supporting cell-cell interactions as hypothesized in previous work that showed biofilm formation to be sensitive to proteinase K treatment (Fernández Ramírez et al. 2015). Further work will be required to determine the role in *L. plantarum* biofilm formation of sortase A dependent protein(s) in a range of conditions that may contribute to cell-cell interactions and biofilm formation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.02.013.

Acknowledgements

The project is funded by TI Food and Nutrition, a public-private partnership on precompetitive research in food and nutrition. The public partners are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The private partners have contributed to the project through regular discussion.

References

Abee, T., Kovacs, A.T., Kuipers, O.P., van der Veen, S., 2011. Biofilm formation and dispersal in gram-positive bacteria. Curr. Opin. Biotechnol. 22, 172–179.
Andre, G., Deghorain, M., Bron, P., van Swam, I., Kleerebezem, M., Hols, P., Dufrene, Y., 2011. Fluorescence and atomic force microscopy imaging of wall teichoic acids in *Lactobacillus plantarum*. ACS Chem. Biol. 6, 366–376.
Bartowsky, E.J., Henschke, P.A., 2008. Acetic acid bacteria spoilage of bottled red wine - a review. Int. J. Food Microbiol. 125, 60–70.
Bayles, K.W., 2007. The biological role of death and lysis in biofilm development. Nat. Rev. Microbiol. 5, 721–726.
Bjorkroth, K.J., Korkeala, H.J., 1996. *Lactobacillus fructivorans* spoilage of tomato ketchup. J. Food Prot. 60, 505–509.
Boekhorst, J., de Been, M.W.H.J., Kleerebezem, M., Siezen, R.J., 2005. Genome-wide detection and analysis of cell wall-bound proteins with LPxTG-like sorting motifs. J. Bacteriol. 187, 4928–4934.
Boesten, R.J., de Vos, W.M., 2008. Interactomics in the human intestine: lactobacilli and Bifidobacteria make a difference. J. Clin. Gastroenterol. 42 (Suppl 3 Pt 2), S163–167.
Branda, S.S., Vik, S., Friedman, L., Kolter, R., 2005. Biofilms: the matrix revisited. Trends Microbiol. 13, 20–26.
Bron, P.A., Meijer, M., Bongers, R.S., de Vos, W.M., Kleerebezem, M., 2007. Dynamics of competitive population abundance of *Lactobacillus plantarum ivi* gene mutants in faecal samples after passage through the gastrointestinal tract of mice. J. Appl. Microbiol. 103, 1424–1434.
Caplice, E., Fitzgerald, G.F., 1999. Food fermentations: role of microorganisms in food production and preservation. Int. J. Food Microbiol. 50, 131–149.
De Angelis, M., Siragusa, S., Campanella, D., Di Cagno, R., Gobbetti, M., 2015. Comparative proteomic analysis of biofilm and planktonic cells of *Lactobacillus plantarum* DB200. Proteomics 15, 2244–2257.
Du, L., He, X., Zhang, H., Liu, F., Ju, X., Yuan, J., 2015. First two domains at the lp_1643 protein N terminus inhibit pathogen adhesion to porcine mucus *in vitro*. J. Food Prot. 78, 370–375.
Fernández Ramírez, M.D., Smid, E.J., Abee, T., Nierop Groot, M.N., 2015. Characterisation of biofilms formed by *Lactobacillus plantarum* WCFS1 and food spoilage isolates. Int. J. Food Microbiol. 207, 23–29.
Fischetti, V.A., Pancholi, V., Schneewind, O., 1990. Conservation of a hexapeptide sequence in the anchor region of surface proteins from gram-positive cocci. Mol. Microbiol. 4, 1603–1605.
Francius, G., Lebeer, S., Alsteens, D., Wildling, L., Gruber, H.J., Hols, P., Keersmaecker, S.D., Vanderleyden, J., Dufrêne, Y.F., 2008. Detection, localization, and conformational analysis of single polysaccharide molecules on live bacteria. ACS Nano 2, 1921–1929.
Fredriksen, L., Mathiesen, G., Moen, A., Bron, P.A., Kleerebezem, M., Eijsink, V.G.H., Egge-Jacobsen, W., 2012. The major autolysin Acm2 from *Lactobacillus plantarum* undergoes cytoplasmic O-glycosylation. J. Bacteriol. 194, 325–333.
Frese, S.A., Mackenzie, D.A., Peterson, D.A., Schmaltz, R., Fangman, T., Zhou, Y., Zhang, C., Benson, A.K., Cody, L.A., Mulholland, F., Juge, N., Walter, J., 2013. Molecular characterization of host-specific biofilm formation in a vertebrate gut symbiont. PLoS Genet. 9, e1004057.
Guiton, P.S., Hung, C.S., Kline, K.A., Roth, R., Kau, A.L., Hayes, E., Heuser, J., Dodson, K.W., Caparon, M.G., Hultgren, S.J., 2009. Contribution of autolysin and Sortase a during *Enterococcus faecalis* DNA-dependent biofilm development. Infect. Immun. 77, 3626–3638.
Jakubovics, N.S., Shields, R.C., Rajarajan, N., Burgess, J.G., 2013. Life after death: the critical role of extracellular DNA in microbial biofilms. Lett. Appl. Microbiol. 57, 467–475.
Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W., Stiekema, W., Lankhorst,

R.M., Bron, P.A., Hoffer, S.M., Groot, M.N., Kerkhoven, R., de Vries, M., Ursing, B., de Vos, W.M., Siezen, R.J., 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U. S. A. 100, 1990–1995.
Kleerebezem, M., Hols, P., Bernard, E., Rolain, T., Zhou, M., Siezen, R., Bron, P., 2010. The extracellular biology of the lactobacilli. FEMS Microbiol. Rev. 34, 199–230.
Kubota, H., Senda, S., Nomura, N., Tokuda, H., Uchiyama, H., 2008. Biofilm formation by lactic acid bacteria and resistance to environmental stress. J. Biosci. Bioeng. 106, 381–386.
Kubota, H., Senda, S., Tokuda, H., Uchiyama, H., Nomura, N., 2009. Stress resistance of biofilm and planktonic *Lactobacillus plantarum* subsp. *plantarum* JCM 1149. Food Microbiol. 26, 592–597.
Landersjö, C., Yang, Z., Huttunen, E., Widmalm, G., 2002. Structural studies of the exopolysaccharide produced by *Lactobacillus rhamnosus* strain GG (ATCC 53103). Biomacromolecules 3, 880–884.
Lebeer, S., Verhoeven, T., Francius, G., Schoofs, G., Lambrichts, I., Dufrene, Y., Vanderleyden, J., De Keersmaecker, S., 2009. Identification of a gene cluster for the biosynthesis of a long, galactose-rich exopolysaccharide in *Lactobacillus rhamnosus* GG and functional analysis of the priming glycosyltransferase. Appl. Environ. Microbiol. 75, 3554–3563.
Lee, I.-C., Caggianiello, G., van Swam, I.I., Taverne, N., Meijerink, M., Bron, P.A., Spano, G., Kleerebezem, M., 2016. Strain-specific features of extracellular polysaccharides and their impact on *Lactobacillus plantarum*-host interactions. Appl. Environ. Microbiol. 82, 3959–3970.
Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. 15, 67–78.
Lyhs, U., Korkeala, H., Vandamme, P., Björkroth, J., 2001. *Lactobacillus alimentarius*: a specific spoilage organism in marinated herring. Int. J. Food Microbiol. 64, 355–360.
Malik, S., Petrova, M.I., Claes, I.J.J., Verhoeven, T.L.A., Busschaert, P., Vaneechoutte, M., Lievens, B., Lambrichts, I., Siezen, R.J., Balzarini, J., Vanderleyden, J., Lebeer, S., 2013. The highly autoaggregative and adhesive phenotype of the vaginal *Lactobacillus plantarum* strain CMPG5300 is sortase dependent. Appl. Environ. Microbiol. 79, 4576–4585.
Malik, S., Petrova, M.I., Imholz, N.C.E., Verhoeven, T.L.A., Noppen, S., Van Damme, E.J.M., Liekens, S., Balzarini, J., Schols, D., Vanderleyden, J., Lebeer, S., 2016. High mannose-specific lectin Msl mediates key interactions of the vaginal *Lactobacillus plantarum* isolate CMPG5300. Sci. Rep. 6, 37339.
Mercier, C., Durrieu, C., Briandet, R., Domakova, E., Tremblay, J., Buist, G., Kulakauskas, S., 2002. Positive role of peptidoglycan breaks in lactococcal biofilm formation. Mol. Microbiol. 46, 235–243.
Merritt, J.H., Kadouri, D.E., O'Toole, G.A., 2005. Growing and Analyzing Static Biofilms. In: Current Protocols in Microbiology. John Wiley & Sons, Inc..
Metselaar, K.I., Saá Ibusquiza, P., Ortiz Camargo, A.R., Krieg, M., Zwietering, M.H., den Besten, H.M.W., Abee, T., 2015. Performance of stress resistant variants of *Listeria monocytogenes* in mixed species biofilms with *Lactobacillus plantarum*. Int. J. Food Microbiol. 213, 24–30.
Moscoso, M., Garcia, E., Lopez, R., 2006. Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. J. Bacteriol. 188, 7785–7795.
Muñoz-Elías, E.J., Marcano, J., Camilli, A., 2008. Isolation of *Streptococcus pneumoniae* biofilm mutants and their characterization during nasopharyngeal colonization. Infect. Immun. 76, 5049–5061.
O'Toole, G., Kaplan, H.B., Kolter, R., 2000. Biofilm formation as microbial development. Annu. Rev. Microbiol. 54, 49–79.
Pratt, L.A., Kolter, R., 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. 30, 285–293.
Pretzer, G., Snel, J., Molenaar, D., Wiersma, A., Bron, P.A., Lambert, J., de Vos, W.M., van der Meer, R., Smits, M.A., Kleerebezem, M., 2005. Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. J. Bacteriol. 187, 6128–6136.
Qin, Z., Ou, Y., Yang, L., Zhu, Y., Tolker-Nielsen, T., Molin, S., Qu, D., 2007. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology 153, 2083–2092.
Remus, D., van Kranenburg, R., van Swam, I., Taverne, N., Bongers, R., Wels, M., Wells, J., Bron, P., Kleerebezem, M., 2012. Impact of 4 *Lactobacillus plantarum* capsular polysaccharide clusters on surface glycan composition and host cell signaling. Microb. Cell Factories 11, 149.
Remus, D.M., Bongers, R.S., Meijerink, M., Fusetti, F., Poolman, B., de Vos, P., Wells, J.M., Kleerebezem, M., Bron, P.A., 2013. Impact of *Lactobacillus plantarum* sortase on target protein sorting, gastrointestinal persistence, and host immune response modulation. J. Bacteriol. 195, 502–509.
Rice, K.C., Mann, E.E., Endres, J.L., Weiss, E.C., Cassat, J.E., Smeltzer, M.S., Bayles, K.W., 2007. The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U. S. A. 104, 8113–8118.
Rolain, T., Bernard, E., Courtin, P., Bron, P., Kleerebezem, M., Chapot-Chartier, M.-P., Hols, P., 2012. Identification of key peptidoglycan hydrolases for morphogenesis, autolysis, and peptidoglycan composition of *Lactobacillus plantarum* WCFS1. Microb. Cell Factories 11, 137.
Samelis, J., Kakouri, A., Rementzis, J., 2000. Selective effect of the product type and the packaging conditions on the species of lactic acid bacteria dominating the spoilage microbial association of cooked meats at 4°C. Food Microbiol. 17, 329–340.
Siezen, R., Francke, C., Renckens, B., Boekhorst, J., Wels, M., Kleerebezem, M., van Hijum, S., 2012. Complete resequencing and reannotation of the *Lactobacillus plantarum* WCFS1 genome. J. Bacteriol. 194, 195–196.
Silhavy, T., Kahne, D., Walker, S., 2010. The bacterial cell envelope. Cold Spring Harb. Perspect. Biol. 2, a000414.
Somers, E.B., Johnson, M.E., Wong, A.C., 2001. Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. J. Dairy Sci. 84, 1926–1936.
Stewart, P.S., Franklin, M.J., 2008. Physiological heterogeneity in biofilms. Nat. Rev. Microbiol. 6, 199–210.
Sturme, M.H., Fujii, T., Vaughan, E.E., Kleerebezem, M., de Vos, W.M., 2005. A Regulator Containing an AraC-type Helix-Turn-Helix DNA-binding Domain is Involved in Regulation of Cell Surface Properties in *Lactobacillus plantarum*. Wageningen Centre of Food Sciences, Wageningen.
Thomas, V.C., Thurlow, L.R., Boyle, D., Hancock, L.E., 2008. Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development. J. Bacteriol. 190, 5690–5698.
Turroni, F., Ventura, M., Buttó, L., Duranti, S., O'Toole, P., Motherway, M.C., van Sinderen, D., 2014. Molecular dialogue between the human gut microbiota and the host: a *Lactobacillus* and *Bifidobacterium* perspective. Cell. Mol. Life Sci. 71, 183–203.
van der Veen, S., Abee, T., 2011. Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. Int. J. Food Microbiol. 144, 421–431.
Watnick, P., Kolter, R., 2000. Biofilm, city of microbes. J. Bacteriol. 182, 2675–2679.