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RESEARCH ARTICLE

Exposure to specific fungal lectins during adhesion impairs biofilm formation of *Listeria* on polystyrene

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Abstract

Listeria monocytogenes is a pathogenic bacterium that can form biofilms in food processing plants, allowing the bacteria to survive despite the control measures applied. As the surface of the bacteria is covered with versatile polysaccharides and proteins, these influence the interactions of the bacterium with any surface. The unique properties and high stability of fungal proteins make them good candidates for the control of bacteria by targeting surface structures. We screened a group of fungal lectins and protease inhibitors from different fungal species, protein folds and known targets for their antibacterial and antibiofilm activity against model strains of *Listeria innocua* and *Listeria monocytogenes*. Several of them significantly decreased the viability of biofilm bacteria, but had no effect on bacterial growth parameters at 37°C and thus had no antibacterial activity. Fungal lectins significantly impaired biofilm development even at room temperature, which was attributed to exposure to lectins during adhesion. The tested fungal proteins also reduced biofilm development on biological model surfaces. The observed antibiofilm activity of fungal proteins suggests that they have the potential to modulate interactions between bacteria and/or between bacteria and surfaces, which could be used in the future to reduce surface contamination by *Listeria*.

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen associated with the consumption of contaminated ready-to-eat foods of animal and plant origin, frequently leading to food recalls and outbreaks in the European Union (EFSA, 2023). While the incidence of *Listeria* infection (listeriosis) is moderate, mortality among infected individuals is very high (20%–30%) (EFSA, 2023; Radoshevich & Cossart, 2017). And although regulatory controls have been in place for many years, cases of *L. monocytogenes* are not decreasing (Spanu & Jordan, 2020). This bacterium is extremely tolerant to elevated acidity and osmolarity, which are the basis for traditional food preservation methods, and grows in a temperature range between −0.4 and 45°C. Its persistence in the food production environment is attributed to its ability to attach firmly to various types of surfaces and develop a biofilm that provides further protection against environmental stressors (Mazaheri et al., 2021). Due to the pathogenicity and persistence of *L. monocytogenes* in the environment, testing antimicrobial strategies is challenging, especially on a large scale (Mohan et al., 2019). Because *Listeria innocua* is genetically related to *L. monocytogenes* and both naturally inhabit similar environments, continuous efforts are being made to find suitable surrogate strains within this species (Glaser et al., 2001). To date, there have been conflicting results on this approach, but the suitability of a surrogate strain may depend on the antimicrobial strategy and the similarity of targets between species (Janež et al., 2021).

Bacterial surface properties are the key determinant for the initial contact with abiotic surface (Zheng et al., 2021). The structures found on the surface of Gram-positive bacteria are peptidoglycan, lipoteichoic acids (LTA), cell wall teichoic acids (WTA) and cell wall proteins, which play a key role in bacterial survival and interaction with the environment (Rajagopal & Walker, 2017). The distribution or abundance of the surface structures determines the local surface properties that are important for bacterial attachment and biofilm development on abiotic surfaces (Bos et al., 1999; Park et al., 2022; Renier et al., 2014). These structures are also potential targets for preventing or interfering with surface colonization.

Fungi are a rich source of proteins, including lectins and protease inhibitors with unique specificity and ability to withstand extremes of temperature and pH and digestion by proteases, offering great potential for biotechnological applications (Künzler, 2018; Sabotič et al., 2016). Fungal lectins and protease inhibitors serve as natural defence systems of fungi: fungal lectins act on invaders by binding glycoepitopes in their intestinal system but do not recognize their own glycans, and protease inhibitors regulate the activity of proteolytic enzymes (proteases) that are important for metabolic and regulatory processes (Künzler, 2018; Sabotič et al., 2016).

Many fungal lectins and protease inhibitors have nematotoxic and/or entomotoxic activity, and continue to be investigated as an alternative pest control strategy (Bleuler-Martinez et al., 2011, 2022; Sabotič et al., 2012; Schubert et al., 2012; Šmid et al., 2013; Wohlschlager et al., 2011, 2020; Žurga et al., 2014). Since much less is known about their antimicrobial potential, a set of fungal lectins and protease inhibitors with different folds and known binding motifs (Tables 1 and 2) were selected in this study for analysis of their antibacterial and antibiofilm activity against *L. innocua* and *L. monocytogenes*. Their effects on the growth parameters and viability of biofilm bacteria on polystyrene were determined, followed by room temperature application time tests to further decipher their activity. In addition to polystyrene, the biological model surfaces mucin and fibronectin were also used to evaluate the effects of the fungal proteins on biofilm development.

EXPERIMENTAL PROCEDURES

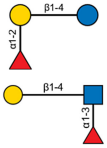
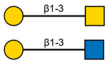
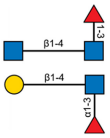
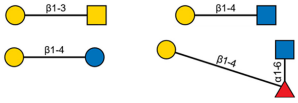
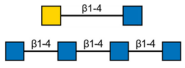
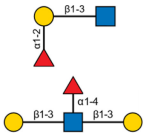
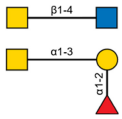
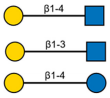
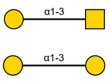
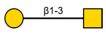
Strains and cultivation

Model strains *L. innocua* ŽM39 (Culture Collection of the Laboratory for Food Microbiology, Biotechnical Faculty, University of Ljubljana) and *L. monocytogenes* ATCC 19115 were grown in Luria broth (Miller modification: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl pH 7.5) at 37°C either on agar plates or in broth.

Heterologous expression of lectins and protease inhibitors

Escherichia coli expression system was used for production of recombinant fungal lectins and protease inhibitors tested in this study (Tables 1 and 2). Recombinant protease inhibitors clitocypin (Clt, UniProt ID: Q9P4A2), cospin (PIC, Uniprot ID: D0EWJ0) and macrocypins Macrocypin 1 (Mcp1, Uniprot ID: B9V973), Macrocypin 3 (Mcp3, Uniprot ID: B9V979), Macrocypin 4 (Mcp4, Uniprot ID: B9V982), were expressed using pET vectors as described previously (Sabotič et al., 2007, Sabotič et al., 2009, Sabotič et al., 2012). Similarly, recombinant lectins *Clitocybe nebularis* lectin (CNL, UniProt ID: B2ZRS9) and *Macrolepiota procera* lectin (MpL, Uniprot ID: F6KMV5) were prepared as described using pET expression system in *Escherichia coli* (Pohleven et al., 2012; Žurga et al., 2014). Coding sequences of lectins AAL (*Aleuria aurantia* lectin, UniProt ID: P18891), CCL2 (*Coprinopsis cinerea* lectin 2, UniProt ID: B3GA02), CGL2 (*Coprinopsis* galectin 2, UniProt ID: Q9P4R8), CGL3 (*Coprinopsis* galectin 3, UniProt ID: Q206Z5CML1) and MOA (*Marasmius*

TABLE 1 Effect of the fungal lectins tested in this study on the viability of *Listeria innocua* and *Listeria monocytogenes* within the biofilm, their sensitivity to proteinase K digestion and their known binding motifs.

Protein name	Known binding motif	Proteinase K sensitivity	Antibiofilm activity	
			Listeria innocua	*Listeria monocytogenes*
Aleuria aurantia lectin (AAL) (Wimmerova et al., 2003)		+++	+	+
Agaricus bisporus lectin (ABL) (Carrizo et al., 2005)		+++	+	+
Coprinopsis cinerea lectin 2 (CCL2) (Bleuler-Martinez et al., 2017)		–	–	–
Coprinopsis galectin 2 (CGL2) (Walser et al., 2004)		+	+	–
Coprinopsis galectin 3 (CGL3) (Wälti et al., 2008)		+++	-	–
Coprinopsis cinerea mucin-binding lectin 1 (CML1) (Bleuler-Martinez et al., 2022)		–	+	–
Clitocybe nebularis lectin (CNL) (Pohleven et al., 2012)		–[1]	+	+
concanavalin A (ConA)		+	–	–
Laccaria bicolor tectonin 2 (LbTec2) (Wohlschlager et al., 2014)		ND	ND	ND
Macrolepiota procera lectin (MpL) (Žurga et al., 2014)		–[1]	–	–
Marasmius oreades agglutinin (MOA) (Grahn et al., 2009)		+++	–	–
Sordaria macrospora transcript associated with perithecial development (TAP1) (Nowrousian & Cebula, 2005)		+++	+	+

Note: The symbolic nomenclature for glycans (SNFG) is used.

Abbreviations and Symbols: Glucose (Glc, blue circle), N-acetylglucosamine (GlcNAc, blue squares), fucose (Fuc, red triangles), galactose (Gal, yellow circles), N-acetylgalactosamine (GalNAc, yellow squares). Sensitivity to proteinase digestion was classified as follows: − no degradation, + partially degraded, +++ fully degraded protein. Antibiofilm activity is labelled + if the fungal protein statistically significantly ($p < 0.05$) reduced the viability of biofilm bacteria, and − if no effect was observed.

[1]Previously published by Žurga et al. (2015).

TABLE 2 Effect of the fungal protease inhibitors tested in this study on the viability of *Listeria innocua* and *Listeria monocytogenes* within the biofilm, their sensitivity to proteinase K digestion and their known in vitro specificity.

Protein name	Known binding motif	Proteinase K sensitivity	Antibiofilm activity	
			Listeria innocua	*Listeria monocytogenes*
Clitocypin (Clt) (Sabotič et al., 2007; Renko et al., 2009)	C1 and C13 cysteine peptidase family inhibitor	–[1]	ND	ND
Macrocypin 1 (Mcp1) (Renko et al., 2009; Sabotič et al., 2009)	C1 and C13 cysteine peptidase family inhibitor	–[1]	+	+
Macrocypin 3 (Mcp3) (Renko et al., 2009; Sabotič et al., 2009)	C1 and C13 cysteine peptidase family inhibitor	–[1]	–	–
Macrocypin 4 (Mcp4) (Renko et al., 2009; Sabotič et al., 2009)	C1 cysteine peptidase family and trypsin inhibitor	–[1]	-	+
Cospin (PIC) (Sabotič et al., 2012)	Trypsin inhibitor	–[1]	+	–

Note: Sensitivity to proteinase digestion was classified as follows: − no degradation, + partially degraded, +++ fully degraded protein. Antibiofilm activity is labelled + if the fungal protein statistically significantly ($p < 0.05$) reduced the viability of biofilm bacteria, and − if no effect was observed.

[1]Previously published by Žurga et al. (2015).

oreades agglutinin, UniProt ID: Q8X123) were cloned into pMCSG7 and/or pMCSG9 expression vectors using LIC protocols (Bleuler-Martinez et al., 2022; Boulianne et al., 2000; Kruger et al., 2002; Olausson et al., 2008; Schubert et al., 2012; Stols et al., 2002; Wälti et al., 2008). In reference to pMCSG7 vector, pMCSG9 contains an additional maltose-binding protein (MBP) solubility tag. Coding sequences for lectins ABL (*Agaricus bisporus* lectin, UniProt ID: Q00022), CML1 (*Coprinopsis cinerea* mucin-binding lectin 1, UniProt ID: B3VS76), TAP1 (*Sordaria macrospora* transcript associated with perithecial development, UniProt ID: F7VWP8), and LbTec2 (*Laccaria bicolor* tectonin 2, Uniprot ID: B0CZL6) were designed and codon-optimized based on an amino acid sequence from UniProt database and their synthetic genes obtained as gblocks (Integrated DNA Technologies) for cloning into expression vector pMCSG7 using LIC protocols. Heterologous expression conditions were optimized, and lectins were produced using different *E. coli* host strains, either BL21(DE3) or BL21-CODONPLUS (DE3)-RIL (Agilent Technologies), in ZYM-5052 autoinduction medium for 4 h at 37°C and 20 h at 18°C (Bleuler-Martinez et al., 2022; Nowrousian & Cebula, 2005; Studier, 2005; Wohlschlager et al., 2014). Bacteria were harvested with centrifugation and sonicated in buffer A (30 mM Tris pH 7.5, 400 mM NaCl) with 1 mg/mL lysozyme and cOMpLete™ EDTA-free Protease Inhibitor Cocktail (Roche). Lectins were purified using a two-step purification protocol with His-tag affinity chromatography (HisTrap FF 5 mL column (Cytiva) in buffer A with 10 mM imidazole for binding and 300 mM imidazole for elution) and size-exclusion chromatography (HiPrep 26/60 Sephacryl S-200 HR or HiPrep 26/60 Sephacryl S-100 HR column (Cytiva) in buffer A). To remove the MBP tag additional steps were performed with proteins produced using pMCSG9 vector as described below. Recombinant His6-tobacco etch virus protease (TEV protease) (1:50 [w/w]) was used to remove the His and MBP tag over the course of a 24 h incubation at 4°C with 10 mM β-mercaptoethanol. In a secondary NiNTA step using HisTrap FF 5 mL column the flow-through fraction containing the protein of interest was collected, concentrated and dialyzed against PBS pH 7.4. The recombinant proteins were stored in a PBS buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl) in aliquots at −20°C until use. Lectin Concanavalin A (ConA) (L-1000) was purchased from Vector Labs.

The purity and molecular masses of the fungal proteins (Figure S7) were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a homogeneous 15% (*w*/*v*) acrylamide gel with a thickness of 0.75 mm on a mPAGE Mini Gel System (Millipore). For SDS-PAGE, samples were dissolved in a sample buffer containing 300 mM Tris, 3% SDS, 38 mM EDTA, 0.6 M DTT, 60% glycerol and 4.5 mM bromophenol blue and incubated at 95°C for 10 min. The gels were stained with 0.1% (*w*/*v*) Coomassie Brilliant Blue R-350. Molecular mass was estimated using standard low molecular weight proteins of 14.4–97 kDa (Amersham GE Healthcare).

Growth curve measurement

Growth curves were determined as described before (Sterniša et al., 2022). Briefly, the inoculum (~10^5 CFU/mL) and fungal protein stocks were aliquoted in wells of 96-well microtiter plates (Nunc, Thermo Fisher Scientific) to a final volume of 200 μL and protein concentration 250 μg/mL. The microtiter plates were then incubated in a microtiter plate reader (Varioskan Lux, Thermo Fisher Scientific) at 37°C, with measurements of absorbance at 600 nm (OD_{600}) taken every 20 min for 20 h.

Agglutination assay

Overnight cultures of model strains were harvested by centrifugation and the pellet was resuspended in phosphate buffer (PBS). The OD_{600} of the bacterial suspension was adjusted to 0.2. Fifty microliters of the bacterial suspension were mixed with 50 μL of fungal protein at a concentration of 500 μg/mL in 96-well U-bottom microtiter plates (Nunc, Thermo Fisher Scientific). The mixture was mixed by pipetting and incubated for 15 min at room temperature to observe the formation of pellets. As agglutination did not reveal any visible aggregates, the experiments were repeated using μCLEAR microtiter plates (Greiner BioOne) so that the aggregates could be detected by bright-field microscopy at 100× magnification with oil immersion on the Olympus microscope CKX53. The experiments were repeated three times separately. The fungal protein was considered agglutinating if aggregates were observed in all three replicates.

Protease digestion sensitivity assay

Lectins and protease inhibitors were diluted in buffer (20 mM Tris–HCl, 0.3 M NaCl, 5 mM $CaCl_2$ pH 5) to a concentration of 0.4 mg/mL and were mixed with proteinase K (0.01 mg/mL). After the digestion for 2 h at 40°C, proteins were resolved on SDS-PAGE gel and the bands were visualized by silver staining.

Antibiofilm activity evaluated by viability assay

Experiments were performed as previously described (Klančnik et al., 2020). In summary, 96-well polystyrene microtiter plates (Nunc, Thermo Fisher Scientific) were inoculated with bacterial inoculum (~10^5 CFU/mL) mixed with fungal proteins to achieve final concentration of 250 μg/mL in a 1:1 ratio to final volume 200 μL and incubated at 37°C for 24 h. Quantification of biofilm cells was performed as described previously (Šikić Pogačar et al., 2016). Briefly, after incubation, the biofilms were rinsed three times and the remaining cells were detached by sonication at room temperature for 5 min at frequency 28 kHz and power 300 W (Iskra Pio). The number of bacteria was determined by plating (CFU/mL). Experiments were performed in three biological repetitions.

Antibiofilm activity evaluated by fluorescence microscopy (application time test)

Two different treatments were used to study the antibiofilm activity by fluorescence microscopy: (i) addition of fungal proteins at the time of inoculation of the biofilm (during adhesion) or (ii) addition of lectins to the 24-h-old biofilm to test its ability to disperse the biofilm. Overnight cultures of model strains were harvested by centrifugation and the OD_{600} of the bacterial suspension was adjusted to 0.2. Fifty microliters of the bacterial suspension were mixed with 50 μL of fungal protein at a concentration of 500 μg/mL on μCLEAR microtiter plates (Greiner BioOne) to test the effect on adhesion. The bacteria were allowed to adhere for 1 h at room temperature. Non-adherent bacteria were then removed and fresh broth was added, followed by overnight incubation at room temperature. Phosphate buffer was used to prepare the untreated control. To test dispersion, the biofilm was grown overnight in a similar way to the untreated control. Then fresh broth containing fungal protein at a concentration of 250 μg/mL was added and incubation was continued for 18 to 24 h. For titration experiments, the fungal proteins were diluted twofold (from 500 to 62.5 μg/mL) and treated as described above. After incubation, the wells were washed three times with PBS and the bacteria were labelled with the DNA probe SYTO9 (Thermo Fisher Scientific) at a final concentration of 3 μM. The unbound probe was removed after 30 min, and the remaining biofilm was immediately examined by fluorescence microscopy using Zeiss Observer.Z1 microscope with a 10x objective. For each experiment, the optimal settings for image acquisition were determined based on the untreated controls, and then the images were acquired. The raw images were compared visually, and the number of biofilm bacteria was analysed using CellProfiler3 (McQuin et al., 2018). To calculate the percentage of remaining biofilm bacteria, the quantitatively analysed images of the treated biofilms were compared with the untreated controls of the experiment. A reduction in biofilm bacteria of more than 50% was considered antibiofilm effective. The experiments were repeated three times independently.

The brightness of the images shown in Figure 2 was adjusted using Fiji to highlight the features of the biofilm for better visual assessment (Schindelin et al., 2012).

Antibiofilm activity evaluated on collagen, mucin and fibronectin

Microtiter plates were coated with 50 μL of mucin type II (50 μg/mL), fibronectin (10 μg/mL), or collagen type I (10, 25, 50 μg/mL) and incubated overnight at 4°C. Then, the excess solution was removed, and the wells were washed once with PBS. Biofilms were formed and analysed as described above for viability assay and fluorescence microscopy.

Cell viability assay

The viability of the human colorectal adenocarcinoma cell line (CaCo-2, ATCC; HTB-37) was examined after addition of selected fungal proteins having antibiofilm on biological model surfaces using the MTS assay CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Cells were maintained in the ATCC recommended culture medium containing heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco). Cells were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. For the MTS cell viability assay, 3×10^4 cells/well were seeded 12 h before the addition of the fungal proteins at a concentration of 125 μg/mL, and cell viability was determined after 48 h. Absorbance was measured at 490 nm using the Infinite M1000 microplate reader (Tecan). The experiments were repeated twice separately.

Adhesion assay on Caco-2 cell line

The adhesion experiment was performed as previously described with slight adjustments (Reddy & Austin, 2017). Overnight cultures of *L. monocytogenes* were harvested by centrifugation, and the OD_{600} of the bacterial suspension was adjusted to 20 in DMEM supplemented with FBS. A fungal protein and bacterial suspension were mixed in a 1:1 ratio to reach the final protein concentration used for the cell viability assay and incubated for 15 min at room temperature to allow lectin to bind to the bacterial surface. Phosphate buffer was used as a control. For the adhesion assay, two different treatments were performed: (i) the lectin was removed from the mixture by centrifugation or (ii) the lectin was not removed from the mixture prior to exposure to the Caco-2 cell line. Then, 30 μL of the treated or untreated bacterial suspension in 1 mL DMEM was added to the Caco-2 cell line layer to achieve a multiplicity of infection (MOI) of 10. The plates were then briefly centrifuged and incubated for 30 min at 37°C and 5% CO_2. The cells were then washed five times with PBS. To lyse the cells and detach adherent bacteria, 500 μL of cold 0.1% Triton X100 was added. The resulting lysates were serially diluted in PBS, and the dilutions were spotted onto Tryptone-soy (TS) agar plates. After 48 h, colonies were counted and CFU/mL were calculated. The experiments were repeated twice separately.

Statistical analysis

A one-way ANOVA was used to evaluate antibiofilm effects of fungal proteins determined with the viability test and Dunnett's test was used to determine whether there were statistically significant differences between fungal protein treated and untreated (control) samples. A p-value of $p < 0.05$ was considered statistically significant. The bacterial growth kinetics data were fitted using the model:

$$N = N_0 + \frac{h}{(1 + \exp(-r * (t - i)))} + \varepsilon,$$

where N represents the dependent variable indicating the number of bacteria, N_0 is the initial number of bacteria, h denotes the difference in OD_{600} between N_0 and the capacity (k), r is the growth rate, i represents the time at the inflexion point, t denotes the independent variable time and ε is the normally distributed error term of the model with zero expectation. The nonlinear growth model was fitted by improving the estimations of the four parameters (N_0, h, r and i) while minimizing the residual sum of squares. Regression diagnostics were performed in three steps: (1) calculating the explained variance rate (R^2) and testing its significance using a t test, (2) testing the model accuracy using an F test and (3) evaluating the significance levels of the parameter estimations by t tests. The models were accepted only if all the tests were significant ($p < 0.05$). The carrying capacity ($k = N_0 + h$), and the maximal specific growth rate ($\mu_{max} = h * r / 4$) were assessed from the estimated parameters. Finally, the estimated parameter values (r and μ_{max}) gained for the control and fungal protein treatment conditions were compared using t tests specifically developed for model parameter comparisons (Motulsky & Christopoulos, 2004).

A three-way ANOVA was performed to evaluate the effects of fungal proteins and application time on the remaining live biofilm of *L. innocua* and *L. monocytogenes* as determined by fluorescence microscopy. In addition, the effect of application time for all fungal proteins was investigated separately for each bacterial species.

Two-way ANOVA was used to compare biofilm bacteria grown on mucin or fibronectin versus polystyrene.

The normality of the residuals was deemed acceptable based on the absolute values of their skewness and kurtosis being below 1. The homogeneity of variances was assessed using Levene's test ($p < 0.05$).

Statistical analysis was conducted using IBM SPSS Statistics v29 (IBM Corp, Armonk, NY, 2022).

RESULTS

Effect of fungal proteins on growth parameters and viability of biofilm bacteria

Model strain of *L. innocua* was exposed to fungal proteins for 24 h and its growth was monitored to determine antibacterial activity. The observed differences in bacterial growth parameters were marginally significant, with the lectins AAL, CGL2, ConA, MOA and MpL slightly increasing the specific maximal growth rate (μ_{max}) of *L. innocua*, while CML1 and LbTec2 slightly decreased it (Table S1). Since fungal proteins could be used by bacteria as a nutrient source, leading to an increase in μ_{max}, susceptibility to digestion by proteinase K was tested. The susceptibility to proteinase K and the increase in μ_{max} coincide for the lectins AAL, CGL2, ConA and MOA, while MpL is resistant to proteinase K digestion and ABL, CGL3 and TAP1 are susceptible but have no effect on μ_{max}.

The model strains were then exposed to fungal proteins when they were growing in the form of a biofilm to test their antibiofilm activity. The viability of *L. innocua* biofilm bacteria was significantly reduced by the action of AAL, CGL2, ABL, CNL, TAP1 and CML1. The lectins AAL, ABL, CNL and TAP1 (Table 1) significantly reduced the viability of *L. monocytogenes* biofilm bacteria, while others did not.

None of the fungal protease inhibitors caused changes in the growth parameters of *L. innocua*. Antibiofilm activity against *L. innocua* was observed for Mcp1 and PIC, and against *L. monocytogenes* for Mcp1 and Mcp4 (Table 2).

Effect of fungal proteins on adhesion and biofilm dispersion on polystyrene at room temperature

The effect of fungal proteins on biofilm development was tested by exposing bacteria to the fungal proteins during adhesion (1 h) or 24 h after biofilm maturation to investigate the ability to disperse the biofilm. We detected significant overall fungal protein type effect (lectin versus protease inhibitor) and application time effect (adhesion versus dispersion) together with their interaction effect (lectin: $F(19,160) = 5.60$; application time: $F(1,160) = 46.14$; interaction: $F(19,160) = 4.29$, all with $p < 0.001$). The fungal proteins with effect on *L. innocua* and *L. monocytogenes* thus interfere with biofilm development during the adhesion and the effect of the lectins on the tested species was significantly greater than that of the protease inhibitors (lectin: 0.40; application time: 0.22). The lectins CGL3, LbTec2, MOA affected biofilm development during adhesion of *L. innocua* and CGL2, ConA, LbTec2, MOA and the protease inhibitor Mcp4 affected *L. monocytogenes* ($F(1,4) > 8.5$, $p < 0.05$) (Figure 1). Titration of fungal proteins did not abolish the effect of CGL3 in *L. innocua* (Figure S1) and CGL2, LbTec2 and MOA in *L. monocytogenes* (Figure S1).

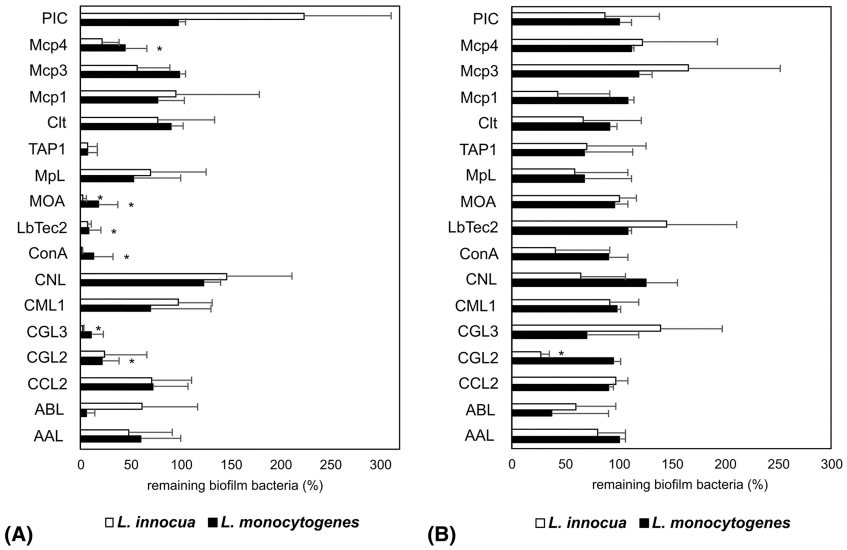


FIGURE 1 Effect of the tested fungal proteins at a concentration of 250 μg/mL on the biofilm development of *Listeria innocua* and *Listeria monocytogenes* on polystyrene at room temperature, distinguishing between the effect on adhesion (A) and the dispersing effect on the mature biofilm (B). Statistically significant differences between the application times (adhesion or dispersion) at $p < 0.05$ are marked with an asterisk.

Fungal lectins with a significant effect on the biofilm caused *L. innocua* to attach only loosely to the polystyrene, while the bacteria in the control group attached firmly and were immobile (Figure S4). Similar results were observed for the attachment of *L. monocytogenes*, with the exception of MOA and CGL2, where no changes were observed compared to the control (Figure S5). The fungal proteins ConA, Mcp4, CGL2 and TAP1, whose effect on the biofilm of *L. innocua* was pronounced but not significant (Figure 1), also caused loose attachment of the bacteria to the polystyrene. The lectin CML1, which had no effect on the biofilm, had no effect on attachment (Figure S4). Similar results were observed for *L. monocytogenes* (Figure S5).

There was no significant difference between the effect of the tested fungal proteins on the bacterial species ($F(1,160) = 0.63$, $p = 0.43$). A slightly significant three-way interaction effect was found ($F(19,160) = 1.61$, $p = 0.06$), which could mask a single significant fungal protein effect of the lectin CGL2, which also dispersed the biofilm of *L. innocua*, while all other fungal proteins only affected adhesion (Figures 1 and 2).

The fungal proteins tested did not lead to agglutination of the model bacteria at room temperature to the extent that visible pellets were formed (Figures S2, S3). Most of the fungal proteins tested do not cause aggregate formation, as only individual aggregates can be observed, but not in a repeatable manner, so their agglutinating activity was not considered conclusive (Table 3). The lectins ConA, LbTec2 and TAP1 tend to be less stable under storage conditions (Figure S7), as analysis on the gel also produced non-targeted bands and these lectins also gave inconclusive results; on the other hand, MOA and Mcp4 are stable but also give inconclusive agglutination results.

Effect of fungal proteins on *L. innocua* and *L. monocytogenes* biofilm on collagen, mucin and fibronectin-coated polystyrene

The model species strains did not form a biofilm on collagen, and no statistical significance was found in the number of biofilm bacteria within the untreated control biofilms on mucin and fibronectin compared to polystyrene independently whether *L. innocua* or *L. monocytogenes* was considered (bacterial species: $F(1,8) = 0.02$, $p = 0.89$; surface: $F(2,8) = 0.06$, $p = 0.94$). Lectins CGL2, CML1, TAP1 abolished *L. innocua* biofilm development on mucin and fibronectin. Additionally, CNL and MpL showed antibiofilm activity on mucin and Mcp4 and MOA on fibronectin (Table S2). The application time test based on microscopy confirmed the observations for TAP1 and CGL2 and for CNL on mucin. The biofilm of *L. monocytogenes* on mucin was prevented by CGL2, CGL3, MpL and TAP1, and by ConA on fibronectin (Table S2). Only the effect of ConA was confirmed by microscopy. Despite the descriptive congruence there was no statistically relevant correlation between the results of the methods used (viability assay, microscopy) due to the large variability (Pearson's $p > 0.05$).

Effect on *L. monocytogenes* adhesion to Caco-2 cell line

Fungal lectins, CGL2, CML1, TAP1 and CCL2, did not affect the viability of the Caco-2 cell line, but MOA decreased it (Figure S6A). Adhesion of *L. monocytogenes* to the Caco-2 cell line was slightly reduced

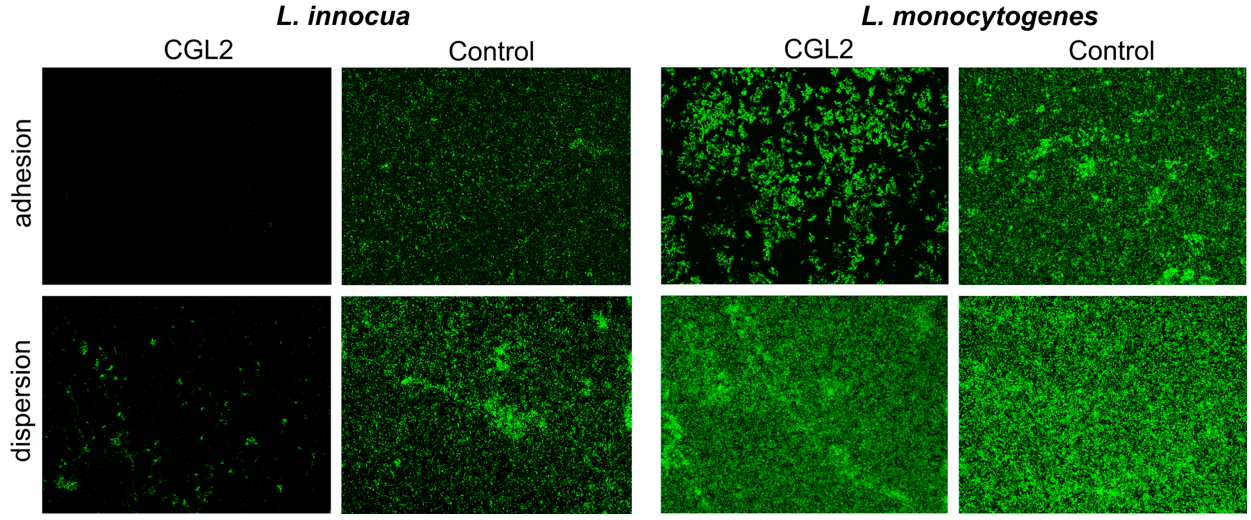


FIGURE 2 Effect of the lectin CGL2 on biofilm development of *Listeria innocua* and *Listeria monocytogenes* at the time of adhesion (1 h) and its effect on the 24-h-old mature biofilm to study its ability to disperse the biofilm. CGL2 was the only lectin tested that affected the mature biofilm. The bacteria of the remaining biofilm were labelled with the DNA probe Syto9, and images were taken at 10× magnification.

TABLE 3 Biochemical properties of the tested fungal proteins and their agglutination activity against *Listeria innocua* and *Listeria monocytogenes*.

Protein name abbreviation	Protein group	Number of binding sites per monomer/protein	Agglutination activity	
			Listeria innocua	*Listeria monocytogenes*
AAL	β-propeller	5/10	–	–
ABL	Actinoporin	2/8	Inc	Inc
CCL2	β-trefoil	1/2	–	Inc
CGL2	Galectin	1/4	–	–
CGL3	Galectin-like	1/4	–	–
CML1	2 antiparallel β-sheets Sandwich	1/6	–	–
CNL	β-trefoil	1/2	–	–
ConA	ConA-like protein	1/4	Inc	–
LbTec2	β -propeller	6/24	Inc	Inc
MpL	β -trefoil	1/2	–	–
TAP1	Actinoporin-like	Unknown	Inc	Inc
MOA	β-trefoil chimerolectin	3/6	Inc	Inc
CLT	β -trefoil	1/1	–	–
Mcp1	β -trefoil	1/1	–	–
Mcp3	β -trefoil	1/1	–	–
Mcp4	β -trefoil	1/1	Inc	–
PIC	β -trefoil	1/1	–	–

Note: Agglutination was evaluated as: − no agglutination, Inc - inconclusive.

in the presence of LbTec2 but could not be prevented by other lectins tested, CGL2, CML1 and TAP1, which have been shown to prevent biofilm development on model mucin or fibronectin surfaces (Figure S6B).

DISCUSSION

Biofilms are the most widespread form of bacterial growth in nature and provide a protective barrier against environmental stressors including antibiotics, disinfectants and other antimicrobial agents. As a result, they are difficult to remove, leading to continuous cross-contamination. Biofilms have therefore attracted a lot of attention, as preventing their formation would minimize the number of food recalls and possibly also the number of illnesses in the long term. Here, we consider fungal proteins as an alternative approach to prevent biofilm formation of *L. innocua* and *L. monocytogenes*. The fungal proteins tested are lectins, carbohydrate-binding proteins and protease inhibitors, which differ in their biochemical structures and known binding motifs (Tables 1, 2). The tested fungal lectins had only a minor effect on bacterial growth parameters, but caused a significant reduction in the viability of the biofilms of the tested bacteria at 37°C (Tables 1, 2). Both assays, antibacterial and antibiofilm, reflect the effect on the viability of bacteria, but differ in their execution. Antibacterial activity was evaluated using growth curves, which does not require sample preparation prior to measurement and allows conclusions to be drawn based on calculations of growth parameters. This is in contrast to the biofilm viability test, which requires extensive sample washing before plating out the bacteria. The decrease in bacterial viability of the biofilm could therefore be interpreted as a result of either antibacterial activity or increased destruction of the biofilm during washing, as the fungal proteins interfere with biofilm formation. An antibacterial activity can be excluded here, as the growth parameters change only slightly and the decrease in viability can therefore be attributed mainly to the activity of the fungal proteins on the biofilm formation itself. Lectins that have T-antigen (β-Gal-1,3-GalNAc) as a known target (ABL, TAP1 and CGL2) impaired biofilm development of *L. innocua* as well as *L. monocytogenes,* with the exception of CGL2. This could be the result of differences in protein folding or weaker binding to the T-antigen than in the other two (Walser et al., 2004). In contrast to CGL2, which has a galectin fold, ABL and TAP1 structurally resemble the pore-forming proteins actinoporins, which may be important for activity against *L. monocytogenes*. Lectin CNL also caused a reduction in the viability of biofilm bacteria of both species, but since it shares the known glycoepitope (β-GalNAc-1,4-GlcNAc) with

CGL3, which had no effect on the tested bacteria, this suggests either novel targets of this lectin or that slight differences in glycan-binding specificity play an important role. The antibiofilm effect of the protease inhibitors (Table 2) implies that inhibition of proteases or other protein–protein interactions are involved as important factors. Due to the 24-h exposure, the inhibition of surface proteases such as sortases, autolysins or signal peptidases as well as intracellular proteases could play a role. However, many of the surface proteins could also be targeted by these fungal protease inhibitors, as it has been shown that they can also bind via protein–protein interactions, altering their own activity and the activity of their binding partner (Žurga et al., 2015).

At room temperature, lectins had a significant effect on biofilm development when bacteria were exposed to them during adhesion. However, these differed from those that affected biofilm development at 37°C (Table 1, Figure 1). The observed effect at room temperature could be attributed to interference of the lectin with the attachment of bacteria, but this does not explain the activity of the lectins CGL2 and MOA on *L. monocytogenes* (Figure 1, Figures S4, S5). We can hypothesize that the effect of some of the tested fungal proteins is broader, thus affecting the bacterial surface in the long term or influencing their metabolism in yet unknown ways, eventually leading to impaired biofilm development. A possible mechanism of action could be the impairment of chemotaxis by disruption of motility, as observed in *Salmonella enterica* treated with mannose-binding lectin (MBL) (Xu et al., 2016). Motility of *Listeria monocytogenes* is known to be important for invasion of epithelial cells as well as biofilm formation during initial attachment and subsequent biofilm growth (Lemon et al., 2007; O'Neil & Marquis, 2006). This is of particular importance for the observations at room temperature, as *Listeria* down-regulates motility at 37°C, although there are some differences between strains (Gründling et al., 2004). In addition, it is known that the flagella of *Listeria* are glycosylated by β-O-linked N-acetylglucosamine, which is a potential target for lectins (Schirm et al., 2004). Lectins with antibiofilm activity at room temperature have no known common target, as appears to be the case for T- antigen at 37°C. Only the lectin ConA has already been shown to have an antibiofilm effect on *L. monocytogenes* (Facinelli et al., 1994). Here, we observed that ConA impairs adhesion of a model strain that has a similar glycosylation pattern of WTA as the strain used in Facinelli et al. (1994) and thus probably recognizes terminal glucose.

Although no pronounced agglutination activity was observed, the occasional formation of loosely bound aggregates could result in a large proportion of the biofilm being removed during washing, suggesting a reduced biofilm formation. Washing is essentially a standard practice in the preparation of biofilm samples to remove unbound bacteria that are not part of the biofilm and to remove background that interferes with subsequent biofilm detection, and is therefore still used in clinical biofilm research today. Even if sample preparation by washing may lead to this type of discrepancy, the effect of fungal proteins on intact biofilms is difficult to detect unless the proteins are labelled, as they affect the interactions between bacteria and the surface or between bacteria, but not the structure of the biofilm itself. Therefore, washing steps in sample preparation can be helpful in revealing these differences, as the biofilm can be broken up more easily by washing if the fungal proteins loosen the interactions within the biofilm.

Antibiofilm tests on biological model surfaces, fibronectin and mucin, were chosen to mimic the extracellular matrix relevant for the pathogenesis of *Listeria*. Binding to biological surfaces is thought to be mediated by specific interactions via proteins with fibronectin-binding domains or mucin-binding domains or other bacterial lectins, although the latter have not yet been identified in *Listeria* according to the Unilectin database (Bonnardel et al., 2020). The results of the two methods used (viability assay, microscopy) were mostly not congruent. The reason for the observed differences between the results of the viability assay and fluorescence microscopy is not clear, but since washing affects the structure of the biofilm, its effect may not be reproducible, leading to the observed discrepancies as discussed above.

The effect of some lectins and protease inhibitors on biofilm enhancement, which could be the disadvantage of their use against biofilms, could also be due to their potential sensitivity to protease digestion, as it has been shown that protein fragments resulting from proteolysis can enhance the biofilm phenotype as *Listeria* integrates the resulting peptides into the biofilm (Franciosa et al., 2009). However, sensitivity to proteolytic degradation by proteinase K is not a general indication of biofilm enhancement potential, as the lectins AAL, ABL, TAP1 and MOA were also digested by proteinase K but had no enhancing effect on biofilm development (Table 1).

CONCLUSIONS

The fungal proteins tested are thought to act on the bacterial surface through a specific interaction with cell wall components, either carbohydrate or protein moieties or both, leading to a long-term impairment of biofilm development without having an antibacterial effect. They are effective when the bacteria are exposed during adhesion, but have no effect on the

mature biofilm. Although biofilm methodology generally needs to be improved to achieve reproducible results and clearly demonstrate antibiofilm properties, we conclude that the observed effects of fungal proteins on the development of *Listeria* biofilms on different types of surfaces suggest that they have the potential to modulate interactions between bacteria and/or between bacteria and surfaces. These effects could be used to reduce the bacterial load on various abiotic surfaces or biological systems, either alone or in combination with established antimicrobial compounds.

AUTHOR CONTRIBUTIONS

Nika Janež: Conceptualization; data curation; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Márta Ladányi:** Data curation; formal analysis; writing – review and editing. **Meta Sterniša:** Investigation; writing – review and editing. **Blaž Jug:** Investigation; writing – review and editing. **Tanja Zupan:** Investigation; writing – review and editing. **Tjaša Peternel:** Investigation; writing – review and editing. **Aleksandar Sebastijanović:** Investigation; writing – review and editing. **Milica Perišić Nanut:** Investigation; writing – review and editing. **Katarina Karničar:** Investigation; writing – review and editing. **Ajda Taler-Verčič:** Investigation; writing – review and editing. **Dušan Turk:** Resources; funding acquisition; writing – review and editing. **Anja Klančnik:** Resources; funding acquisition; writing – review and editing. **Janez Štrancar:** Funding acquisition; resources; writing – review and editing. **Jerica Sabotič:** Conceptualization; methodology; funding acquisition; project administration; resources; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have no competing interests to declare that are relevant to the content of this article.

DATA AVAILABILITY STATEMENT

The authors declare that the data supporting the findings of this study are available within the manuscript and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

DECLARATION OF GENERATIVE AI IN SCIENTIFIC WRITING

During the preparation of this work the author(s) used Instatext in order to improve readability and language. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

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REFERENCES

Bleuler-Martinez, S., Butschi, A., Garbani, M., Wällti, M.A., Wohlschlager, T., Potthoff, E. et al. (2011) A lectin-mediated resistance of higher fungi against predators and parasites. *Molecular Ecology*, 20, 3056–3070.

Bleuler-Martinez, S., Stutz, K., Sieber, R., Collot, M., Mallet, J.M., Hengartner, M. et al. (2017) Dimerization of the fungal defense lectin CCL2 is essential for its toxicity against nematodes. *Glycobiology*, 27, 486–500.

Bleuler-Martinez, S., Varrot, A., Olieric, V., Schubert, M., Vogt, E., Fetz, C. et al. (2022) Structure-function relationship of a novel fucoside-binding fruiting body lectin from *Coprinopsis cinerea* exhibiting nematotoxic activity. *Glycobiology*, 32, 600–615.

Bonnardel, F., Perez, S., Lisacek, F. & Imberty, A. (2020) Structural database for lectins and the UniLectin web platform. *Methods in Molecular Biology*, 2132, 1–14.

Bos, R., van der Mei, H.C. & Busscher, H.J. (1999) Physico-chemistry of initial microbial adhesive interactions—its mechanisms and methods for study. *FEMS Microbiology Reviews*, 23, 179–230.

Boulianne, R.P., Liu, Y., Aebi, M., Lu, B.C. & Kues, U. (2000) Fruiting body development in *Coprinus cinereus*: regulated expression of two galectins secreted by a non-classical pathway. *Microbiology (N Y)*, 146, 1841–1853.

Carrizo, M.E., Capaldi, S., Perduca, M., Irazoqui, F.J., Nores, G.A. & Monaco, H.L. (2005) The antineoplastic lectin of the common edible mushroom (*Agaricus bisporus*) has two binding sites, each specific for a different configuration at a single epimeric hydroxyl. *The Journal of Biological Chemistry*, 280, 10614–10623.

EFSA. (2023) The European Union one health 2022 zoonoses report. *EFSA Journal*, 21, e8442.

Facinelli, B., Giovanetti, E., Casolari, C. & Varaldo, P.E. (1994) Interactions with lectins and agglutination profiles of clinical, food, and environmental isolates of *Listeria*. *Journal of Clinical Microbiology*, 32, 2929–2935.

Franciosa, G., Maugliani, A., Scalfaro, C., Floridi, F. & Aureli, P. (2009) Expression of internalin A and biofilm formation among *Listeria monocytogenes* clinical isolates. *International Journal of Immunopathology and Pharmacology*, 22, 183–193.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F. et al. (2001) Comparative genomics of *Listeria* species. *Science*, 294, 849–852.

Grahn, E.M., Winter, H.C., Tateno, H., Goldstein, I.J. & Krengel, U. (2009) Structural characterization of a lectin from the mushroom *Marasmius oreades* in complex with the blood group B trisaccharide and calcium. *Journal of Molecular Biology*, 390, 457–466.

Gründling, A., Burrack, L.S., Bouwer, H.G.A. & Higgins, D.E. (2004) *Listeria monocytogenes* regulates flagellar motility gene expression through MogR, a transcriptional repressor required for virulence. *Proceedings of the National Academy of Sciences*, 101, 12318–12323.

Janež, N., Škrlj, B., Sterniša, M., Klančnik, A. & Sabotič, J. (2021) The role of the *Listeria monocytogenes* surfactome in biofilm formation. *Microbial Biotechnology*, 14, 1269–1281.

Klančnik, A., Gobin, I., Jeršek, B., Možina, S.S., Vučković, D., Žnidarič, M.T. et al. (2020) Adhesion of *Campylobacter jejuni* is increased in association with foodborne bacteria. *Microorganisms*, 8(2), 201.

Kruger, R.P., Winter, H.C., Simonson-Leff, N., Stuckey, J.A., Goldstein, I.J. & Dixon, J.E. (2002) Cloning, expression, and characterization of the Galalpha-1,3-gal high affinity lectin from the mushroom *Marasmius oreades*. *The Journal of Biological Chemistry*, 277, 15002–15005.

Künzler, M. (2018) How fungi defend themselves against microbial competitors and animal predators. *PLoS Pathogens*, 14(9), e1007184.

Lemon, K.P., Higgins, D.E. & Kolter, R. (2007) Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. *Journal of Bacteriology*, 189, 4418–4424.

Mazaheri, T., Cervantes-Huamán, B.R.H., Bermúdez-Capdevila, M., Ripolles-Avila, C. & Rodríguez-Jerez, J.J. (2021) *Listeria monocytogenes* biofilms in the food industry: is the current hygiene program sufficient to combat the persistence of the pathogen? *Microorganisms*, 9, 1–19.

McQuin, C., Goodman, A., Chernyshev, V., Kamentsky, L., Cimini, B.A., Karhohs, K.W. et al. (2018) CellProfiler 3.0: Next-generation image processing for biology. *PLoS Biology*, 16, e2005970.

Mohan, V., Wibisono, R., de Hoop, L., Summers, G. & Fletcher, G.C. (2019) Identifying suitable *Listeria innocua* strains as surrogates for *Listeria monocytogenes* for horticultural products. *Frontiers in Microbiology*, 10, 2281.

Motulsky, H. & Christopoulos, A. (2004) *Fitting models to biological data using linear and nonlinear regression: a practical guide to curve fitting*. New York: Fitting models to biological data using linear and nonlinear regression, 2004; online edn, Oxford.

Nowrousian, M. & Cebula, P. (2005) The gene for a lectin-like protein is transcriptionally activated during sexual development, but is not essential for fruiting body formation in the filamentous fungus *Sordaria macrospora*. *BMC Microbiology*, 5, 1–10.

Olausson, J., Tibell, L., Jonsson, B.H. & Påhlsson, P. (2008) Detection of a high affinity binding site in recombinant *Aleuria aurantia* lectin. *Glycoconjugate Journal*, 25, 753–762.

O'Neil, H.S. & Marquis, H. (2006) *Listeria monocytogenes* flagella are used for motility, not as adhesins, to increase host cell invasion. *Infection and Immunity*, 74, 6675–6681.

Park, M., Kim, J., Horn, L., Haan, J., Strickland, A., Lappi, V. et al. (2022) Sugar modification of wall teichoic acids determines serotype-dependent strong biofilm production in *Listeria monocytogenes*. *Microbiology Spectrum*, 10(5), e0276922.

Pohleven, J., Renko, M., Magister, Š., Smith, D.F., Künzler, M., Štrukelj, B. et al. (2012) Bivalent carbohydrate binding is required for biological activity of *Clitocybe nebularis* lectin (CNL), the N,N′-Diacetyllactosediamine (GalNAcβ1–4GlcNAc, LacdiNAc)-specific lectin from Basidiomycete *C. nebularis*. *The Journal of Biological Chemistry*, 287, 10602–10612.

Radoshevich, L. & Cossart, P. (2017) *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis. *Nature Reviews. Microbiology*, 16, 32–46.

Rajagopal, M. & Walker, S. (2017) Envelope structures of Gram-positive bacteria. *Current Topics in Microbiology and Immunology*, 404, 1–44.

Reddy, S. & Austin, F. (2017) Adhesion and invasion assay procedure using Caco-2 cells for *Listeria monocytogenes*. *Bio-Protocol*, 7(9), e2267.

Renier, S., Chagnot, C., Deschamps, J., Caccia, N., Szlavik, J., Joyce, S.A. et al. (2014) Inactivation of the SecA2 protein export pathway in *Listeria monocytogenes* promotes cell aggregation, impacts biofilm architecture and induces biofilm formation in environmental condition. *Environmental Microbiology*, 16, 1176–1192.

Renko, M., Sabotič, J., Mihelič, M., Brzin, J., Kos, J. & Turk, D. (2009) Versatile loops in mycocypins inhibit three protease families. *The Journal of Biological Chemistry*, 285, 308–316.

Sabotič, J., Bleuler-Martinez, S., Renko, M., Caglič, P.A., Kallert, S., Štrukelj, B. et al. (2012) Structural basis of trypsin inhibition and entomotoxicity of cospin, serine protease inhibitor involved in defense of *Coprinopsis cinerea* fruiting bodies. *The Journal of Biological Chemistry*, 287, 3898–3907.

Sabotič, J., Galeša, K., Popovič, T., Leonardi, A. & Brzin, J. (2007) Comparison of natural and recombinant clitocypins, the fungal cysteine protease inhibitors. *Protein Expression and Purification*, 53, 104–111.

Sabotič, J., Ohm, R.A. & Künzler, M. (2016) Entomotoxic and nematotoxic lectins and protease inhibitors from fungal fruiting bodies. *Applied Microbiology and Biotechnology*, 100, 91–111.

Sabotič, J., Popovič, T., Puizdar, V. & Brzin, J. (2009) Macrocypins, a family of cysteine protease inhibitors from the basidiomycete *Macrolepiota procera*. *The FEBS Journal*, 276, 4334–4345.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T. et al. (2012) Fiji: an open-source platform for biological-image analysis. *Nature Methods*, 9(7), 676–682.

Schirm, M., Kalmokoff, M., Aubry, A., Thibault, P., Sandoz, M. & Logan, S.M. (2004) Flagellin from *Listeria monocytogenes* is glycosylated with β-O-linked N-acetylglucosamine. *Journal of Bacteriology*, 186, 6721–6727.

Schubert, M., Bleuler-Martinez, S., Butschi, A., Wälti, M.A., Egloff, P., Stutz, K. et al. (2012) Plasticity of the β-trefoil protein fold in the recognition and control of invertebrate predators and parasites by a fungal defence system. *PLoS Pathogens*, 8, e1002706.

Šikić Pogačar, M., Klančnik, A., Bucar, F., Langerholc, T. & Smole Možina, S. (2016) Anti-adhesion activity of thyme (*Thymus vulgaris* L.) extract, thyme post-distillation waste, and olive (*Olea europea* L.) leaf extract against *Campylobacter jejuni* on polystyrene and intestine epithelial cells. *Journal of the Science of Food and Agriculture*, 96, 2723–2730.

Šmid, I., Gruden, K., Buh Gašparič, M., Koruza, K., Petek, M., Pohleven, J. et al. (2013) Inhibition of the growth of Colorado potato beetle larvae by macrocypins, protease inhibitors from the parasol mushroom. *Journal of Agricultural and Food Chemistry*, 61, 12499–12509.

Spanu, C. & Jordan, K. (2020) *Listeria monocytogenes* environmental sampling program in ready-to-eat processing facilities: a practical approach. *Comprehensive Reviews in Food Science and Food Safety*, 19, 2843–2861.

Sterniša, M., Sabotič, J. & Klančnik, A. (2022) A novel approach using growth curve analysis to distinguish between antimicrobial and anti-biofilm activities against *Salmonella*. *International Journal of Food Microbiology*, 364, 109520.

Stols, L., Gu, M., Dieckman, L., Raffen, R., Collart, F.R. & Donnelly, M.I. (2002) A new vector for high-throughput, ligation-independent cloning encoding a tobacco etch virus protease cleavage site. *Protein Expression and Purification*, 25, 8–15.

Studier, F.W. (2005) Protein production by auto-induction in high density shaking cultures. *Protein Expression and Purification*, 41, 207–234.

Walser, P.J., Haebel, P.W., Künzler, M., Sargent, D., Kües, U., Aebi, M. et al. (2004) Structure and functional analysis of the fungal galectin CGL2. *Structure*, 12, 689–702.

Wälti, M.A., Walser, P.J., Thore, S., Grünler, A., Bednar, M., Künzler, M. et al. (2008) Structural basis for chitotetraose-coordination by CGL3, a novel galectin-related protein from *Coprinopsis cinerea*. *Journal of Molecular Biology*, 379, 146–159.

Wimmerova, M., Mitchell, E., Sanchez, J.F., Gautier, C. & Imberty, A. (2003) Crystal structure of fungal lectin. Six-bladed β-propeller fold and novel fucose recognition mode for *Aleuria aurantia* lectin. *Jl Biol Chem*, 278, 27059–27067.

Wohlschlager, T., Butschi, A., Grassi, P., Sutov, G., Gauss, R., Hauck, D. et al. (2014) Methylated glycans as conserved targets of animal and fungal innate defense. *Proceedings of the National Academy of Sciences*, 111, e2787.

Wohlschlager, T., Butschi, A., Zurfluh, K., Vonesch, S.C., Auf Dem Keller, U., Gehrig, P. et al. (2011) Nematotoxicity of *Marasmius oreades* agglutinin (MOA) depends on glycolipid binding and cysteine protease activity. *The Journal of Biological Chemistry*, 286, 30337–30343.

Wohlschlager, T., Titz, A., Künzler, M. & Varrot, A. (2020) Expression, purification, and functional characterization of tectonin 2 from *Laccaria bicolor*: a six-bladed beta-propeller lectin specific for O-methylated glycans. *Methods in Molecular Biology*, 2132, 669–682.

Xu, J., Nakamura, S., Islam, M.S., Guo, Y., Ihara, K., Tomioka, R. et al. (2016) Mannose-binding lectin inhibits the motility of pathogenic *Salmonella* by affecting the driving forces of motility and the chemotactic response. *PLoS One*, 11(4), e0154165.

Zheng, S., Bawazir, M., Dhall, A., Kim, H.E., He, L., Heo, J. et al. (2021) Implication of surface properties, bacterial motility, and hydrodynamic conditions on bacterial surface sensing and their initial adhesion. *Frontiers in Bioengineering and Biotechnology*, 9, 643722.

Žurga, S., Pohleven, J., Kos, J. & Sabotič, J. (2015) β-Trefoil structure enables interactions between lectins and protease inhibitors that regulate their biological functions. *Journal of Biochemistry*, 158, 83–90.

Žurga, S., Pohleven, J., Renko, M., Bleuler-Martinez, S., Sosnowski, P., Turk, D. et al. (2014) A novel β-trefoil lectin from the parasol mushroom (*Macrolepiota procera*) is nematotoxic. *The FEBS Journal*, 281, 3489–3506.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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