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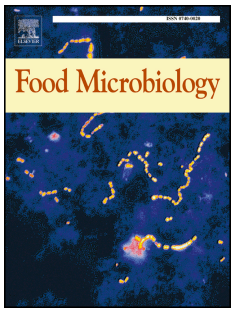

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Lactococcus lactis subsp. *lactis* as a natural anti-listerial agent in the mushroom industry

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Abstract

Mushroom growth substrates from different commercial producers of mushrooms (*Agaricus bisporus*) were screened for the presence of bacteria with potential for use as biocontrol agents for controlling *Listeria monocytogenes* in the mushroom production environment. Eight anti-listerial strains were isolated from different sources and all were identified using 16s rRNA gene sequencing as *Lactococcus lactis* subsp. *lactis*. Whole-genome sequencing of the *Lc. lactis* isolates indicated that strains from different sites and substrate types were highly similar. Colony MALDI-TOF mass spectrometry found that these strains were Nisin Z producers but inhibitory activity was highly influenced by the incubation conditions and was strain dependant. The biofilm forming ability of these strains was tested using a crystal violet assay and all were found to be strong biofilm formers. Growth of *Lc. lactis* subsp. *lactis* using mixed-biofilm conditions with *L. monocytogenes* on stainless steel resulted in a 4-log reduction of *L. monocytogenes* cell numbers. Additional sampling of mushroom producers showed that these anti-listerial *Lc. lactis* strains are commonly present in the mushroom production environment. *Lc. lactis* has a generally regarded as safe (GRAS) status and therefore has potential for use as an environmentally benign solution to control *L. monocytogenes* in order to prevent product contamination and to enhance consumer confidence in the mushroom industry.

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1 Introduction

Listeria monocytogenes is the causative agent of listeriosis in humans and a foodborne pathogen of significant public health concern. It is ubiquitous in nature and it can contaminate food crops and food processing environments (Ferreira et al., 2014). Although there has been an isolated report of *L. monocytogenes* associated septicaemia in an elderly man linked with the consumption of wild-collected salted mushrooms (*Lactarius rufus,* Junttila and Brander, 1989), there have been, to the author's knowledge, no reports of listeriosis due to the consumption of fresh cultivated mushrooms (*Agaricus bisporus*). However, there is concern in the mushroom industry as studies have shown that *L. monocytogenes* can be found in mushroom production facilities which therefore pose a risk of product contamination (Murugesan et al., 2015; Viswanath et al., 2013). In recent years, multiple recalls occurred in Canada for *L. monocytogenes* contaminated sliced mushroom products, although there were no incidents of listeriosis reported (Canadian Food Inspection Agency, 2012, 2014, 2015). Additionally, there have been eleven notifications of mushrooms contaminated with *L. monocytogenes* since 2013 in the European Commission's Rapid Alert System for Food (RASFF) database. These recalls resulted in an economic and reputational loss for the industry. Thus, it is important to take proactive steps to maintain this industry's current reputation for food safety by exploring novel biocontrol agents to provide enhanced assurance of product quality and safety.

The primary concern in food production and processing environments, including the mushroom industry, is that *L. monocytogenes* biofilms can form on approved materials (e.g. stainless steel, rubber and polymers), including food contact surfaces (Beresford et al., 2001; Bridier et al., 2014; Di Bonaventura et al., 2008). Studies of disinfectants or sanitizing agents that are currently used in the food industry (i.e. quaternary ammonium compounds, chlorine based compounds and oxidising agents) have found that they can reduce biofilm on different surfaces, but they are not consistently effective in dealing with *L. monocytogenes* biofilms, as the reduction levels were dependent on biofilm age, type of surface, and substrate on which the biofilms were formed (Amalaradjou et al., 2009; O'Neill et al., 2015; Pan et al., 2006; Yang et al., 2009). In addition, several studies suggest that listerial biofilms that have been exposed to certain antimicrobials, which did not result in complete removal of the pathogen, induced a change in biofilm structure with increased antimicrobial

resistance (Ibusquiza et al., 2011; Pan et al., 2006; Yun et al., 2012). This shows the need for novel agents that can effectively control listerial biofilm and ideally are environmentally benign. As an alternative to chemical disinfectants, biological control (biocontrol) options could be promising alternatives, as they are considered natural, have a "green" image and are environmentally friendly.

Currently, the mushroom industry uses the commercial product Nemasys® (BASF, Germany) as a biocontrol agent which utilises a nematode called *Steinernema feltiae*, to control sciarid fly larvae during mushroom production cycles. However, such an approach against food-borne pathogens that could contaminate the crop has not been considered. One option for this approach during production would be to utilise the bacteriocin nisin, which is used in the food industry as a food additive that acts as a natural preservative, mainly as an anti-botulinic agent in cheese and liquid eggs, sauces and canned foods (Balciunas et al., 2013). Nisin is produced by certain strains of *Lactococcus lactis* subsp. *lactis* and is the best-studied bacteriocin. Another way of utilising bacteriocin-producing bacteria (BPB) is through competitive exclusion, where the BPB could be used to take over the niche occupied by pathogenic bacteria, such as *L. monocytogenes*, to potentially inhibit the growth of the pathogen. Within a food production environment, bacteria that have GRAS status, such as lactic acid bacteria (LAB) are ideal as they pose low risk to public health. Studies investigating such approaches have found that competitive exclusion bacteria can control *L. monocytogenes* biofilms formed on surfaces found in food processing facilities (García-Almendárez et al., 2008; Zhao et al., 2013). The aim of this study was to isolate and evaluate bacteriocins or bacteriocin-producing bacteria naturally present in mushroom growth substrates, which can prevent or eliminate *L. monocytogenes* and *L. monocytogenes* biofilm formation.

2 Materials and Methods

2.1 Strains

The main target strain used for activity tests in this study was *L. monocytogenes* 6179, a persistent strain isolated from a cheese processing plant (Fox et al., 2011). The other indicator strains used for activity spectrum tests are listed in Table 1. *L. monocytogenes* strain 2081, isolated from a mushroom production facility, was identified as a strong biofilm

former (unpublished results), while strains 1698 and 1721 were identified by Bolocan *et al.* (2016). All bacterial strains were stored on Protect beads (Technical Service Consultants Ltd., UK) with 50% glycerol solution at -80 °C. All the stored cultures from Table 1 were resuscitated by streaking a bead on to Tryptone Soya Agar (TSA, Oxoid UK) and incubating at 37°C for 18-24 hours.

2.2 Isolation of anti-listerial bacteria

The three types of mushroom growth substrates that were used for isolating anti-listerial bacteria are as follows: fresh mushroom casing (a nutrient-poor peat based substrate), phase-3 substrate (a nutrient-rich straw and manure based substrate that has been composted, pasteurised and then colonised with mushroom mycelium) and spent mushroom substrate (SMS), which is what remains at the end of the crop. Batches of fresh mushroom casing were obtained from four different farms, phase-3 substrate from one farm and SMS from two farms. All the farms were located in different regions of Ireland. For the preliminary screening for anti-listerial bacteria (ALB), 25 g of substrate was diluted in 225 ml of maximum recovery diluent (MRD, Oxoid, UK) and homogenised in a stomacher for five minutes. Ten-fold serial dilutions of the homogenised samples were then carried out and 100 µl of each dilution were spread plated on different media: LB agar (Oxoid, UK), TSA supplemented with 0.6% yeast extract (TSAYE, Oxoid, UK), Brain Heart Infusion (BHI) agar (Oxoid, UK), de Man, Rogosa and Sharpe agar (MRS, Oxoid, UK), GM17 (M17 supplemented with 0.5% glucose, Oxoid, UK), LM17 (M17 supplemented with 0.5% lactose, Oxoid, UK) and Mueller-Hinton agar (Oxoid, UK). The plates were then incubated at a number of temperatures and atmospheres to promote ideal conditions for antagonistic activity: aerobically for 24 hours at 37°C, anaerobically for 24 hours at 37°C, aerobically for 24 hours at 25°C and anaerobically for 24 hours at 25°C. After incubation, the plates were overlaid with soft TSAYE (0.75% agar) seeded with approximately $\log_{10}$ 6 CFU ml^{-1} of *L. monocytogenes* 6179 and then incubated again aerobically for 24 hours at 37°C. Positive results for inhibition were identified as visually detectable zones of clearing around a colony or colonies after incubation.

2.3 Identification of anti-listerial agents in cell-free supernatants

Cell-free supernatants (CFS) of isolates with antagonistic activity from the previous test were analysed for their anti-listerial activity using a sensitive well diffusion assay described by Alvarez-Ordóñez *et al.* (2013), with minor modifications. Briefly, two types of solid media were utilised for this assay: an underlay (1% agarose [Sigma-Aldrich, UK], 0.03% TSB [Oxoid, UK] and 0.02% Tween 20 [Sigma-Aldrich, UK]) and overlay (1% agarose, 1X TSB). Wells with a diameter of 8 mm were made on the underlay that was seeded with approximately $\log_{10}$ 6 CFU ml^{-1} of indicator *L. monocytogenes* 6179 strain. Then, 50 µl aliquots of neutralised CFS were added in to the wells and allowed to diffuse for two hours. The overlay was poured over the underlay media, allowed to solidify and incubated at 37°C for 24 hours. Other *L. monocytogenes* strains and foodborne contaminants such as *Escherichia coli* (NCTC 9001), *Staphylococcus aureus* (NCTC 6571) and *Salmonella* Typhimurium (SARB 67) were also tested for inhibition (Table 1). This experiment was repeated three times and the activity was interpreted as the presence or absence of a zone of clearance. The proteinaceous nature of the anti-listerial agents in the CFS was then confirmed using proteolytic enzymes and heat treatments, as shown in Supplementary Table 1. Colonies with anti-listerial activity were selected and the bacteriocins produced were identified using colony mass spectrometry according to the method described by Field et al. (2015). The resulting peptide masses were then compared to a web-based database (BAGEL) to identify putative bacteriocins (de Jong et al., 2006).

2.4 Determination of anti-biofilm activity of ALB

A modified crystal violet assay was used to test the anti-biofilm efficacy of ALB CFS on pre-formed 72h biofilms of *L. monocytogenes* strains (1698, 1721 and 2081) based on the method used by Bolocan et al. (2016), with minor modifications. In brief, 72h biofilms grown in microtitre plates were treated with ALB CFS for 24h, stained with crystal violet and the absorbance results compared with the biofilms treated without bacteriocins.

2.5 Identification of anti-listerial bacteria

The potential anti-listerial bacteria were initially identified according to their 16S rRNA gene sequence. This was carried out according to the method described by Chopra et al.,

(2014), with minor modifications. Genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Germany). Sequencing of the amplicons was carried out by Eurofins (Germany), after which the sequence was analysed using the BLAST tool at the National Centre for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6 Competitive exclusion assay

The method for competitive exclusion (CE) was based on the methodology of García-Almendárez *et al.* (2008), with minor modifications. The CE bacteria, *Lc. lactis* subsp. *lactis* (Ca55), was chosen for this study based on its ability to grow better on plates under aerobic conditions, strong biofilm formation and its anti-listerial activity (Figure 1). The *L. monocytogenes* strain used for this study was *L. monocytogenes* 2081. The material chosen to grow the biofilms on was sterile stainless steel coupons (Type 304, measuring 3×0.8×0.1 cm). Liquid cultures of *L. monocytogenes* and *Lc. lactis* were prepared in BHIYE and incubated for 24 hours at different temperatures; 37°C for *L. monocytogenes* and 30°C for *Lc. lactis.* Anti-listerial biofilm efficacy was tested by utilising the *Lc. lactis* in mixed biofilm with *L. monocytogenes* and its cell-free supernatant on listerial biofilms. To test the effect of *Lc. lactis*, 100 µl of liquid culture ($\log_{10}$ 8 CFU ml^{-1}) was inoculated on three sterile stainless steel coupons. For testing the anti-biofilm efficacy of the CFS from Ca55, 100 µl of CFS was added to three stainless steel coupons instead. Then, 100 µl of *L. monocytogenes* (LM 2081) liquid culture ($\log_{10}$ 4- $\log_{10}$ 5 CFU ml^{-1}) was added to all of the coupons, including three control coupons with *L. monocytogenes* alone. Cell adhesion was allowed by incubating all coupons for 6 hours at 25°C. Afterwards, the coupons were washed with 5 ml of PBS, 200 µl of BHIYE pipetted on to the surface, placed in sterile petri dishes and incubated for 24 hours at 25°C. For the CFS treated coupons, 100 µl of BHIYE and 100 µl of CFS were added after the washing step. The washing and replacement of media was then repeated every 24 hours for five days.

To enumerate the biofilms after treatment, the coupons were washed with PBS to remove unattached cells and the biofilms were dislodged by using a 45 kHz ultrasonic bath (VWR, Ireland) for 15 minutes; followed by vortexing for 1 minute and serial dilutions in MRD (Oxoid, UK). *L. monocytogenes* was enumerated from all of the coupons by spread plating on to Oxford selective media (Oxoid, UK), while *Lc. lactis* was pour-plated in MRS

agar. The MRS plates were incubated at 30°C for 24h, while Oxford plates were incubated at 37°C for 24h. Both experiments were repeated three times and the results expressed in $\log_{10}$ CFU cm^{-2}.

2.7 Whole-genome sequencing and data analysis

In order to assess strain similarity, genomic DNA from the eight *Lc. lactis* subsp. *lactis* strains were prepared using the DNeasy blood and tissue kit (Qiagen, Germany) and were quantified using the NanoDrop ND-1000 (ThermoFisher Scientific, UK). Whole-genome sequence data was generated and assembled by MicrobesNG (https://microbesng.uk) using the lllumina MiSeq platform, including a standard analysis pipeline. Chromosomal organisation of the resulting contigs were predicted by aligning them against the *Lc. lactis* subsp. *lactis* IL1403 reference genome, obtained from the NCBI database (https://www.ncbi.nlm.nih.gov), using the Mauve Contig Mover algorithm (MCM) (Rissman et al., 2009). The progressiveMauve algorithm was then used to align all of the concatenated sequences with the reference genome (Darling et al., 2010). Overall genomic relatedness between the different genomes were then assessed using the Orthologous Average Nucleotide Identity (OrthoANI) tool (Lee et al., 2016), which is the modified version of the average nucleotide identity (ANI) algorithm (Richter and Rossello-Mora, 2009).

2.8 Statistical analysis

One-way ANOVA was used to compare between different groups, followed by the Tukey test to determine significant differences between the means ($P \leq 0.05$). These tests were performed using the IBM SPSS software (version 24.0, SPSS Inc., Chicago IL, USA).

3 Results & Discussion

3.1 Isolation and identification of anti-listerial bacteria and bacteriocin produced

In this study, LAB with anti-listerial activity were found to exist in growth substrates used in the mushroom production environment. During the initial screening, 234 colonies exhibiting anti-listerial activity were isolated from the different media and growth conditions used. However, after the well diffusion assay experiment, only eight isolates, shown in Table 1, retained their activity; Ca27, Ca29, Ca30, Ca46 and Ca55 were isolated

from mushroom casing, while Sp32, Sp34 and Sp41 were isolated from SMS. The high level of false positive inhibition may have been due to zone of inhibition overlap between different colonies and lactic acid production. Molecular identification of the isolates with anti-listerial activity, by 16S rRNA gene sequencing, using the BLAST program, showed that the eight anti-listerial isolates had greatest homology to *Lc. lactis* subsp. *lactis* IL1403. This is similar to the study by Chen *et al.* (2005) that isolated LAB from soil samples in Japan, including *Lc. lactis* subsp. *lactis* strains, but a follow-up study found that those isolates were not bacteriocin-producing strains (Yanagida et al., 2006). Kim and Kwak (2012) also isolated LAB from SMS but they mainly found *Lactobacillus spp.* The eight strains found in this study were able to demonstrate a broad spectrum of inhibition against a bank of *L. monocytogenes* strains, including several from the mushroom production environment (Table 1). These *Lc. lactis* isolates were found to have similar anti-listerial activity to strains isolated in other studies (Bolocan et al., 2016; Heo et al., 2012; Noonpakdee et al., 2002; Olasupo et al., 1999).

Whole-genome comparison of the *Lc. lactis* subsp. *lactis* strains, aligned to the reference genome, showed that most of the locally collinear blocks (LCBs) are highly homologous between all of the assemblies (Supplementary Figure 1). The OrthoANI values indicated that there was a high level of similarity between the genomic sequences of the *Lc. lactis* isolates (Supplementary Figure 2). Additionally, a cluster can also be observed between Ca29, Ca30, Ca46 and Sp34, with OrthoANI values of 100% similarity between the strains. As a follow-up experiment for this study, LAB were detected and *Lc. lactis* isolates were presumptively identified (data not shown) from five additional mushroom production facilities. This reiterates the results from the initial screening step and the OrthoANI values, which suggests that LAB and bacteriocin-producing *Lc. lactis* are naturally present in the mushroom production environment. To identify if these additional isolates are bacteriocin producers, further analysis would be needed.

The proteinaceous nature of the antibacterial agents, present in the CFS of the *Lc. lactis* strains, was confirmed by the effects of enzymes and heat treatment on their activity (as shown in Supplementary Table 1), while colony mass spectrometry detected a molecular mass of 3331 Da, which corresponds to the mass of nisin Z, from all eight *Lc. lactis* subsp. *lactis* mushroom isolates (Supplementary Figure 3). Despite all being nisin Z producers and

the high levels of strain similarity, the different strains exhibited varying incubation conditions for optimum levels of antimicrobial activity, with aerobic conditions being the preferable option for possible industry application. Whole-genome sequencing analysis showed no difference in the nisin operon sequence between strains. Bolocan *et al.* (2016) also reported that the growth environment had a differential ability to influence the *Lc. lactis* strains' anti-listerial activity. Rattanachaikunsopon and Phumkhachorn (2008) proposed that the sensitivity of the indicator strain used in the activity assays may be a factor, but it could be mainly due to varying concentrations of bacteriocins produced. Kim *et al.* (1997) found that, incorporation of an identical nisin-production transposon into different *L. lactis spp.* strains did not yield identical nisin concentrations, as one of the key determinants for final nisin concentrations were the endogenous features of the producing organism. Further studies would be needed to identify these endogenous features that influence bacteriocin production.

3.2 Competitive exclusion

As shown in Table 2, the competitive exclusion treatments had different effects on the biofilms formed by *L. monocytogenes* in either multi-species biofilms or with *Lc. lactis* Ca55 supernatant. *L. monocytogenes* was able to form $\log_{10}$ 5 CFU cm^{-2} of mono-culture biofilm on stainless steel. *Lc. lactis* Ca55 exhibited an anti-biofilm effect against *L. monocytogenes,* as it was able to significantly reduce (P<0.05) the *L. monocytogenes* biofilm by approximately 4 logs, while exhibiting strong biofilm formation on co-culture biofilm on stainless steel. This is similar to previous studies carried out on *Lc. lactis* subsp. *lactis* strains isolated from floor drains at different food processing plants, which found them to be effective at controlling *L. monocytogenes* biofilms for long periods (28 days) and at low temperatures (4°C) (Zhao et al., 2004, 2013). Guerrieri *et al.* (2009) found that biofilms of other LAB (*Lb. plantarum* 35d) can control *L. monocytogenes* in a small-scale model where they found a 3.9 log reduction of *L. monocytogenes* biofilm during a 10-day experiment. These observed inhibitions of pathogenic bacteria by LAB biofilms cannot only be attributed to the production of bacteriocins but also by possible biosurfactants, such as xylolipid that is produced by a *Lc. lactis* strain found by Saravanakumari and Mani (2010). Additionally, Gómez et al. (2016) observed an anti-biofilm adhesion effect in both bacteriocin producer and non-bacteriocin producer strains of *Lc. lactis.*

In contrast to the co-culture study, the supernatant of the *L. lactis* Ca55 strain had no effect on biofilm formed on stainless steel and showed no significant difference on the amount of mono-species biofilm of *L. monocytogenes* 2081. This differs to the results using microtitre plates shown in Figure 1, where the CFS from the different *Lc. lactis* strains caused a significant reduction (P<0.05) on most of the 72h biofilms formed by the three *L. monocytogenes* strains, when compared to the results from the GM17 controls. Bolocan *et al.* (2016) reported similar results, from *L. lactis* ALB79, for the competitive exclusion assay (co-culture and supernatant), where they saw a 3.6 log reduction of *L. monocytogenes* biofilm in co-culture but none from supernatant with *L. monocytogenes*. The possibility of more harbourage sites on stainless steel compared to polystyrene could be promoting increased resistance of *L. monocytogenes* biofilm against nisin but on the contrary, Ibusquiza *et al.* (2011) found that biofilm formed on polystyrene had higher resistance to nisin. Based on the results of Messens *et al.* (2003), this loss of bacteriocin activity could also be attributed to endogenous proteases produced during the growth phase, allowing *L. monocytogenes* 2081 to grow and form biofilms during the three 24-hour cycles of washing, media replacement and incubation. Overall, these results suggest that the utilisation of the competitive exclusion strains themselves in the mushroom production environment, where they can continuously produce anti-listerial agents during the mushroom production cycle, may be the most effective approach for minimising *L. monocytogenes*.

4 Conclusion

Based on the results of this study, it was found that *Lc. lactis* subsp. *lactis* strains naturally present in mushroom growth substrates have potential for application as biocontrol agents against *L. monocytogenes* in the mushroom production environment. Their GRAS status, natural presence, and ability to produce bacteriocins and biofilms at industry relevant temperatures make them suitable candidates. They could be either utilised as the strains themselves, their bacteriocins could be isolated and used, or either could be used as part of a hurdle technology to help control *L. monocytogenes* biofilms in the mushroom production environment. Further investigation is required in pilot scale mushroom growing trials.

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Table 1: Spectrum of activity of CFS from anti-listerial bacteria (ALB)

Organisms[a]	Isolated from[b]:	Serotype/ Serogroup[c]	Cell-free supernatants of ALB isolates[d]							
			Ca27	Ca29	Ca30	Ca46	Ca55	Sp32	Sp34	Sp41
L. monocytogenes strains:										
63/21	Milk sock filter	3a*	+ + +	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +
105	Hay in cow barn	3b*	+	+ +	+ +	+	+	-	+	+ +
1628	Mushroom industry	4a*	+ +	+	+ +	+	+	-	+ +	+
1634	Mushroom industry	7*	+ +	+ + +	+ +	+	+	+	+ +	+ + +
6179	Cheese isolate	1/2a*	+	+	+	+	+	+	+	+ +
ATCC 15313	Rabbit	1/2a*	+	+ +	+ +	+	+ + +	-	+ +	+ +
ATCC 19112	Clinical isolate	1/2c*	+	+	+	+	+	-	+	+
ATCC 19116	Chicken	4c*	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
ATCC 19117	Sheep	4d*	+	+ +	+	+ +	+	+	+	+ +
ATCC 19118	Chicken	4e*	+	+	+	+	+	-	+	+ + +
DPC 4605	Unknown	3c*	+	+ + +	+ +	+	+ + +	-	+ +	+
EGD-e	Rabbit	1/2a*	+ +	+ + +	+ +	+ +	+ + +	+	+ +	+ +
NCTC 11994	Soft cheese	4b*	+	+ + +	+ +	+ +	+ + +	+	+	+ +
Scott A	Clinical isolate	4b*	+ +	+ +	+ +	+	+	+	+ +	+ +
1698	Mushroom industry	1/2a-3a**	+	+	+	+	+	-	+	+
1702	Mushroom industry	1/2b-3b-7**	+ +	+ +	+ +	+	+ + +	+	+ +	+
1703	Mushroom industry	1/2a-3a**	+ +	+ + +	+ + +	+	+ + +	-	+ + +	+
1721	Mushroom industry	1/2a-3a**	++	++	++	+	++	+	+	++
1722	Mushroom industry	4b-4d-4e**	+ +	+ + +	+ +	+	+ +	+ +	+ +	+ + +
2081	Mushroom industry	1/2a-3a**	++	++	+++	+	+	-	++	+
Other organisms:			-	-	-	-	-	-	-	-
Escherichia coli NCTC 9001	Clinical isolate		-	-	-	-	-	-	-	-
Salmonella Typhimurium SARB 67			-	-	-	-	-	-	-	-
Staphylococcus aureus NCTC 6571	Lake water		-	-	+	-	-	-	+	+

[a] ATCC: American Type Culture Collection; DPC: Teagasc Food Research Centre Moorepark Culture Collection; NCTC: National Collection of Type Cultures; SARB: Salmonella Reference Collection B.

[b] ALB: anti-listeria bacteria; All strains were obtained from the Teagasc Food Research Centre culture collection

[c] *, serotype; **, serogroup

[d] -, no zone of inhibition; +, inhibition zone <3 mm; ++, inhibition zone 3–6 mm; +++, inhibition zone >6 mm

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Table 2: Competitive exclusion assay on 120h *L. monocytogenes* biofilms formed on stainless steel by *Lc. lactis*.

	Biofilm after 120h (Log_{10} CFU/cm^2 ± SD)[a]	
Tests	*L. monocytogenes*	*Lc. lactis*
L. monocytogenes only	5.1 ± 1.0[A]	
L. monocytogenes and *Lc. lactis* co-culture biofilm	1.1 ± 1.2[B]	6.3 ± 0.6
L. monocytogenes biofilm and *Lc. lactis* supernatant	5.0 ± 0.4[A]	

[a] Values with different letters (A-B) are significantly different ($P \leq 0.05$)
CFU: colony forming units; SD: standard deviation

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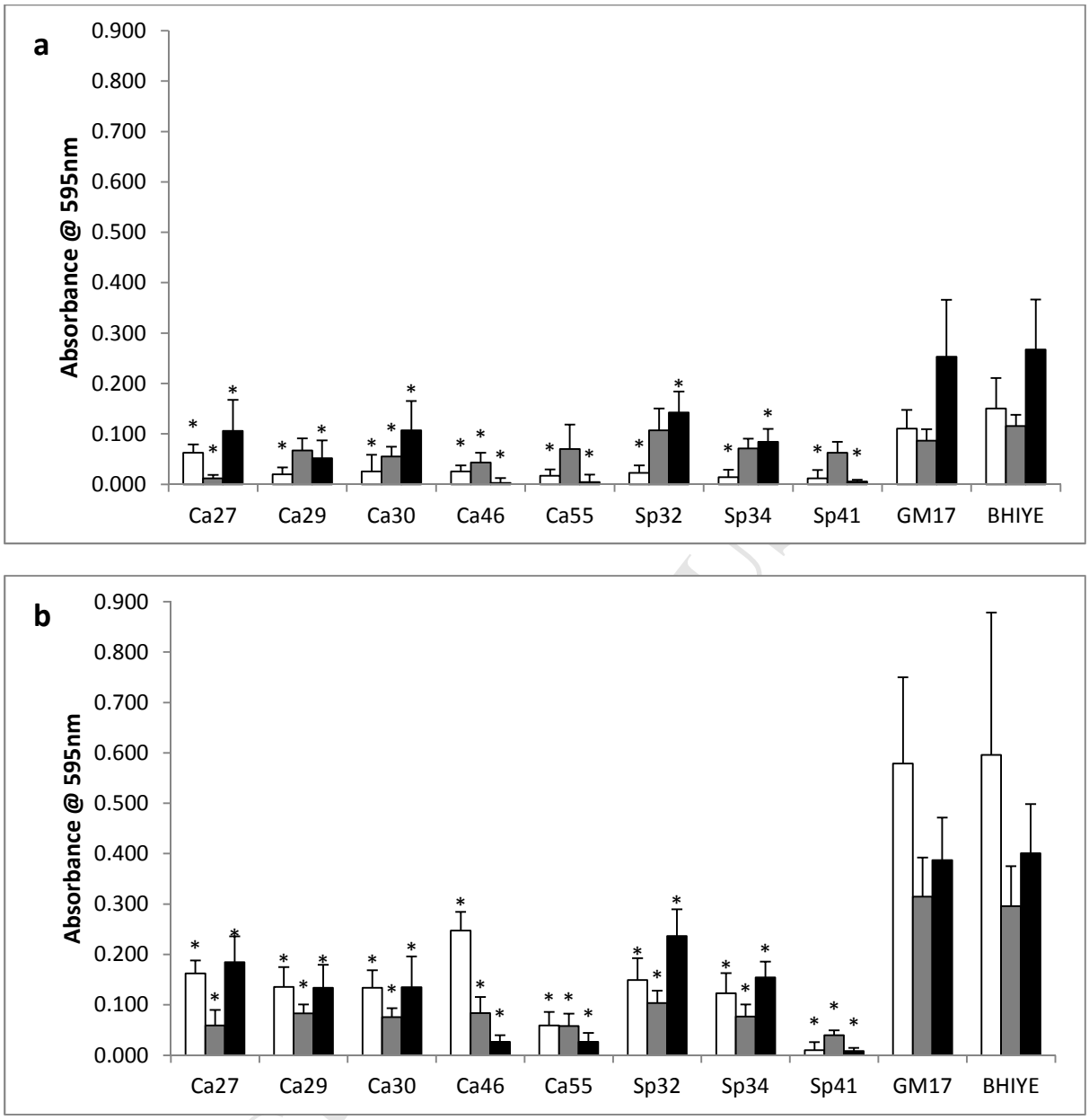

Figure 1: Absorbance values for crystal violet assay for determination of biofilm reduction by LAB CFS on 72h biofilms of three *L. monocytogenes* strains (□LM 1698, ▒LM 1721 and ■ LM 2081) formed at 18°C (**a**) and 25°C (**b**). For each graph, columns with an asterisk (*) on top indicate a significant difference (P<0.05) between the value from the CFS treatment and its respective GM17 control, for each *L. monocytogenes* strain. A reduction of biofilm is determined as an average value from LAB CFS treated biofilm which is significantly less (P<0.05) than the GM17 and BHIYE values.

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Highlights

- Lactic acid bacteria are naturally present in the mushroom production environment.
- *Lactococcus lactis* subsp. *lactis* strains isolated from growth substrates had anti-listerial activity.
- Co-culture biofilm experiments with *Lc. lactis* resulted in a four log reduction of *L. monocytogenes*.

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