SHORT COMMUNICATION

Biofilm formation in various conditions is not a key factor of persistence potential of *Listeria monocytogenes* in food-processing environment

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Summary

In this study, a hypothesis was tested that biofilm formation ability, or ability to form biofilm in specific conditions, is responsible for persistence of *Listeria monocytogenes* in food processing environment. Thirty-three *L. monocytogenes* strains isolated from food-processing environment and food products were tested for biofilm-forming ability in various conditions. Biofilm-forming ability was tested with exponential-phase cultures at 20 °C, 37 °C and 40 °C, with stationary-phase cultures at 20 °C, 37 °C and 40 °C, with cultures grown in limited-nutrient conditions and with cultures grown in high-salt conditions at 4 °C. Biofilm was formed in 96-well polystyrene plates and quantified by the method based on staining with crystal violet. The results showed that biofilm-forming ability of *L. monocytogenes* strains was variable, individual strains exhibiting different relative biofilm-forming ability in various conditions. No statistically significant differences between the groups of persistent and sporadic strains in biofilm-forming ability in any of the conditions were determined. Based on our results, biofilm-forming ability of *L. monocytogenes* is not a key factor that determines its persistence potential in food-processing environment.

Keywords

Listeria monocytogenes; food-processing environment; biofilm; persistence

Listeria monocytogenes is a food-borne pathogen, which is known to contaminate food products in a primary way, i.e. to pass from the raw materials along the processing to the final product, or in a secondary way, i.e. to contaminate the product during or after processing. Contamination by the latter way is mainly caused by strains, which are able to resist routine cleaning and sanitation procedures, and persist in the food-processing environments [1–3]. Although this phenomenon has been widely studied, it is still not clear which phenotypic properties are crucial for a *L. monocytogenes* strain to become persistent. Based on theoretical considerations, biofilm formation ability has been identified as the hottest candidate, based on excessive data on increased resistance of bacterial biofilms to environmental stress, including increased resistance to disinfection [2, 4, 5]. Alternative related hypotheses include ability to form biofilm at low temperatures, at low availability of nutrients or at high salt concentrations, which might help *L. monocytogenes* to persist in the food-processing environment [6, 7]. Nevertheless, reliable experimental evidence for these hypotheses is lacking, as no correlation could be identified between biofilm-forming ability (in various conditions) and persistence of *L. monocytogenes*, if extensive panels of strains were studied; persistence could neither be linked to genotype of strains [2, 8, 9].

In this study, a hypothesis was tested that biofilm formation ability, or ability to form biofilm

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in specific conditions, is responsible for persistence of *L. monocytogenes* in food processing environment. In order to obtain reliable results, a panel of 33 genotypically well characterized strains was tested for biofilm formation in a broad range of conditions involving different growth phase, different temperatures, low availability of nutrients or at high salt concentration. These conditions were previously reported as relevant to biofilm formation in *L. monocytogenes* and could be linked to conditions in various food-processing environments [6, 7]. Biofilm formation was tested in polystyrene 96-well plates, which is established as a representative surface for measuring biofilm-forming ability in bacteria [10, 11].

L. monocytogenes strains were isolated from food-processing environment and from food products, mostly originating in Central Europe. Strains were serotyped by slide agglutination using antisera from Denka Seiken (Tokyo, Japan) and genotyped by AscI macrorestriction and pulsed-field gel electrophoresis [12]. For testing biofilm-forming ability, basic cultures were prepared in stationary or in exponential growth phase in tryptone soya broth (TSB; Merck, Darmstadt, Germany). Stationary-phase culture (absorbance at 600 nm, A_{600} = 0.5) was centrifuged at 3 200 ×*g* for 20 min at 20 °C and the sedimented cells were added fresh medium to A_{600} = 0.5 (approx. 10^{10} CFU·ml^{-1}). Exponential-phase culture (A_{600} = 0.2) was centrifuged at 3 200 ×*g* for 20 min at 20 °C and the sedimented cells were added fresh medium to A_{600} = 0.2 (10^8–10^9 CFU·ml^{-1}). A sample of the culture was pipetted in a well of a 96-well polystyrene plate with flat bottoms (Cat. No. 82.1581.001; Sarstedt, Nümbrecht, Germany) and incubated at 20 °C, 37 °C or 40 °C for 24 h.

The formed biofilm was quantified by crystal violet staining with two washing cycles and spectrophotometric measurement [9]. In case of studying biofilm formation in conditions of nutrient limitation and low temperature, suspension of exponential-phase

Tab. 1. Biofilm formation of *Listeria monocytogenes* strains in various conditions.

Strain	Origin	Supplier	Phenotype	Recovery	Serotype	Pulsotype	Relative biofilm formation							
							Exponential phase			Stationary phase			Limited nutrients, 4°C	
							20 °C	37 °C	40 °C	20 °C	37 °C	40 °C	NaCl 0 g·l^{-1}	NaCl 100 g·l^{-1}
6179*	Cheese	A	Persistent	a	1/2a	nd	100	100	100	100	100	100	100	100
4423	Food-processing environment	B	Persistent	a	1/2a	nd	40	61	62	89	89	72	92	122
R479a	Smoked salmon	C	Persistent	a	1/2a	nd	68	120	91	65	124	96	111	70
CZ48	Food-processing environment	D	Persistent	a	1/2a	nd	44	54	85	35	72	61	79	202
2566	Food-processing environment	B	Persistent	a	1/2a	nd	70	97	198	68	120	140	89	111
L 2008	Food-processing environment	D	Persistent	b	1/2a	719	275	134	103	126	148	137	117	70
L 2182	Food-processing environment	D	Persistent	b	1/2a	719	119	100	91	57	115	85	40	109
L 943	Blue veined cheese	D	Persistent	b	1/2a	719	114	218	150	59	273	135	88	68
L 1069	Blue veined cheese	D	Persistent	b	1/2a	719	76	142	57	100	165	99	83	83
L 1438	Blue veined cheese	D	Persistent	b	1/2a	719	77	129	49	38	196	93	93	90
L 2563	Food-processing environment	D	Persistent	b	1/2a	719	69	146	80	19	214	136	67	73
L 2699	Food-processing environment	D	Persistent	b	1/2a	719	84	133	91	22	178	118	65	58
L 2024	Food-processing environment	D	Persistent	b	1/2a	713	106	89	49	57	81	71	38	nd
L 2325	Food-processing environment	D	Persistent	b	1/2a	713	108	113	100	53	110	91	98	nd

Tab. 1. *continued*

Strain	Origin	Supplier	Phenotype	Recovery	Serotype	Pulsotype	Relative biofilm formation							
							Exponential phase			Stationary phase			Limited nutrients, 4°C	
							20 °C	37 °C	40 °C	20 °C	37 °C	40 °C	NaCl 0 g·l^{-1}	NaCl 100 g·l^{-1}
L 2708	Food-processing environment	D	Persistent	b	1/2a	713	119	113	100	nd	107	101	100	53
L 1037	Cheese	D	Persistent	b	1/2a	713	229	70	58	54	124	138	64	92
L 1303	Cheese	D	Persistent	b	1/2a	713	nd	73	96	60	128	143	53	40
L 1499	Cheese	D	Persistent	b	1/2a	713	70	57	23	47	96	34	nd	64
L 2611	Cheese	D	Persistent	b	1/2a	713	79	110	139	56	114	215	109	79
L 2705	Environment	D	Persistent	b	1/2a	713	89	94	146	nd	113	231	115	71
L 2510	Ham	D	Sporadic	c	1/2a	733	143	91	98	69	109	89	32	nd
L 2531	Food-processing environment	D	Sporadic	c	1/2a	733	99	93	82	63	106	101	22	nd
L 2517	Ham	D	Sporadic	c	1/2a	735	101	54	62	nd	119	86	116	90
L 2750	Environment	D	Sporadic	c	1/2a	701	95	93	73	70	115	94	53	40
L 2778	Environment	D	Sporadic	c	1/2a	774	61	61	68	50	67	36	11	28
L 2748	Environment	D	Sporadic	c	1/2a	762	31	51	63	39	42	48	77	33
29	Cheese, retail	A	Sporadic	d	1/2a	nd	47	68	91	48	90	60	32	nd
N22-2	Food-processing environment	C	Sporadic	d	1/2a	nd	36	45	78	28	49	56	32	109
CZ70	Blue veined cheese, retail	D	Sporadic	d	1/2a	719	58	55	54	16	80	134	16	26
L 2704	Cheese	D	Sporadic	c	1/2b	524	82	72	33	50	60	63	97	nd
L 2712	Food-processing environment	D	Sporadic	c	1/2b	503	83	46	48	68	66	73	55	56
L 2815	Cheese	D	Sporadic	c	1/2b	503	81	89	54	60	61	75	77	nd
L 2808	Environment	D	Sporadic	c	4b	204	56	55	52	29	49	42	45	nd
EGD-e	Animal case (rabbit)	B	na	na	1/2a	nd	33	18	41	26	88	93	11	13
NCTC 11994	Soft cheese	B	na	na	4b	nd	37	58	19	28	45	28	37	28

Relative biofilm formation: Biofilm-forming ability relative to reference strain 6179 in the given experiment, measured by crystal violet staining [11], calculated as $A_S/A_R \times 100$ (where A_S is absorbance at 570 nm for the given strain and A_R is absorbance at 570 nm for reference strain), taking into account average value of six replicates with one or no replicate excluded on the basis of Dixon's Q-test for outliers.

Supplier: A – obtained from Irish Agriculture and Food Development Authority (Cork, Ireland), B – obtained from University of Veterinary Medicine (Vienna, Austria), C – obtained from Technical University of Denmark (Copenhagen, Denmark), D – isolated and characterized by Centre for Food Chain Hygiene (National Institute of Public Health, Brno, Czech Republic).

Recovery: a – recovered on multiple occasions from defined samples or sites during a 12-month period, b – recovered on multiple occasions in 2004–2010, c – sporadically recovered from defined samples or sites in 2008–2010, d – sporadically recovered from defined samples or sites during a 12-month period.

* – reference strain, na – not applicable, nd – not determined.

cells was prepared in 10× diluted TSB, pipetted in a 96-well plate and incubated at 20 °C, 37 °C or 40 °C for 24 h, and at 4 °C for 5 days. In case of studying biofilm formation at increased NaCl concentration, the culture was grown in TSB + 100 $g \cdot l^{-1}$ NaCl at 20 °C for 40 h, centrifuged at 3 200 ×*g* for 20 min at 20 °C and the sedimented cells were washed with 8.5 $g \cdot l^{-1}$ NaCl. Then, 10× diluted TSB was added, the suspension was pipetted in a 96-well plate and incubated at 20 °C, 37 °C or 40 °C for 24 h, and at 4 °C for 5 days. Statistical analysis was performed using ANOVA Tukey's test at $P = 0.05$.

Results summarized in Tab. 1 showed that biofilm-forming ability of *L. monocytogenes* strains was variable, individual strains exhibiting different relative biofilm-forming ability in various conditions. Persistent or sporadic phenotype did not correlate with biofilm-forming ability in any of the conditions. While certain persistent strains (e.g. L2008, L2182, L943, L2708) were strong biofilm formers (relative biofilm formation ≥ 85%) by both exponential- and stationary-phase cultures at all tested temperatures with the exception of stationary cultures at 20 °C, other persistent strains were sensitive to temperature and were strong biofilm formers (relative biofilm formation ≥ 80%) only at 37 °C or 40 °C (e.g. L2563, L2611). However, similar strong ability to form biofilm (relative biofilm formation ≥ 73%) in various conditions was observed in several sporadic strains (e. g. L2510, L2531, L2750) and sporadic strain L2517 was a strong biofilm former (relative biofilm formation ≥ 90%) at limited nutrition at 4 °C. A majority of persistent strains were not consistently strong biofilm-formers (relative biofilm formation ≥ 80%) in various conditions. In conditions of limited nutrient availability and/or in high-salt conditions, no consistent differences between persistent and sporadic strains were determined. Markedly different biofilm-forming ability in various conditions was determined even for strains of identical pulsotype. Statistical analysis did not show any significant differences between the groups of persistent and sporadic strains in biofilm-forming ability in any of the conditions.

Results of this study demonstrate that biofilm formation in various conditions does not facilitate prediction of persistance potential in *L. monocytogenes* from food-processing environments and food products. Our results obtained for strains originating mostly in Central Europe are in agreement with observations for *L. monocytogenes* from Western and Northern Europe. They confirm the opinion that ability to form biofilm (in various conditions) is only one of several phenotypic features that help *L. monocytogenes* to survive in food-processing environment, but is not a prerequisite for a persistent phenotype of this foodborne pathogen [2, 3, 8]. Based on our results, biofilm-forming ability of *L. monocytogenes* is not a key factor that determines its persistence potential in food-processing environment.

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