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Heat-resistance of suspect persistent strains of *Escherichia coli* from cheesemaking plants

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Abstract: Besides its health and spoilage hazards, *Escherichia coli* is a process hygiene indicator for cheeses made from milk that has undergone heat treatment. Hence, its ability to persist in cheesemaking plant environment and equipment is important. In total, 120 samples from two producing plants were analysed and 72 *E. coli* isolates were obtained. The target was to find out whether there is a difference in heat-resistance between persistent and non-persistent *E. coli* strains. The strains were selected using macrorestriction analysis and recurrent detection in cheesemaking plants hereby: one strain persisting in brine for blue-veined cheeses, two strains persisting in brine for hard cheeses and one non-persistent strain from raw material. Their D(50)-values were 196; 417; 370 and 182 min, respectively, D(59)-values ranged from 20 to 32 min and z-values were 7.5; 6.6; 8.1 and 9.0 °C, respectively. The non-persistent strain was the least resistant to heating to 50 °C but it was not the least resistant generally. All tested strains were highly heat-resistant and carried genes of the heat resistance locus LHR1 and/or LHR2. Our results emphasise the need to screen for the presence of LHR genes and the occurrence of heat-resistant *E. coli* in cheese production where they could survive sub-pasteurisation temperatures and contaminate the manufacturing environment and finished products.

Keywords: brine; cheese; hygiene indicator; locus of heat resistance; persistence

According to Commission Regulation (EC) No. 2073 (2005) as amended by later regulations, *Escherichia coli* is a process hygiene indicator for cheeses made from milk that has undergone heat treatment. *E. coli* is able to form biofilms and persist in cheesemaking plant environment and equipment (Kuhtyn et al. 2017) as well as to cause early blowing defect in cheeses (Johnson 2001). Moreover, various *E. coli* strains cause diverse intestinal and extraintestinal diseases by means of virulence factors that affect a wide range of cellular processes (Kaper et al. 2004). These characteristics make *E. coli* undesirable in the dairy industry.

E. coli is generally supposed to be reliably inactivated during milk pasteurisation but it can pollute dairy products as a post-pasteurisation contaminant (Glatz & Brudvig 1980). In that case, *E. coli* resistance to various thermal processes should be considered, e.g. in production technologies of fresh thermised cheeses, cooked and scalded semi-hard and hard cheeses, stretched cheeses and processed cheeses. Furthermore, loci of heat resistance (LHR1 and LHR2) localised in *E. coli,* especially in the chromosome, but even in plasmids have been identified (Mercer et al. 2015; Boll et al. 2017).

Although general information on the heat-resistance of *E. coli* strains originating in milk and dairy products is available (Singh & Ranganathan 1980; Peng et al. 2013), the characteristics of persistent strains are not well explored. The relationship between the risk of persistence and *E. coli* strain characteristics is not well known. Therefore, our aim was to detect *E. coli* strains persisting in cheesemaking plants, assess their heat-

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resistance and compare it with the heat-resistance of a non-persistent strain as well as to discuss strain characteristics in the relationship to the specific conditions of cheese production.

MATERIAL AND METHODS

Detection of *E. coli* in cheesemaking plants. In total, 96 samples from a hard cheese producing plant (producer A) were collected in 2017 and 24 samples from a blue-veined cheese producing plant (producer B) in 2019. Swabs from the processing environment and devices used during various processing steps, raw materials, semi-products, finished products and samples from the staff were analysed. For the detection of *E. coli*, all samples were enriched in buffered peptone water (Oxoid, UK) at 37 °C for 18–24 h and subsequently plated on TBX agar (Biokar Diagnostics, France) and cultivated at 37 °C for 18–24 h. In the samples of finished products before enrichment, the quantification of *E. coli* was performed using a serial dilution and 200 µL aliquots were plated on the surface of TBX agar under the same cultivation conditions as for the detection. A cultivation temperature of 37 °C was chosen to support the growth of environmental strains. Suspect *E. coli* colonies were isolated and identified by mass spectrometry using a MALDI-TOF MS analyser and Biotyper software version 3.1 (Bruker Daltonik, Germany). Afterwards, the isolates were preserved in BHI medium (Merck, Germany) with 20% glycerol and deep frozen at –75 °C.

Pulsed field gel electrophoresis (PFGE). *E. coli* isolates were revitalised on Blood agar (LabMediaServis, Czech Republic) by an aerobic cultivation at 37 °C for 16–18 h. The diversity of isolates was assessed by a macrorestriction analysis using *Xba*I endonuclease with subsequent pulsed field gel electrophoresis (PFGE) according to the PulseNet Europe (2013) protocol for *E. coli*. To construct a dendrogram by the unweighted pair group method with arithmetic mean (UPGMA), BioNumerics version 5.1 (Applied-Maths, Belgium) was used with the following settings: Dice (Opt: 1.10%) (Tol 1.0%–1.0 %) (H > 0.0%, S > 0.0 %) [0.0%–100.0%].

Strain selection for heat-resistance assessment. As persistent strains, *E. coli* of pulsotypes recurrently detected in the processing environment and semi-finished or finished products were selected. As a non-persistent strain, *E. coli* isolated from raw material was chosen. Its pulsotype was not detected in other samples.

Heat-resistance assessment. The whole procedure of heat-resistance assessment was performed two-times; as optimisation of temperature, time and serial dilution in combinations for particular strains and as the data measurement in itself. This attempt allowed to obtain complete datasets containing only quantifiable microbiological results. Although both repetitions provided comparable data we show only the final methodology and results.

Our experimental design was inspired by Nazarowec-White & Farber (1997). Firstly, 1 mL of thawed *E. coli* suspension was plated on BHI agar and revitalised by an aerobic cultivation at 37 °C for 16–18 h. Afterwards, the whole plate was stripped by an inoculation loop into 9 mL of saline to obtain a concentrated stock suspension. *E. coli* counts were determined using serial dilution and cultivation on BHI agar at 37 °C for 24 h and concurrently used in the experiment. Secondly, a 50 mL Falcon tube containing 20 mL of sterile skimmed milk and a sterile magnetic bar was immersed into a water bath with another magnetic bar. The arrangement was placed on a magnetic stirrer with a thermoregulation probe RCT Basic Safety Control and contact thermometer ETS-D5 (both IKA, Germany) to regulate the temperature of water in the bath. The whole system was stabilised at an experimental temperature (50; 53; 56 and 59 °C). Thirdly, 1 mL of stock suspension was added to the Falcon tube with tempered milk. After the lapse of experimental times (10; 30; 60; 90; 120; 150 and 180 min), 1 mL of each sample was taken out and immediately cooled down and surviving *E. coli* counts were determined. D-values and z-values were estimated using the standard regression analysis based on Bigelow and Esty loglinear models as described by Nazarowec-White & Farber (1997). MS Excel 2013 (Microsoft Corporation, USA) was used.

PCR detection of the locus of heat resistance (LHR). A set of strains (n = 4) selected for the phenotypic heat resistance assessment was screened for the presence of the locus of heat resistance 1 (LHR1) on the basis of PCR detection of three separate regions (Mercer et al. 2015). Screening for the presence of LHR2 was performed by the detection of the *clpK2* gene (Boll et al. 2017).

RESULTS AND DISCUSSION

Occurrence of *E. coli* in cheesemaking plants. In hard cheese manufacturing (producer A), *E. coli* was detected in 67% (64/96) of samples, including raw materi-

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Table 1. Analysed and *E. coli* positive samples from the manufacturing of hard cheese (producer A)

Sample type	Description	Analysed	Positive
		(*n*)	
Raw material and semi-products	milk	8	3
	whey	12	7
	curd	5	5
	marine salt for brine preparation	6	0
Swabs and other environmental samples	curd-making vats and equipment	19	15
	moulding and pressing equipment	5	4
	brining vats and equipment	14	11
	brine	7	6
	brine waste sludge	7	6
Staff	rinses from hands	9	7
Products	finished cheeses	4	0

n – number of samples

als and semi-products [48% (15/31) of samples], processing environment [81% (42/52) of samples] and staff hands during curd processing [78% (7/9) of samples]. However, the finished hard cheeses were negative. In the blue-veined cheese processing plant (producer B), *E. coli* was detected in 24% (5/21) of samples from the processing environment, especially associated with brining. All tested finished blue-veined cheeses were *E. coli* positive but contained less than 50 CFU g^{-1} of *E. coli* and fulfilled the demands of Commission Regulation (EC) No. 2073 (2005). More details are given in Tables 1 and 2.

Detection of suspect persistent strains of *E. coli*. PFGE analysis of *E. coli* isolated from the hard cheese processing plant (producer A) revealed the spread of bacterial contamination among raw materials, semi-products, manufacturing environment, technological equipment and the staff. In the hard cheese processing plant, 33 various *E. coli* pulsotypes were identified. The isolates of recurrently detected and the most frequent pulsotype EC-Xba-7 were obtained from a curd-making area and brining room. Another pulsotype repeatedly detected in producer A, especially in the brining room, was EC-Xba-19 (Figure 1). Strains of the EC-Xba-7 and EC-Xba-19 pulsotypes were detected at different sampling dates during one year, which may indicate the presence of a suspect persistent *E. coli* strain in producer A adapted to the production environment. As model persistent strains, isolates LEV 686/17/B (pulsotype EC-Xba-7) and LEV 1282/17 (pulsotype EC-Xba-19) were selected. Isolates of other pulsotypes were detected only sporadically or only during one sampling term. The sporadic isolate LEV 1038/17 of pulsotype EC-Xba-17 (originating in raw material) was selected as a non-persistent model strain.

In the blue-veined cheese processing plant (producer B), all but one isolates were indistinguishable by PFGE

Table 2. Analysed and *E. coli* positive samples from the manufacturing of blue-veined cheese (producer B)

Sample type	Description	Analysed	Positive
		(*n*)	
Swabs and other environmental samples	curd-making vats and equipment	4	0
	brining vats and equipment	8	3
	brine	1	1
	ripened cheese washing water	1	1
	belt conveyer after cheese washing	2	0
	cutting and packaging equipment	5	0
Products	finished cheeses	3	3

n – number of samples

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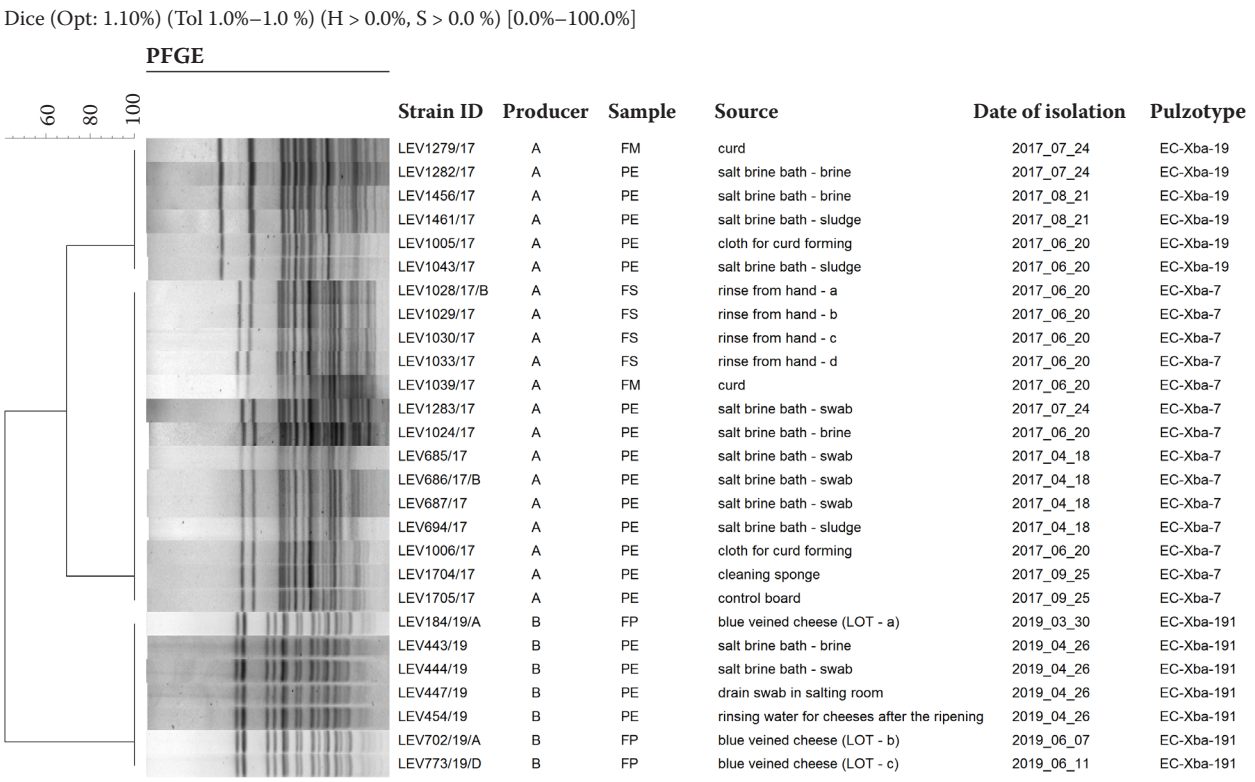

Strain ID	Producer	Sample	Source	Date of isolation	Pulzotype
LEV1279/17	A	FM	curd	2017_07_24	EC-Xba-19
LEV1282/17	A	PE	salt brine bath - brine	2017_07_24	EC-Xba-19
LEV1456/17	A	PE	salt brine bath - brine	2017_08_21	EC-Xba-19
LEV1461/17	A	PE	salt brine bath - sludge	2017_08_21	EC-Xba-19
LEV1005/17	A	PE	cloth for curd forming	2017_06_20	EC-Xba-19
LEV1043/17	A	PE	salt brine bath - sludge	2017_06_20	EC-Xba-19
LEV1028/17/B	A	FS	rinse from hand - a	2017_06_20	EC-Xba-7
LEV1029/17	A	FS	rinse from hand - b	2017_06_20	EC-Xba-7
LEV1030/17	A	FS	rinse from hand - c	2017_06_20	EC-Xba-7
LEV1033/17	A	FS	rinse from hand - d	2017_06_20	EC-Xba-7
LEV1039/17	A	FM	curd	2017_06_20	EC-Xba-7
LEV1283/17	A	PE	salt brine bath - swab	2017_07_24	EC-Xba-7
LEV1024/17	A	PE	salt brine bath - brine	2017_06_20	EC-Xba-7
LEV685/17	A	PE	salt brine bath - swab	2017_04_18	EC-Xba-7
LEV686/17/B	A	PE	salt brine bath - swab	2017_04_18	EC-Xba-7
LEV687/17	A	PE	salt brine bath - swab	2017_04_18	EC-Xba-7
LEV694/17	A	PE	salt brine bath - sludge	2017_04_18	EC-Xba-7
LEV1006/17	A	PE	cloth for curd forming	2017_06_20	EC-Xba-7
LEV1704/17	A	PE	cleaning sponge	2017_09_25	EC-Xba-7
LEV1705/17	A	PE	control board	2017_09_25	EC-Xba-7
LEV184/19/A	B	FP	blue veined cheese (LOT - a)	2019_03_30	EC-Xba-191
LEV443/19	B	PE	salt brine bath - brine	2019_04_26	EC-Xba-191
LEV444/19	B	PE	salt brine bath - swab	2019_04_26	EC-Xba-191
LEV447/19	B	PE	drain swab in salting room	2019_04_26	EC-Xba-191
LEV454/19	B	PE	rinsing water for cheeses after the ripening	2019_04_26	EC-Xba-191
LEV702/19/A	B	FP	blue veined cheese (LOT - b)	2019_06_07	EC-Xba-191
LEV773/19/D	B	FP	blue veined cheese (LOT - c)	2019_06_11	EC-Xba-191

Figure 1. PFGE results on *E. coli* strains belonging to pulsotypes repeatedly detected in the production environment and finished products at the hard cheese producer (A) and the blue-veined cheese producer (B)

PFGE – Pulsed field gel electrophoresis; FM – food material; PE – plant environment; FS – food staff; FP – finished product

(pulsotype EC-Xba-191), which could indicate the presence of a suspect persistent strain (Figure 1). As its representative, isolate LEV 443/19 was selected. All suspect persistent strains LEV 686/17/B, LEV 1282/17 and LEV 443/19 intended for heat-resistance assessment originated in brine or brine vats.

Phenotypic and genotypic heat-resistance of *E. coli*. For heat-resistance assessment, heating conditions were selected with respect to the subsequent analysis; quantifiable counts of viable *E. coli* after a reliably controllable heating time (lasting for at least a few minutes) were needed. Moreover, the used experimental design enabled instantaneous heating and cooling of bacteria without a distinguished reaction time due to small inoculation volumes and continuous stirring which contributed to measurement precision. Experimental design and conditions are factors influencing the obtained results.

Therefore, several authors obtained different results; even if only *E. coli* species was considered. Blackburn et al. (1997) used an immersion heater and obtained D(59.5)-value 1.00 min and D(64.5)-value 0.06 min. In pre-heated test-tubes in a water bath, Murano & Pierson (1992) obtained D(55)-value 8.0 min, while Singh & Ranganathan (1980) calculated for three strains D(50)-values from 20.58 to 51.887 min and D(60)-values from 0.00 to 8.37 min. In another work, Li et al. (2017) reported D(60)-value 0.78 min in an enclosed aluminium testing cell immersed in a water bath. Peng et al. (2013) treated raw cow milk in a continuous plate heat-exchanger for 20 s and for nine strains found D(60)-values of at least 0.37 min (but some strains were not significantly inactivated at all) and D(65)-values from 0.06 to 1.56 min.

Compared to these results, our data suggested even about one decimal order higher D-values at relevant temperatures (Table 3). Conversely, our z-values (Table 4) were comparable with previously published data. Singh & Ranganathan (1980) found z-values from 4.61 to 10.44 °C, Peng et al. (2013) from 3.4 to 6.1 °C. It means that although some *E. coli* strains were more resistant to particular sub-pasteurisation temperatures, their sensitivity to an increase in temperature was comparable with previous findings. However, our strains with the highest D-values and with the highest z-value differed. In other words, the strains more resistant to 50 °C (suspect persistent strains from producer A)

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Table 3. Determined D-values of *E. coli* strains

Strain	Temperature (°C)	D-value (min)	Regression equation*	R^2
LEV 443/19	50	196	$y = -0.0051x + 8.8179$	0.6131
	53	53	$y = -0.0187x + 8.8964$	0.8267
	56	31	$y = -0.0324x + 7.9723$	0.9375
	59	32	$y = -0.0308x + 7.2962$	0.8234
LEV 686/17/B	50	417	$y = -0.0024x + 6.9683$	0.6026
	53	400	$y = -0.0025x + 6.9049$	0.7476
	56	68	$y = -0.0147x + 7.1588$	0.81
	59	23	$y = -0.0426x + 7.2274$	0.9605
LEV 1282/17	50	370	$y = -0.0027x + 8.4188$	0.9834
	53	169	$y = -0.0059x + 8.459$	0.9779
	56	60	$y = -0.0166x + 8.5689$	0.9618
	59	30	$y = -0.0332x + 8.6546$	0.9286
LEV 1038/17	50	182	$y = -0.0055x + 8.4761$	0.945
	53	132	$y = -0.0076x + 8.5922$	0.9743
	56	49	$y = -0.0205x + 8.8055$	0.8449
	59	20	$y = -0.0511x + 8.6908$	0.9267

*x – time (min); y – *E. coli* count (log CFU mL^{-1}); R^2 – coefficient of determination

responded to an increase in temperature more sensitively, which provides a good presumption that such adapted strains could be inactivated during pasteurisation as effectively as other *E. coli* strains.

The differences in D-values could have two reasons. 1) It can be explained by the differences in experimental designs. As discussed above, we made an effort to use as precise heating time and temperature as possible. 2) Heat resistance in *E. coli* can be highly variable and some strains exhibit thermotolerance. In this work, such thermotolerant strains were obtained and tested. An isolate of *E. coli* which had D(60)-value greater than 60 min and survived the temperature of 71 °C has been described (Dlusskaya et al. 2011). According to Mercer et al. (2015), our tested strains could be classified as highly heat resistant because the results of D(59)-value suggested that the strains would exhibit D(60)-value of more than 6 min. The phenotype was in accordance with the genotype, a complete ~14 kb genomic island containing 16 predicted open reading frames encoding putative heat shock proteins and proteases (LHR1) was identified in all suspect persistent strains of *E. coli* in this study. The suspect persistent strains from hard cheese manufacturing (producer A) unlike the strain from producer B carried the *clpk2* gene which was used as a marker for the detection of the ~19 kb genomic island LHR2. Boll et al. (2017) described significantly increased survival in their phenotypic heat resistance assay in strains positive for both *clpK1* (LHR1) and *clpk2* (LHR2) genes. The control strain of *E. coli* LEV 1038/17 isolated sporadically from raw material had incomplete LHR1 (Table 5). The presence of truncated LHR1 or cloning of fragments of this locus does not influence the heat resistance of *E. coli* (Mercer et al. 2015). Similar heat resistance in the non-persistent strain LEV 1038/17 as in the tested persistent strains can be explained by the presence of the *clpk2* gene encoding the heat resistance locus LHR2.

To discuss non-persistent and suspect persistent strains of *E. coli* in detail: The non-persistent strain LEV 1038/17 had the lowest D(50)-value but it was not the least resistant generally.

Table 4. Determined z-values of *E. coli* strains

Strain	z-value (°C)	Regression equation*	R^2
LEV 443/19	7.5	$y = -0.0861x + 6.4454$	0.7946
LEV 686/17/B	6.6	$y = -0.1506x + 10.312$	0.9063
LEV 1282/17	8.1	$y = -0.124x + 8.7696$	0.9947
LEV 1038/17	9.0	$y = -0.1112x + 7.8985$	0.9625

*x – temperature (°C); y – log D-value (min); R^2 – coefficient of determination

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Table 5. Presence of LHR1 and LHR2 in tested *E. coli* strains detected by PCR

Strain	Producer	Persistence	LHR1 region A	LHR1 region B	LHR1 region C	*clpk2* gene LHR2
LEV 443/19	B	yes	+	+	+	–
LEV 686/17/B	A	yes	+	+	+	+
LEV 1282/17	A	yes	+	+	+	+
LEV 1038/17	A	no	+	+	–	+

LHR – the locus of heat resistance; *clpk2* gene – a marker for the detection of LHR2

Among all tested strains, the highest D(50)- and D(53)-values were observed for strains LEV 686/17/B and LEV 1282/17 persisting in hard cheese manufacturing (producer A). These strains were isolated from brine or brine vats but the same pulsotypes were detected e.g. in curd, in clean cheesemaking cloths and on staff hands. It follows that these strains had both to survive 21–23% NaCl in brine and to adapt to heating. The curd is cooked to 52–53 °C and subsequent scalding lasts for 7 min. Cheesemaking clothes are washed at 60 °C but not all parts of the washing machine may undergo the full heating process. The optimal temperature for *E. coli* growth is about 40–42 °C (Gonthier et al. 2001), while maximum growth temperatures are about 47–48 °C (Rosso et al. 1993), which is close to both temperatures used by producer A and temperatures with a low lethal effect on *E. coli*. Nevertheless, long-term ripening of hard cheeses for six months leads to significant microbial changes. Both according to our findings and manufacturer´s knowledge concerning *E. coli*, this type of hard cheese is hazardless.

Strain LEV 443/19 was isolated from brine at producer B. The same pulsotype was detected in finished blue-veined cheeses but not in samples taken before brining. Thus, this strain had to resist above all the elevated NaCl concentrations. The content of NaCl was 12–15 % in brine, 3.7 % in the cheeses and 7.7% in cheese moisture. The suspect persistent strain of *E. coli* from producer B was not exposed to any obvious heat pressure in brine vats and during the following steps of manufacturing. However, we hypothesize in accordance with Marti et al. (2016), that the milk and cheese production environment could provide selective advantage to *E. coli* carrying LHRs enabling them to survive better the pasteurisation of raw milk. It seems that LHR positive strains of *E. coli* can be isolated more frequently from raw milk cheeses (Marti et al. 2016) than from meat or clinical isolates (Mercer et al. 2015). However, there are only a few studies to support such conclusions and deeper research on this issue is needed.

CONCLUSION

We detected three suspect persistent strains of *E. coli* in blue-veined cheese and hard cheese processing plants and identified their possible sources. We focused on their heat-resistance determination in comparison with a non-persistent strain from raw material. All tested strains were classified as highly heat-resistant according to the obtained D-values and the carriage of heat resistance loci LHR1 and/or LHR2. Although we expected a difference in the heat-resistance of non-persistent and persistent strains, or even the relation of heat-resistance with the heating processes used in the place of strain occurrence, this hypothesis was not proved. However, our work surprised by the finding that such highly heat-resistant *E. coli* strains can commonly occur and contaminate the manufacturing environment and finished products. Such *E. coli* strains could be eradicated more hardly than expected which potentially could mean the development of new microbiological hazard for the food industry.

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