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Behaviour of an *Escherichia coli* strain deleted in the salt stress response gene *kdpA* during production and ripening of a semi-hard cheese

Verhalten eines im Salzstressantwort-Gen kdpA deletierten Escherichia coli Stammes während der Produktion und Reifung eines Halbhartkäses

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Summary

In order to survive cheese production and cheese ripening *Escherichia coli* needs to adapt to the encountered stress such as the raised salt concentrations. Such adaptation typically involves the induction of stress response genes. Therefore, impaired or lacking activity in such genes may considerably affect survival of *E. coli* upon stress exposure such as encountered during cheese production. In previous *in vitro* studies, an association between *E. coli* stress response gene expression and survival observed in raw milk cheese was found, indicating that reduced or absent induction of the salt stress response gene *kdpA* might have an impact on survival of an *E. coli* strain during raw milk cheese production. In this study, therefore, the survival of an *E. coli* strain deleted in the *kdpA* gene was investigated in comparison to its wild-type strain during production and ripening of semi-hard cheese. After an initial increase in *E. coli* counts a slow decrease of 1.80 and 1.38 $\log_{10}$ cfu/g until the end of the 16 week ripening period was observed for the *kdpA*-deletion and the wild-type strain, respectively, without significant differences between these strains. This indicates that lack of the *kdpA* gene did not considerably affect *E. coli* survival in cheese and therefore other stress response mechanisms are probably of higher importance for survival of *E. coli* in raw milk cheese.

Keywords: Stress response mechanism, raw milk cheese, KdpA-ATPase system

Zusammenfassung

Um die Käseproduktion und -reifung zu überleben, müssen sich *Escherichia coli* unterschiedlichen Stressfaktoren wie beispielsweise einer erhöhten Salzkonzentration anpassen. Diese Anpassung geschieht vor allem durch die Induktion von Stressantwort-Genen. Deshalb könnte eine reduzierte oder fehlende Aktivität eines solchen Genes das Überleben von *E. coli* während einer Stressexposition, wie sie zum Beispiel während der Käseproduktion auftritt, beträchtlich beeinflussen. In bisherigen *in-vitro*-Studien wurde eine Verbindung zwischen der Expression von Stressantwort-Genen in *E. coli* und dem Überleben in Rohmilchkäse gefunden, welche darauf hinweist, dass eine reduzierte oder fehlende Induktion des Salzstressantwort-Genes *kdpA* einen Einfluss auf das Überleben eines *E. coli*-Stammes während der Rohmilchkäseproduktion haben könnte. In dieser Studie wurde nun das Überleben eines *E. coli*-Stammes, welchem das *kdpA*-Gen deletiert wurde, im Vergleich zu seinem Wildtypstamm während der Produktion und Reifung eines Halbhartkäses untersucht. Nach einem anfänglichen Anstieg der *E. coli* Zahlen wurde eine langsame Reduktion von 1,80 beziehungsweise 1,38 $\log_{10}$ cfu/g bis zum Ende der 16-wöchigen Reifungszeit für den *kdpA*-deletierten und den Wildtypstamm beobachtet, ohne dass signifikante Unterschiede zwischen diesen Stämmen aufgetreten sind. Dies weist darauf hin, dass das Fehlen des *kdpA* Gens keinen beträchtlichen Einfluss auf das Überleben von *E. coli* im Käse hatte und somit andere Stressantwortmechanismen wahrscheinlich von grösserer Bedeutung für das Überleben von *E. coli* im Rohmilchkäse sind.

Schlüsselwörter: Stressantwortmechanismus, Rohmilchkäse, KdpA-ATPase system

Introduction

In raw milk cheese, *Escherichia (E.) coli* encounters different stresses such as raised salt concentrations and presence of organic acids. In order to survive, *E. coli* demands to adapt to these conditions, which typically involves the induction of different stress response genes encoding mechanisms that can counteract the stress present (Peng et al., 2011). Survival of Shiga toxin-producing *E. coli* (STEC) in raw milk cheese represents a risk in view of food safety. Several studies reported on STEC outbreaks that were associated with raw milk products including cheese (Baylis, 2009; Farrokh et al., 2012). Therefore it is important to understand the stress response mechanisms that might contribute to the survival of *E. coli* in raw milk cheese. In a previous study, the survival of five *E. coli* strains was investigated during raw milk cheese production and ripening, which showed variations in survival among these strains (Peng et al., 2013a). The observed variations might result from differences in transcriptional induction of stress response genes. Therefore, in a recent study, the transcriptional induction of two acid and five salt stress response genes were investigated upon exposure to lactic acid or sodium chloride stress exposure mimicking conditions encountered in cheese (Peng et al., 2014). Thereby it was observed that the *E. coli* strain, which weakly survived in raw milk cheese, was the only strain that did not show a significant induction in the salt stress response gene *kdpA*. This gene encodes for the binding and translocation subunit KdpA of the Kdp-ATPase system, which is involved in the uptake and accumulation of potassium as initial stress response caused by a raise in external salt concentrations (Ballal et al., 2007). In addition, the *kdpA* gene in *Salmonella enterica* serovar Typhimurium has been shown to promote long-term persistence in dehydrated environments (Gruzdev et al., 2012). Therefore, KdpA activity might considerably contribute to the survival of *E. coli* in raw milk cheese. In this study, the *kdpA* gene was deleted in an *E. coli* strain by homologous recombination to subsequently investigate the survival of the *kdpA*-deletion mutant in comparison to its wild-type strain during the production and ripening of semi-hard cheese.

Materials and methods

Deletion of the *kdpA* gene

The *E. coli* strain FAM21807, which was previously isolated from raw milk cheese, was used to construct a strain with a deleted *kdpA* gene. Using the "Quick & Easy *E. coli* Gene Deletion Kit" (Gene Bridges GmbH, Heidelberg, Germany) the *kdpA* gene in FAM21807 was disrupted by homologous recombination with an ampicillin-resistance cassette flanked by FRT sites according to manufacturers instructions. Thereafter, a pCP20 plasmid was electroporated into the modified strain to remove the antibiotic-resistance cassette by FLP site-specific recombination leaving a single FRT site as footprint. Successful deletion of the *kdpA* gene was controlled by conventional PCR using primers flanking the *kdpA* gene locus, followed by agarose gel analysis and sequencing of the amplicon.

Challenge test in semi-hard cheese

To investigate the survival phenotypes of FAM21807 wild-type (wt) and *kdpA* gene deletion mutant *(Δkdp A)* in cheese, a challenge test study was conducted. Therefore the *E. coli* strains were spiked into milk that was subsequently used to produce foil-ripened Tilsiter cheese. Initially, 2 l pasteurized milk warmed to 32 °C were spiked with either wt or Δ*kdpA* by the addition of 200 µl from a 1:100 dilution of a culture grown for 16–18 h in skimmed milk at 37 °C. Subsequently, 0.4 ml of a 34 % (w/v) calcium chloride solution, 143 ml pasteurized (past.) water and 1.5 ml each of starter culture MK401 and MK150 were added (MK401 and MK150 are raw mixed cultures of *Lactobacillus delbrueckii* ssp. *lactis, Streptococcus thermophilus,* and *Lactococcus lactis* ssp. *lactis;* Agroscope, Liebefeld, Switzerland). The mixture was rested at 32 °C with occasional stirring, and after 30 min, 0.4 ml of rennet (Winkler GR orange, Winkler AG, Konolfingen, Switzerland) diluted in 5 ml of past. water was added. After 35 min of coagulation, the curd was cut into 4–8 mm cubes and 570 ml of past. water were added. The curd was rested for 20 min at 32 °C and thereafter warmed to 42 °C within 15 min and subsequently rested for 15 min at 42 °C while stirring. The curd was poured into a perforated circular plastic mold (diameter of 9.5 cm) and incubated according to the following temperature programme: 36 °C for 4 h, 34 °C for 4 h, 30 °C for 10 h and 26 °C for 4–8 h. The cheese was pressed during the first 1.5 h in the incubator, then turned and pressed again for 1.5 h by placing an 850 g weight (corresponding to a pressure of app. 1176 Pa) on top of the plastic mold. After incubation for 18–22 h, the cheese was put into 22 % (w/v) brine at 14 °C, dry salt was put on the surface and the cheese rested for 20 min. Then the cheese was turned, dry salt applied to the surface and rested for additional 20 min in brine. Thereafter, the cheese was dried for 1 h in a desiccator at 14 °C before it was put into foil, vacuumed and then ripened at 14 °C for 112 days. Three cheeses were produced for each spiked FAM21807 variant and in addition, three cheeses were produced without addition of an *E. coli* strain to monitor the cheese production and ripening process. Samples were taken after spiking of the milk, after pouring the curd into the mold, and from the cheese after drying in the desiccator as well as after 2, 4, 6, 8, 10, 12, 14 and 16 weeks of ripening. Curd and cheese samples were homogenized in dipotassium hydrogenphosphate solution (115 mmol/l dipotassium hydrogenphosphate, pH 7.5) by using a stomacher. The milk and homogenized curd or cheese samples were subsequently serially diluted and spread plated on MacConkey agar plates (BD Difco™ MacConkey Agar, Becton Dickinson, Heidelberg, Germany), which were then incubated for 16–18 h at 37 °C to enumerate colony forming units (cfu). In addition, the acidification of the cheese was checked during the ripening period by using a pH meter.

Behaviour in different Luria-Bertani broth variants

To investigate the behaviour of wt and Δ*kdpA* strains in different Luria-Bertani broth (LB, Becton Dickinson) variants, regular LB broth was inoculated with either wt or Δ*kdpA* and grown for 16–18 h at 37 °C. These cultures were subsequently used to inoculate a) regular LB, b) LB containing 5 % (w/v) additional sodium chloride or c) LB containing 10 % (w/v) additional sodium chloride at an inoculation level of approximately 3.5 $\log_{10}$ cfu/ml. The LB variants were subsequently incubated at 22 °C or 37 °C and regularly sampled to determine cfu/ml by spread plating on LB agar plates (LB Agar Miller, Becton Dickinson).

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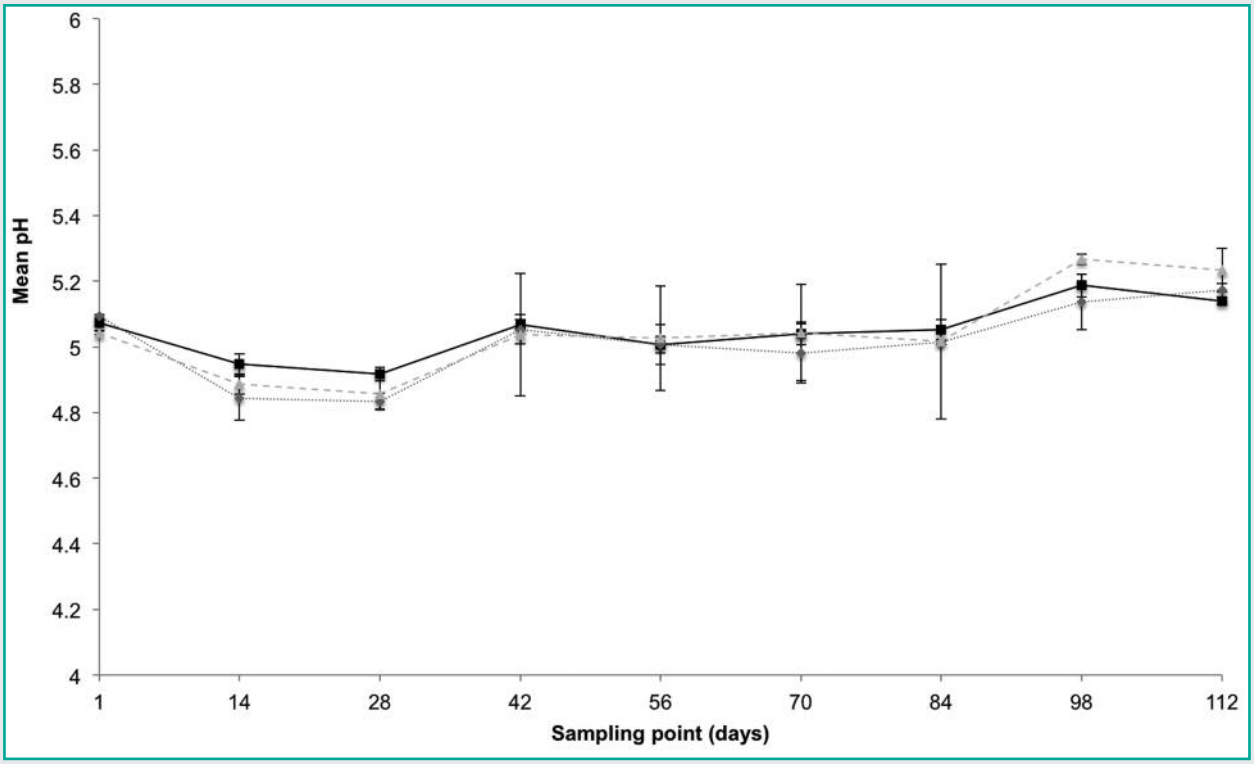

FIGURE 1: *Course of pH during ripening of semi-hard cheese. Mean pH and standard deviations were determined based on three independent experiments. Control cheese without spiked E. coli (■, solid line), cheese spiked with wild-type strain (◆, dotted line), cheese spiked with ΔkdpA strain (▲, dashed line).*

Results & Discussion

Challenge test in semi-hard cheese

The cheese production process was monitored by measuring the pH curve throughout ripening, which showed no differences between control cheeses and the cheeses spiked with the *E. coli* strains and a typical pH curve for the produced cheese type (Fig. 1). The survival of FAM21807 wt and Δ*kdpA* was investigated during production and ripening of the semi-hard cheese (Fig. 2). After spiking, wt and Δ*kdpA* were counted in milk at 2.9 ± 0.0 and 2.8 ± 0.1 $\log_{10}$ cfu/g, respectively, before an increase of 4.9 and 5.2 $\log_{10}$ cfu/g was observed in the cheese before salting of the loaf. This increase is attributed to a physical concentration effect from milk to the curd, which is estimated to contribute about 1 $\log_{10}$ cfu/g, and growth of the *E. coli* strains during the cheese production process. Such an increase was also similarly observed in other challenge test studies in cheese (Peng et al., 2013a, Peng et al., 2013b). Thereafter a slow decrease in *E. coli* counts of 1.80 and 1.38 $\log_{10}$ cfu/g occurred for wt and Δ*kdpA*, respectively, until the end of the 16 week ripening period. On average the reduction of wt and Δ*kdpA* was 0.11 and 0.09 $\log_{10}$ cfu/g per week, respectively. The inactivation occurred more slowly than observed for *E. coli* strains in a

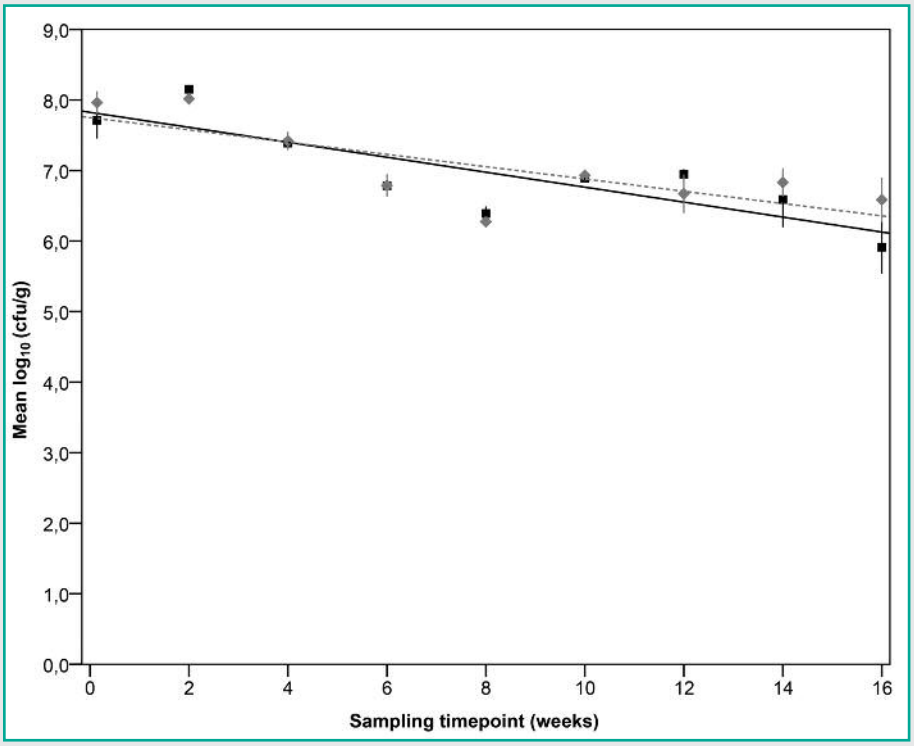

FIGURE 2: *Survival of E. coli FAM21807 wild-type and ΔkdpA strains in semi-hard cheese. Linear regression, mean $\log_{10}$ cfu/g and standard deviations were determined based on three independent experiments. Wild-type (■, solid line), ΔkdpA (◆, dashed). Fit of linear regression was $R^2 = 0.711$ (wt) and $R^2 = 0.602$ (ΔkdpA).*

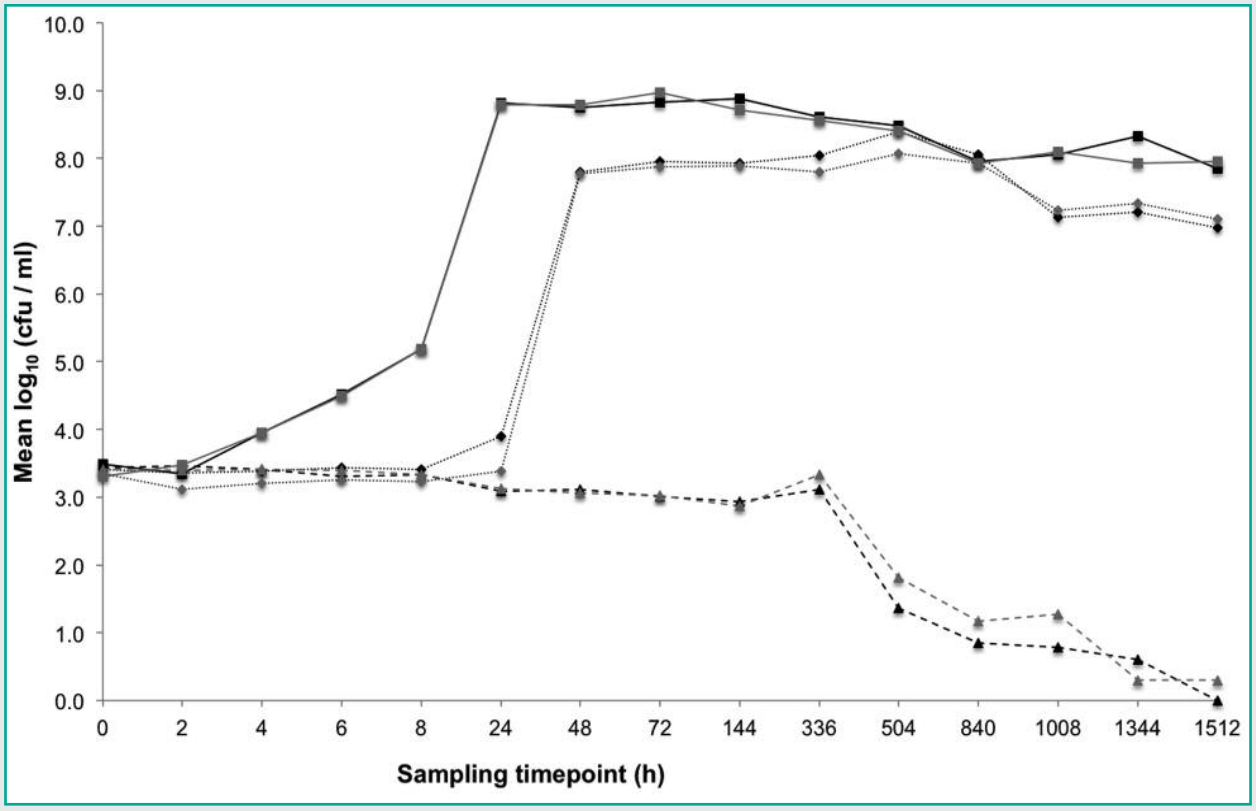

FIGURE 3: *Behaviour of E. coli FAM21807 wild-type and ΔkdpA strains in different Luria-Bertani (LB) broth variants containing 0, 5, or 10 % (w/v) additional sodium chloride incubated at 22 °C. 0 % sodium chloride (■, ■, solid line), 5 % sodium chloride (◆, ◆, dotted line), 10 % sodium chloride (▲, ▲, dashed line); wild-type (black), ΔkdpA strain (grey).*

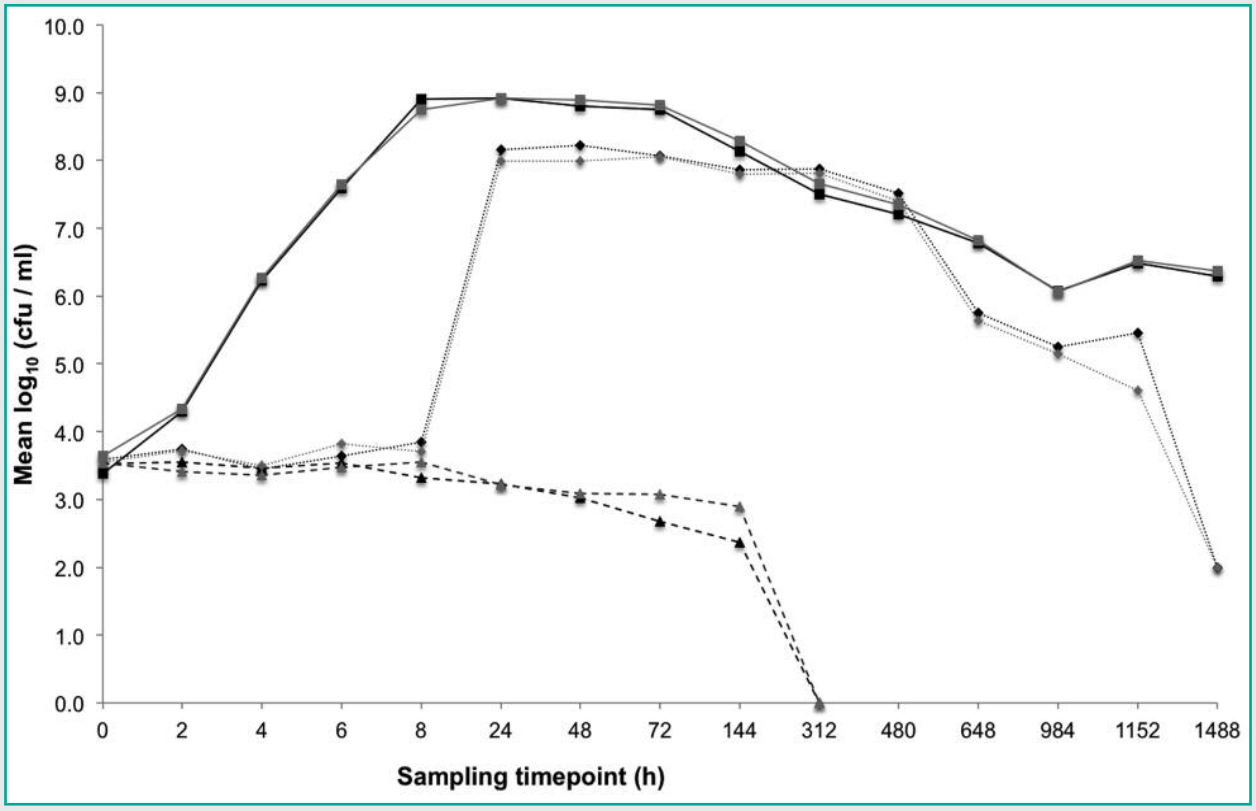

FIGURE 4: *Behaviour of E. coli FAM21807 wild-type and ΔkdpA strains in different Luria-Bertani (LB) broth variants containing 0, 5, or 10 % (w/v) additional sodium chloride incubated at 37 °C. 0 % sodium chloride (■, ■, solid line), 5 % sodium chloride (◆, ◆, dotted line), 10 % sodium chloride (▲, ▲, dashed line); wild-type (black), ΔkdpA strain (grey).*

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recent cheese challenge test study (Peng et al, 2013a). This indicates that the overall stress encountered was lower in the foil-ripened cheese than in the semi-hard raw milk cheese previously assayed or that strain FAM21807 is better able to survive in cheese than the *E. coli* strains investigated in the recent study. Nevertheless, the survival of wt and Δ*kdpA* strains was highly similar in the cheese assayed (repeated measures ANOVA p value = 0.256), indicating that lack of the *kdpA* gene did not affect survival of the *E. coli* strain in cheese. In addition to the challenge test study in cheese, the wt and Δ*kdpA* strain were assayed for their behaviour in LB broth variants that contained increased sodium chloride concentrations (Fig. 3 and 4). In regular LB and LB containing 5 % (w/v) additional sodium chloride, wt and Δ*kdpA* grew to a similar maximal level at 22 °C and 37 °C. Thereafter, the reduction in *E. coli* counts observed at 22 °C was lower than at 37 °C, which was in agreement with other studies that reported a higher non-thermal inactivation rate at increasing temperatures (Ross et al. 2008). In LB containing 10 % (w/v) additional sodium chloride, *E. coli* counts decreased slowly after inoculation with a stronger inactivation at 37 °C than at 27 °C. Nevertheless, in all LB variants, there were no differences observed between the wt and the Δ*kdpA* strain like in the cheese challenge test study.

In conclusion, no differences in survival in semi-hard cheese or in behaviour in different LB variants were observed between the wt and the Δ*kdpA* strain. The lack in KdpA activity might have been compensated by the induction of other salt stress response genes such as those responsible for uptake and synthesis of compatible solutes (Wood, 1999). Further investigations are still required to analyse the contribution of different (salt) stress response genes for *E. coli* survival in raw milk cheese.

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