Journal of Food Safety and Food Quality 63, Heft 5 (2012), Seiten 129–164

Arch Lebensmittelhyg 63,
136–141 (2012)
DOI 10.2376/0003-925X-63-136

ISSN 0003-925X

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In vitro characterization of Shiga toxin-producing and generic *Escherichia coli* in respect of cheese production-relevant stresses

In vitro Charakterisierung von Shigatoxin-produzierenden und generischen Escherichia coli im Hinblick auf mit der Käseproduktion assoziierte Stressfaktoren

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Summary

Forty-one *Escherichia (E.) coli* strains from raw milk cheese, including 26 Shiga toxin-producing strains (STEC), and six STEC strains from cattle feces were characterized in respect of cheese production-relevant stress (thermal inactivation, glucose-repressed oxidative acid resistance system, acidic and osmotic stress). Of all 47 *E. coli* isolates, seven generic *E. coli* strains showed an increased heat tolerance (55 °C, 15 min) and 20 strains (STEC and generic *E. coli*) exhibited a survival rate of ≥10 % (pH 2.5, 2 h) with induced oxidative acid resistance system. Besides, growth was observed for all *E. coli* strains at pH or a_w values commonly found in (semi-hard) cheese (pH=5.2, a_w=0.970), whereas counts tended to decrease at a pH value of 4.5 or an a_W value of 0.942. Overall, no clear and universal differences between STEC and generic *E. coli* strains were found. The large strain variations observed in respect of the applied stresses within this strain collection do not indicate a shared feature amongst our *E. coli* strains that may be beneficial for their survival in raw milk cheese. It remains to be elucidated if there are key factors enabling the survival of *E. coli* and in particular STEC during production of raw milk cheese or if differences in the production process are more important.

Keywords: Shiga toxin-producing *Escherichia coli,* heat stress, acid stress, osmotic stress, raw milk cheese

Zusammenfassung

In der vorliegenden Arbeit wurden 41 *Escherichia (E.) coli*-Stämme aus Rohmilchkäse, einschliesslich 26 Stämmen von Shigatoxin-bildenden *Escherichia coli* (STEC), sowie sechs STEC-Stämme aus Rinderkot auf das Verhalten gegenüber Hitzestress, Säurestress und osmotischem Stress untersucht. Eine erhöhte Hitzetoleranz (55 °C, 15 min) wurde bei sieben generischen *E. coli*-Stämmen festgestellt. Mit induziertem oxidativem Säureresistenzsystem zeigten 20 Stämme (STEC und generische *E. coli*) nach 2 h bei pH 2.5 Überlebensraten von ≥10 %. Zudem lag bei einem pH-Wert von 5.2 oder einem a_w-Wert von 0.970 für alle *E. coli*-Stämme Wachstum vor, während sich die Stämme bei einem pH-Wert von 4.5 oder einem a_w-Wert von 0.942 nicht mehr an den jeweiligen Stress adaptieren konnten. Insgesamt zeigten sich dabei keine eindeutigen Unterschiede zwischen STEC und generischen *E. coli.* Die grossen Stammvariabilitäten gegenüber den untersuchten Stressfaktoren innerhalb dieses Stammkollektivs sprechen gegen einen gemeinsamen Faktor, der das Überleben von *E. coli* in Rohmilchkäse begünstigt. Es bleibt abzuklären, ob solche Schlüsselfaktoren für das Überleben von *E. coli* und insbesondere STEC während der Rohmilchkäseproduktion existieren oder ob Unterschiede im Herstellungsprozess diesbezüglich von grösserer Bedeutung sind.

Schlüsselwörter: Shigatoxin-bildende *Escherichia coli,* Hitzestress, Säurestress, osmotischer Stress, Rohmilchkäse

Introduction

Many cheeses throughout Europe are typically made from unpasteurized milk with the natural enzymes and microflora responsible for enhancing desirable flavor characteristics. However, along with poor hygienic practices, pathogenic bacteria as Shiga toxin-producing *Escherichia coli* (STEC) can gain access to the raw milk by fecal contamination during the milking process since ruminants represent an important natural reservoir of STEC. Recently, several studies also reported the occurrence of STEC in raw milk cheese (Mora et al., 2007; Pradel et al., 2000; Madic et al., 2011; Rey et al., 2006; Vernozy-Rozand et al., 2005; Zweifel et al., 2010). An increased potential to carry STEC and other bacteria pathogenic for humans has thereby been demonstrated for soft and semi-soft raw milk cheese (Almeida et al., 2007; Andreoletti et al., 2007). Dairy products including cheese have also been linked to STEC outbreaks (Deschênes et al., 1996; Espié et al., 2006; Gaulin et al., 2012; Honish et al., 2005)

During the cheese production process, *Escherichia (E.) coli* encounter different stress conditions (Peng et al., 2011). Major stresses comprise the cooking temperature causing heat shock, low water activity (a_w) causing osmotic stress, and the acidification due to the metabolic activity of lactic acid bacteria. For the survival of *E. coli* during production and ripening of raw milk cheese, the corresponding stress response mechanisms are therefore of great importance. In the present study, STEC and generic *E. coli* strains originating from raw milk cheese, which might have adapted to respective stresses or might possess special properties enabling them to survive the cheese production, as well as STEC strains originating from cattle feces were investigated. The latter were selected because fecal contamination constitutes the common source of STEC in raw milk. These *E. coli* strains were *in vitro* characterized in respect of cheese production-relevant stresses. The aims were thereby to compare the properties of STEC and generic *E. coli* strains and to identify features enhancing the survival during production and ripening of raw milk cheese.

Materials and methods

Collection of *E. coli* strains

The origin and selected characteristics of the 47 investigated *E. coli* strains investigated are shown in Table 1. Phenotypically, only two strains (3164/1, 3721/2) from cattle feces showed hemolytic activity on sheep blood agar plates and six strains (FAM19196; FAM19197; FAM21804; FAM21807; K133;

TABLE 1: *Selected characteristics and origin of the 47 investigated E. coli strains.*

Serotype	Strain	*stx*1	*stx*2	*hlyA*	*eae*	Cheese-type	Milk-type
O2:H27	K156/1	–	+	–	–	semi-hard	cows' milk
O2:H27	K170/3	–	+	–	–	semi-hard	cows' milk
O2:H27	K342	–	+	–	–	semi-hard	cows' milk
O2:H27	K356	–	+	–	–	semi-hard	cows' milk
O2:H27	K381	–	+	–	–	semi-hard	cows' milk
O2:H27	312	–	+	–	–	–	cattle feces
O2:[H27][a])	K435	–	+	–	–	semi-hard	cows' milk
O2:H45	4191/1	+	–	–	–	–	cattle feces
O8:H20	K572	–	+	–	–	semi-hard	goat's milk
O8:H21	FAM19195	–	–	–	–	semi-hard	cows' milk
O9:[H21][a])	K303	–	–	–	–	semi-hard	cows' milk
O11:H11	FAM21808	–	–	–	–	semi-hard	cows' milk
O15:H16	K332	–	+	–	–	semi-hard	goat's milk
O15:H16	K345	–	+	–	–	semi-hard	cows' milk
O16:H21	FAM21846	–	–	–	–	semi-hard	cows' milk
O21:NT	K5712	–	–	–	–	semi-hard	cows' milk
O22:H8	K172/2	–	+	–	–	semi-hard	cows' milk
O22:H16	K280	–	+	+	–	semi-hard	cows' milk
O26:H11	N09-1208	+	–	–	+	vat milk	cows' milk
O27:NM	FAM21802	–	–	–	–	semi-hard	cows' milk
O68:H14	FAM19201	–	–	–	–	semi-hard	cows' milk
O68:H14	FAM21805	–	–	–	–	soft	cows' milk
O68:H14	FAM21807	–	–	–	–	semi-hard	cows' milk
O68:[H14][a])	FAM21845	–	–	–	–	semi-hard	cows' milk
O82:H17	FAM19196	–	–	–	–	semi-hard	cows' milk
O86:[H21][a])	K388	+	–	–	–	semi-hard	cows' milk
O91:[H10][a])	K124	–	+	–	–	semi-hard	cows' milk
O91:H21	K307/4	–	+	+	–	semi-hard	cows' milk
O91:H21	K331/4	+	+	+	–	semi-hard	cows' milk
O91:H21	3721/2	+	+	+	–	–	cattle feces
O103:H2	3164/1	+	–	+	–	–	cattle feces
O113:H4	K133	–	+	–	–	semi-hard	cows' milk
O113:H4	STM3	+	–	–	–	–	cattle feces
O116:[H28][a])	K406	–	+	–	–	soft	goat's milk
O116:NM	3943/1	–	+	–	–	–	cattle feces
O132:H21	FAM21803	–	–	–	–	semi-hard	cows' milk
O136:H16	FAM19197	–	+	–	–	semi-hard	cows' milk
O146:H21	FAM21806	–	–	–	–	semi-hard	cows' milk
O148:H8	K376	–	+	–	–	semi-hard	cows' milk
O174:H21	K386	–	+	–	–	semi-hard	cows' milk
O174:[H21][a])	K125	–	+	–	–	semi-hard	cows' milk
O178:H12	FAM21843	–	–	–	–	semi-hard	cows' milk
NT:H9	K138/1	–	+	–	–	soft	goat's milk
NT:H9	K145/2	–	+	–	–	soft	goat's milk
NT:H16	K35413	–	+	–	–	semi-hard	cows' milk
NT:H17	FAM21804	–	–	–	–	semi-hard	cows' milk
NT:NM	K25	–	+	–	–	soft	cows' milk

[a]): phenotypically non-motile

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N09-1208) grew on cefixime-tellurite (0.05/2.5 mg/l) sorbitol MacConkey agar (CT-SMAC agar, Oxoid AG, Pratteln, Switzerland). Serotypes determined using monospecific antisera (O1 to O181 and H1 to H56) and the presence or absence of *stx1* and *stx2* (encoding Shiga toxins of the respective group), *eae* (encoding intimin), and *hlyA* (encoding EHEC hemolysin) are shown in Table 1. The 41 *E. coli* strains from raw milk cheese comprised 26 STEC strains and the majority of them originated from semi-hard cheese. Twenty-five STEC strains have been described previously (Zweifel et al., 2010) and one strain (FAM19197) was provided by M. Contzen (Chemisches und Veterinäruntersuchungsamt Stuttgart, Germany). In addition to the strains from raw milk cheese, six STEC strains previously isolated from cattle feces (Zweifel et al., 2005) were included in the present study.

RpoS phenotype

For indirect screening of the allelic state of *rpoS,* the hydrogen peroxidase II (HPII) assay was used (Large et al., 2005). Twenty-four colonies of each isolate from Luria-Bertani (LB) agar plates (LB Agar Miller, Beckton Dickinson AG) were tested for catalase activity by using 3 % (w/v) hydrogen peroxide and for glycogen production by using iodine.

Thermal inactivation

After incubation (16–18 h, 37 °C) of the isolates in 10 ml of brain heart infusion (BHI) broth (Oxoid AG), 0.1 ml of the 10^{-2} dilutions were inoculated in 0.9 ml of BHI broth, which had been pre-warmed (55 °C) in a thermoblock using 1.5 ml plastic tubes. To evaluate the thermal inactivation, samples of 0.1 ml were removed immediately and after 15 min and plated on BHI agar plates (Oxoid AG). This assay was performed twice for each strain. For calculation of $\log_{10}$ reduction between the sampling points, colony counts were logarithmized ($\log_{10}$). D values (time to inactivate 90 % of the population at a defined temperature) were calculated by D value = time / $\log_{10}$ reduction.

Glucose-repressed oxidative acid resistance (OXI) system

Conditions inducing and repressing the OXI system were tested according to Lin et al. (1996) and Large et al. (2005). After incubation (16–18 h, 37 °C) of the isolates in 10 ml of LB broth, 50 µl were added (i) to 10 ml of LB broth buffered to pH 5.5 with 0.1 mol/l morpholineethanesulfonic acid (LBMES) and (ii) to 10 ml of minimal E (EG) medium buffered to pH 7.0 (0.073 mol/l dipotassium hydrogen phosphate, 0.017 mol/l ammonium sodium hydrogen phosphate, 0.0008 mol/l magnesium sulfate, 0.01 mol/l citrate and 4 % (w/v) glucose, pH was adjusted using 4 mol/l hydrochloric acid solution). After incubation of LBMES pH 5.5 (induced OXI system) and EG pH 7.0 media (repressed OXI system) for 24 h at 37 °C, aliquots of 10 µl were added (in duplicate for LBMES media) to 10 ml of EG medium pH 2.5 at 37 °C. Immediately and after 2 h, samples of 0.1 ml were plated on LB agar plates for colony counting. For each strain, percentage of survival between the sampling points was calculated.

Acidic and osmotic stress

After incubation (16–18 h, 37 °C) of the isolates in 10 ml of BHI broth, 0.1 ml of the 10^{-2} dilutions were inoculated in four variants of pre-warmed (37 °C) BHI broth (9 ml): a) BHI broth pH 5.2, b) BHI broth pH 4.5 (adjusted using 90 % lactic acid), c) BHI broth with 5 % (w/v) sodium chloride (a_w of 0.970) and d) BHI broth with 10 % (w/v) sodium chloride (a_w of 0.942). These conditions were selected to represent typical and extreme pH and aw values found in (semi-hard) cheese. Immediately and after 2, 4, 6, 8, and 24 h of incubation (37 °C), samples of 0.1 ml were plated on BHI agar plates. Colony counts were logarithmized ($\log_{10}$) and compared using repeated measurement analysis of variance (ANOVA).

Results and discussion

RpoS phenotype

Forty-six of the 47 *E. coli* strains were positive for catalase activity and glycogen accumulation. The lacking of catalase activity in the generic *E. coli* strain K303 might indicate a potential RpoS dysfunction, which could affect the stress response abilities (Large et al., 2005). In accordance, *E. coli* strain K303 was strongly inactivated in the thermal inactivation and the OXI system assays.

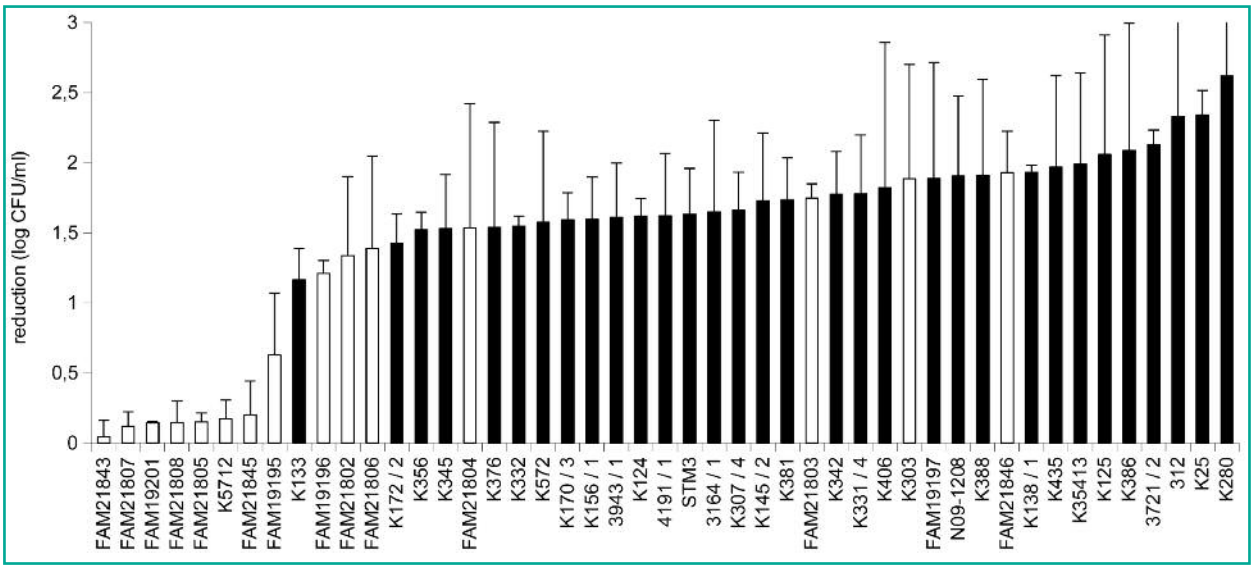

FIGURE 1: *Thermal inactivation of the E. coli strains in BHI broth at 55 °C after 15 min (Average and standard deviation of two replicates. STEC strains: full bars, generic E. coli strains: empty bars).*

Thermal inactivation

After 15 min in BHI broth at 55 °C, an average reduction of at least one order of magnitude was observed for all 32 STEC strains and seven of the 15 generic *E. coli* strains (Fig. 1; average D_{55} value = 8.8±1.5 min; average D_{55} value of only STEC = 8.6±1.3 min). The D_{55} values are comparable to those reported by Whiting and Golden (2002) for different O157:H7 *E. coli* strains. Of the remaining eight generic *E. coli* strains (Fig. 1), strain FAM19195 was reduced on average by 0.6 orders of magnitude (D_{55} value = 23.9 min), whereas seven strains, including four strains of serotype O68:H14, were reduced by less than 0.2 orders of magnitude (average D_{55} value = 133±91 min; P>0.05). In the literature, a similar heat tolerance has only been rarely described for *E. coli* isolates (Dlusskaya et al., 2011). The mechanisms for the increased heat tolerance remain to be elucidated. E. g., the ATPase ClpK, described in *Klebsiella pneumoniae,* was associated with high thermotolerance and shares extensive amino acid sequence identity with uncharacterized Clp proteins in *E. coli* genomes (Bojer et al., 2010).

Glucose-repressed oxidative acid resistance (OXI) system

The survival rate of the *E. coli* strains with induced OXI system was between 0.4 % and 44.9 % at pH 2.5 after 2 h (Fig. 2). Twenty strains exhibited a survival rate of ≥10 %, including both STEC and generic *E. coli* strains. Five of the six STEC strains from cattle feces thereby belonged to this group. On the other hand, when the OXI system was repressed, the survival rate of 34 strains was ≤1 % or lower. But two generic *E. coli* strains, both belonging to the serotype O68:H14, showed a survival rate of more than 10 % under these conditions. Our results from strains with induced or repressed OXI system indicate an important role of the OXI system for the survival of acidic conditions, albeit the relevance of this system might vary between different food types and production processes. E. g., Montet et al. (2009a, 2009b) compared the survival of acid-resistant and non-acid-resistant STEC in Camembert cheese and fermented raw meat sausages. No differences in survival were thereby observed in Camembert cheese between acid-resistant and non-acid-resistant STEC strains (Montet et al. 2009b). But in raw meat sausage, only acid-resistant STEC strains persisted during the whole ripening period (Montet et al. 2009a).

Acidic and osmotic stress

In BHI broth acidified to pH 5.2, the *E. coli* strains grew by two orders of magnitude within 8 h (Fig. 3a). Higher counts were thereby observed during the growth phase for STEC strains from raw milk cheese. These isolates have probably adapted faster to the acidic conditions than the other strains. In BHI broth acidified to pH 4.5, counts of all *E. coli* strains slightly decreased (by about 0.2 orders of magnitude) within 24 h (Fig. 3b). In BHI broth containing 5 % sodium chloride (a_W=0.970), after a slight reduction within 2 h, all *E. coli* strains grew by about two orders of magnitude within 24 h (Fig. 3c). Higher counts were thereby observed during the growth phase for the STEC strains from cattle feces, but this was probably related to the higher inoculation level. In BHI broth containing 10 % sodium chloride (a_W=0.942), an average reduction of 0.8 - orders of magnitude was observed for all *E. coli* strains within 24 h (Fig. 3d). High salt concentrations cause osmotic upshifts, which have the potential to disrupt bacteria. The response to osmotic stress includes e.g. the accumulation of compatible solutes to compensate the osmotic difference (Wood, 1999). Our results indicate that the investigated *E. coli* strains could adapt to 5 % sodium chloride and restore growth, while they were not able to compensate the osmotic stress caused by 10 % sodium chloride.

Conclusion

In the present study, we characterized 32 STEC strains (from raw milk cheese and cattle feces) and 15 generic *E. coli* strains (from raw milk cheese) in respect of cheese production-relevant stress. No clear and universal differences were thereby found between the STEC and generic *E.*

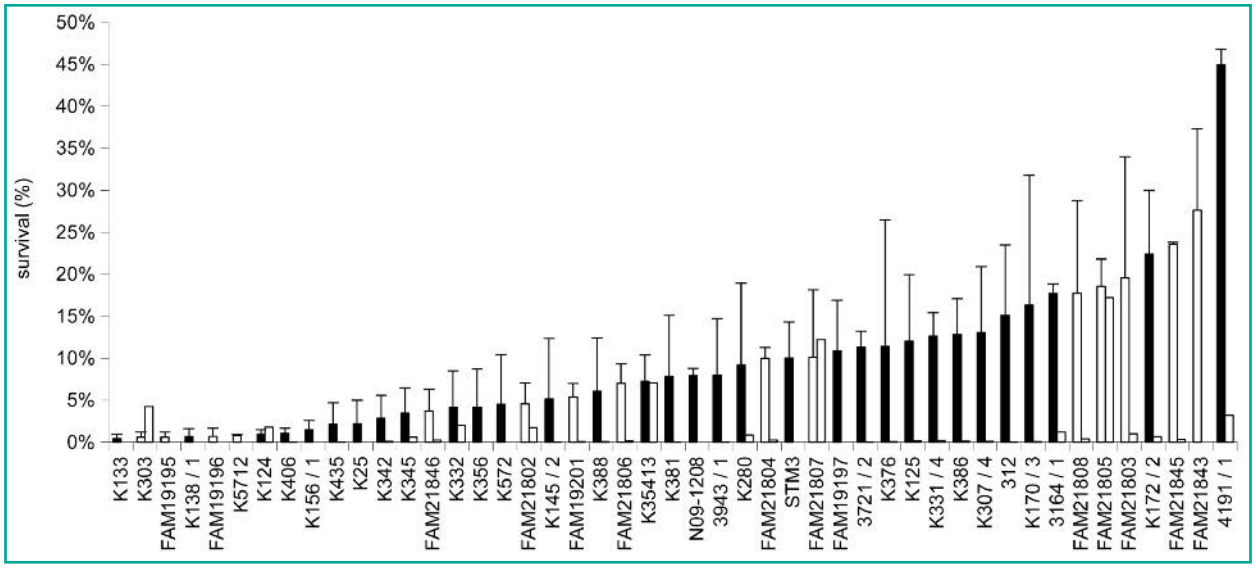

FIGURE 2: *Survival (percentage) of the E. coli strains with induced or repressed OXI system (STEC strains with induced OXI system: full bars; generic E. coli with induced OXI system strains: empty bars; average and standard deviation of two replicates; repressed OXI system: dashed bars).*

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coli strains. Of the 47 *E. coli* isolates, seven generic *E. coli* strains showed an increased heat tolerance (55°C, 15 min) and 20 strains (STEC and generic *E. coli*) exhibited a survival rate of more than ≥10 % (pH 2.5, 2 h) with induced OXI system. Besides, growth was observed for all *E. coli* strains at pH or a_w values commonly found in (semi-hard) cheese (pH=5.2, a_w=0.970), while they were not able to adapt to the acidic stress at pH 4.5 or the osmotic stress at a_w 0.942. The fact that the majority of our *E. coli* strains, including those showing no increased heat or acid tolerance, were isolated from (semi-hard) raw milk cheese, indicates that (i) conditions during cheese production (cooking temperatures, acidification) were not sufficient for inactivation and (ii) the investigated stress response mechanisms are probably of minor importance for the survival of *E. coli* in raw milk cheese. To inactivate STEC and other unwanted bacteria, evaluation of e. g. effective sub-pasteurization heat-treatments of raw milk is therefore advisable. Besides, the large strain variations observed in respect of the applied stresses do not indicate a shared feature amongst our *E. coli* strains that may be beneficial for their survival in raw milk cheese. It remains to be elucidated if there are key factors enabling the survival of *E. coli* and in particular STEC during production of raw milk cheese or if differences in the production process are more important.

Acknowledgements

We thank Dieter Weik and Javorka Naskova (Agroscope Liebefeld-Posieux) for their technical assistance.

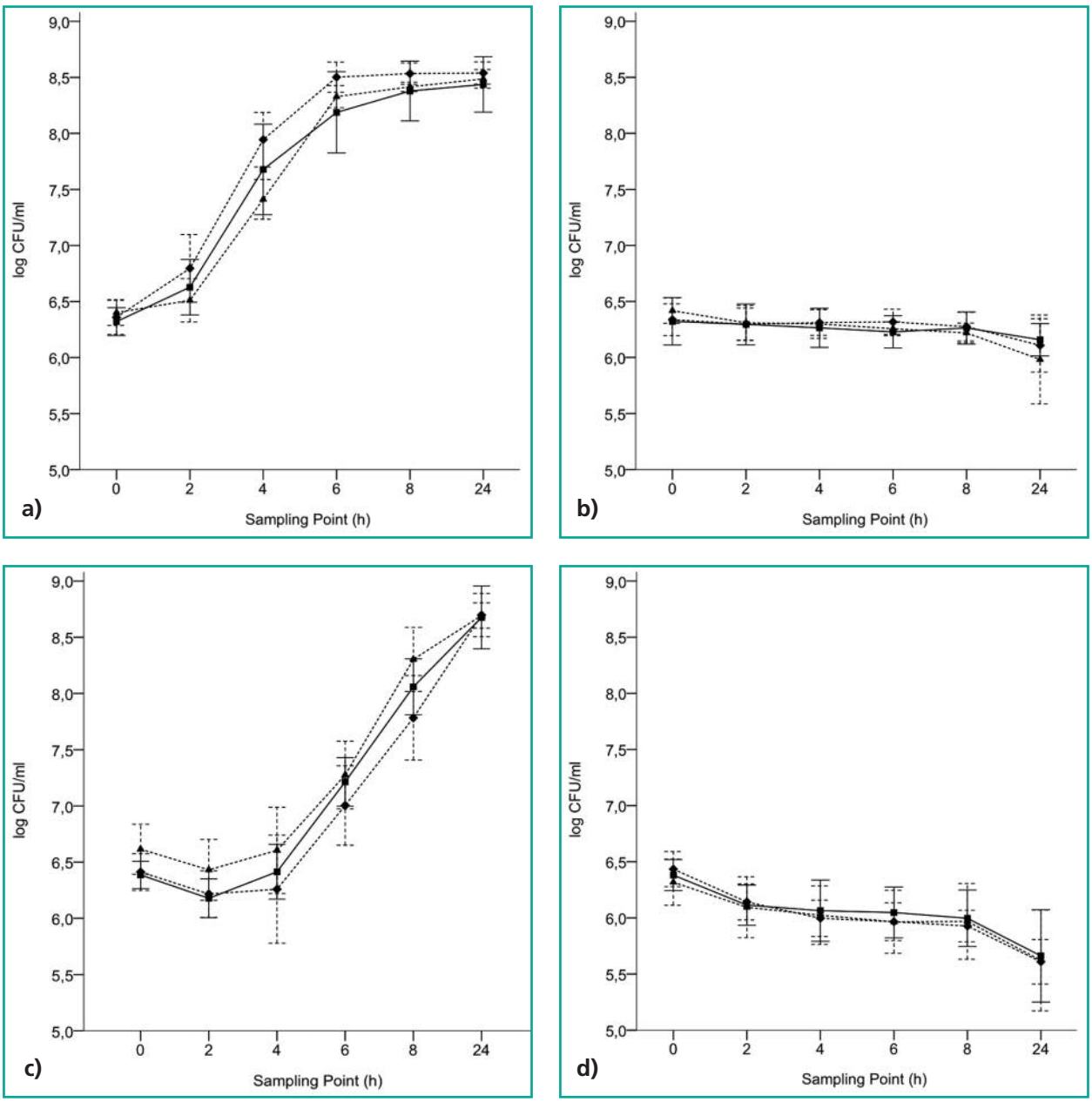

FIGURE 3: *Behavior of the E. coli strains in BHI broth a) acidified to pH 5.2, b) acidified to pH 4.5, c) containing 5 % (w/v) sodium chloride, and d) 10 % (w/v) sodium chloride (STEC strains from raw milk cheese: ◆, dashed line, n=26; generic E. coli strains from raw milk cheese: ■, solid line, n=15; STEC strains from cattle feces: ▲, dashed line, n=6; average and standard deviation of strains within the same group).*

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