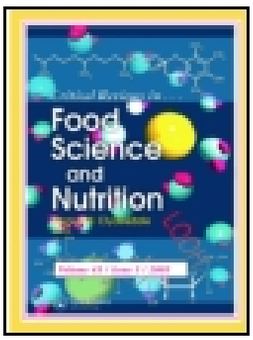

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REVIEW

Everybody loves cheese: crosslink between persistence and virulence of Shiga-toxin *Escherichia coli*

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ABSTRACT

General cheese manufacturing involves high temperatures, fermentation and ripening steps that function as hurdles to microbial growth. On the other hand, the application of several different formulations and manufacturing techniques may create a bacterial protective environment. In cheese, the persistent behavior of Shiga toxin-producing *Escherichia coli* (STEC) relies on complex mechanisms that enable bacteria to respond to stressful conditions found in cheese matrix. In this review, we discuss how STEC manages to survive to high and low temperatures, hyperosmotic conditions, exposure to weak organic acids, and pH decreasing related to cheese manufacturing, the cheese matrix itself and storage. Moreover, we discuss how these stress responses interact with each other by enhancing adaptation and consequently, the persistence of STEC in cheese. Further, we show how virulence genes *eae* and *tir* are affected by stress response mechanisms, increasing either cell adherence or virulence factors production, which leads to a selection of more resistant and virulent pathogens in the cheese industry, leading to a public health issue.

KEYWORDS

Acid stress; cross protection; heat shock; osmotic stress; STEC; stress responses

Introduction

Milk and cheese consumption date from a long time ago, probably when humans understood domestication, following the nutritious and beneficial values such products could provide (Fox et al. 2017a; Gross 2018). Originally, cheese has been created in an attempt to concentrate and conserve milk in a stable state so it could be consumed posteriorly (Yoon, Lee, and Choi 2016). In fact, the development of techniques such as pasteurization, renneting and the utilization of starter cultures brought cheesemaking to another level of processing, thus enabling cheese diffusion around the world (Bennett and Johnston 2004; Johnson 2017; McSweeney, Ottogalli, and Fox 2004).

The cheesemaking process includes different manufacturing steps that work as hurdles, thus averting microbial proliferation. These steps include the filtering/clarification of milk, high temperatures in pasteurization and cooking processes, addition of salt or contact with brine, fermentation processes, decreasing of a_w, addition or production of weak organic acids by lactic acid bacteria, among others (Artursson et al. 2018; Fox and McSweeney 2017). Cheese is considered a ready-to-eat food, not requiring further cooking nor processing prior to its consumption (Moubarac et al. 2014). As the majority of the microorganisms are intended to be inactivated after proper milk pasteurization, the subsequent manufacturing steps together with cheese physicochemical characteristics should be able to guarantee the safety of the final product, unless post contamination occurs (Fromm and Boor 2004; Martin et al. 2016). Therefore, as raw milk cheese lacks the pasteurization step, it should be consumed carefully, especially those of soft and semisoft varieties, which are more prone to harbor foodborne pathogens than those of hard varieties (Andreoletti et al. 2007; Leistner 2000).

Microorganisms such as *Escherichia coli*, a commensal bacterium in the gastrointestinal tract of humans and animals, are known to be indicators of fecal contamination of foods (Conway and Cohen 2015). Although the majority of *E. coli* isolates are incapable of causing disease in the host, diarrheagenic strains can be found in the most diverse types of food (Gomes et al. 2016). The symptoms and mechanisms of pathogenicity of diarrheagenic strains are related to specific characteristics related to the group in which they are classified: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusively-adherent *E. coli* (DAEC), adherent invasive *E. coli* (AIEC), Shiga-toxigenic *E. coli* (STEC) and, within the latter category, enterohemorrhagic *E. coli* (EHEC) (Robins-Browne et al. 2016).

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In this context, Shiga toxin-producing *E. coli* (STEC) is reported for being an important foodborne pathogen, often related to several outbreaks due to the action of encoded potent toxins that can cause intestinal cells damage, as well as other systemic lesions, such as bloody diarrhea (hemorrhagic colitis), and more seriously, thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) (Castro, Teixeira, et al. 2019). Furthermore, young children and elderly people are more prone to develop dangerous complications, such as HUS, acute renal failure, and neurological symptoms as sequelae, which may even result in death (Lee et al. 2016).

The main virulence factors associated with STEC are the Shiga toxins Stx1 and Stx2, whereas STEC strains can produce either one or both toxins. However, Stx2 producers are often related to severe cases of infection, including HUS and death (Steiner 2016). According to the Foodborne Outbreak Database (CDC 2015), dairy and ready-to-eat products are listed as major causes of multistate foodborne outbreaks. In fact, as cattle and other ruminants such as sheeps and goats, are recognized as the main sources of STEC shedding (Chase-Topping et al. 2008; Gonzalez and Cerqueira 2019), milk may be exposed to this pathogen by fecal contamination during milking steps. To overcome this situation, the utilization of pasteurized milk for cheese production is highly indicated, as STEC is very likely to be inactivated in this process (Ahmed and Samer 2017). However, STEC can also contaminate cheese during the manufacturing and processing steps, being able to persist in the final product (Cardoso and Marin 2016). Figure 1 shows the possible routes of contamination in a general pasteurized cheese making process (Modified from Peng et al. 2011). STEC persistence in cheese matrices is achieved through survival response mechanisms that enable the pathogen to surpass harsh conditions found in these products, which can be a risk for human consumption (Peng et al. 2011). Indeed, STEC strains are considered to be more resistant to stress abiotic factors compared to the generic *E. coli* (Vidovic and Korber 2016).

In this context, the aim of this review is to (1) considerate the cell response mechanisms which enable the persistence of *E. coli* and, more importantly, Shiga toxin-producing *E. coli*, in cheese products, and (2) how they could enhance the pathogen virulence in cheese and cheese products. Discussion about heat shock, cold stress, osmotic and acidic stress responses are pointed out, as well as the relation between stress response mechanisms and virulence traits modulation.

Cheese manufacturing: an overview

Cheese is the most consumed dairy product worldwide (Fox and McSweeney 2017). It can be produced from milk of different species in a variety of both taste and shapes, presenting distinct rheological characteristics (Silva and Costa 2019). Although considered a very nourishing product, its nutritional composition can differ not only due to its raw material characteristics (centesimal composition) but also according to the manufacturing steps it is submitted (Skeie 2010).

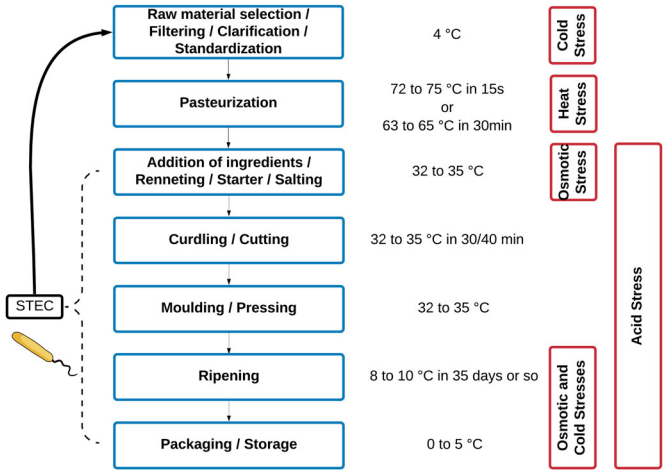

Figure 1. Flow chart of production of a general pasteurized cheese, including stresses conditions. Solid line represents standard contamination of STEC. Dashed line represents post pasteurization contamination of STEC.

Immediately after milking, milk can be stored at the farm, generally at 4 °C, and then transported to the processing plant. In an optimum situation, transportation should be done as soon as possible. Once, the maintenance of raw milk at low temperatures for a long time can favor the psychrotrophic bacteria growth, which may affect the general quality of the final product due to the production of proteolytic and lipolytic enzymes (Oliveira et al. 2015). In a general cheese preparation, milk is usually filtered and clarified before cheesemaking. These steps are responsible for the mechanical removal of milk impurities, such as hay, hair, or even clumps of somatic cells. The filtering step consists in using a cloth or a synthetic material filter to hold such undesirable solids (Artursson et al. 2018).

Similarly, the clarification step uses a two-phase centrifuge to remove materials denser than milk proteins and fat. In addition, milk can undergo separation, which consists of utilizing a three-phase centrifuge that separates milk into cream, skim milk, and a third phase consisting of impurities, which improves milk quality (Johnson 2016). Further, milk can be standardized/homogenized regarding its fat content and then submitted to cheese manufacturing (Fox et al. 2017b).

The cheese production can be performed with pasteurized or raw milk. The milk pasteurization for cheesemaking can be made out of two processes: flash pasteurization (also called "high-temperature-short-time", HTST), and low temperature pasteurization (also called "low-temperature-long-time", LTLT or vat/batch pasteurization). Such processes aim to improve cheese safety by eliminating vegetative forms of pathogenic and spoilage bacteria in milk (Qi et al. 2015). The HTST is the most used process because it reduces costs and improves the process efficacy. In this process, the milk is heated at 72 °C for 15 seconds, usually in a plate heat exchanger pasteurizer. In this pasteurization, the cold milk is heated by the pasteurized milk warmness, whereas a partial cooling of this one is achieved, being 90% more energetically efficient (Meunier-Goddik and Sandra 2016).

Contrasting, the LTLT pasteurization consists of heating the milk at 63 °C for 30 minutes. It can be carried out in a double-jacketed tank, where immediately after the treatment, cold water is circulated for later cooling. Milk must be kept under mechanical agitation to facilitate the heat exchange and prevent the product from burning on the tank walls, but, due to economical and technological aspects, this process is practically out of use in the industry (Meunier-Goddik and Sandra 2016). Moreover, after pasteurization, the milk should be rapidly cooled down again because the process cannot destroy all microorganisms, neither inactivate all enzymes (Watts 2016).

Cheese production from raw milk is only allowed for cheese with maturation step of a minimum of 60 days at high temperatures (≥2 °C) (FDA 2017). This regulation was created as a response of several foodborne outbreaks caused due to the consumption of raw milk cheese before the year of 1949 (Knoll 2005). In fact, raw milk cheese consumption has been shown to be associated with several foodborne outbreaks (Artursson et al. 2018; Costard et al. 2017; Farrokh et al. 2013).

The curdling step is present in all varieties of cheese, being the phase where the transformation of milk into curd occurs (Figure 1). The milk curd can be defined as a gel consisting mainly of the concentration of precipitated casein (milk protein) and fat, whereas the major amount of lactose, whey proteins, and soluble solids are removed together with the whey. The casein precipitation can be achieved over acid coagulation, heat-acid coagulation, or enzymatic coagulation (renneting). Briefly, acid coagulation requires a low pH environment (pH ~4.6), achieved by bacterial production or direct addition of organic acids. Because acid coagulation forms a fragile curd, this technique is used to produce soft cheese (Lucey 2011). Contrasting, enzymatic coagulation requires the addition of a rennet, which breaks the κ-casein fraction in a specific spot, thus culminating in protein precipitation and curdling. This reaction does not need an acidic environment to happen, and it is the most used technique upon cheesemaking (Gregersen and Lucey 2016).

After curdling, the cheese molding is prepared, as this step includes the collection of curds and inclusion in molds to obtain a cohesive form of mass after whey loss (Figure 1). Currently, most of the molds used in cheesemaking are available in plastic material, with perforations to facilitate the drainage of whey, which is also easier to maintain proper hygiene, compared to wooden or metallic molds, originally used (Legg et al. 2017). In addition, the dimensions of the molds (height and diameter) may vary according to the desired cheese type, which can be cosmetical or adequate toward certain varieties. In fact, surface-ripened cheeses may be formed into small and low cylindric molds, which is essential for mold ripening to happen from the surface to the center. On the other hand, cheeses with large eyes are usually large in size, also requiring a larger mold, which helps to keep the pressure of CO_2 formation inside the cheese, required for eye formation (Fox et al. 2017d).

Subsequently, pressing is usually applied to semi-hard and hard varieties aiming to complement the moisture regulation process, along with mass consolidation, which plays a role in texture and gas retention, as aforementioned (Fox et al. 2017e). Pressing methods depends on the cheese shape variety and pressing systems available. It can be performed as simple as the application of a weight on a cheese unit, to the vertical or horizontal pneumatic presses, which can be done individually or collectively (Fox et al. 2017d).

Ripening (maturation) is the step where cheese is kept under controlled temperature and humidity, whereas numerous microbiological, biochemical, physical, and chemical reactions occur (Fox et al. 2017c). Ripening involves not only hydrolysis of protein and fat, and lactose fermentation, but also the synthesis of aromatic compounds formed due to protein and fat degradation (Costa and Conte-Junior 2015; Diezhandino et al. 2015; Fox et al. 2017c). In addition, lactose fermentation is one of the most important reactions that occur during cheesemaking (Costa and Conte-Junior 2016). It can be done by a variety of lactic acid bacteria, generally mesophilic or thermophilic, via starter culture addition, which will result in a decrease of pH caused by lactic acid production, thus affecting the final product characteristics and sensory features (Bekele et al. 2019). Moreover, organic acids production in different types of cheese may vary according to the ripening conditions and time, plus the chosen starter culture (Costa and Conte-Junior 2015). Currently, the most used starter cultures in cheese are *Lactococcus* lactis, *Leuconostoc* sp., *Streptococcus thermophilus*, *Lactobacillus* delbrueckii subsp., *lactis*, *Lb. delbrueckii* subsp., *bulgaricus* and *Lb. helveticus*, which should be used based on the type of cheese wanted (Engels, Dusterhoft, and Huppertz 2017). In addition, cheese is credited to be one of the best matrices for probiotic delivery. Besides considered to be suitable for industrial production, strains of *Lactobacillus* spp. and *Bifidobacterium* spp. are constantly added to cheese in order to improve health quality of consumers (Blaiotta et al. 2017).

Moreover, cheese can be added with spices, extracts, herbs, and condiments to improve taste and aroma. Such addition is important because besides improving the product flavor, some of these ingredients show influence in the microbiological quality of the final product (Hayaloglu and Farkye 2011). As an example, the addition of oregano essential oil (*Origanum vulgare* var *hirtum*) in Argentinian cheese avoided the growth of *Enterobacteriaceae* without changing the cheese sensory acceptance (Marcial et al. 2016).

Finally, the storage step, when cheese is kept refrigerated prior and/or after selling, seems to affect cheese sensory characteristics: changes in pH, acidity, texture, elasticity, and color are frequently observed (Felicio et al. 2016). These effects are caused mainly due to proteolysis, residual lactose fermentation, and also calcium dissociation. As storage time reduces the gradual quality of cheeses, this product should not be kept under storage for extended periods (Delgado-Martínez et al. 2019; Fuentes et al. 2015).

STEC survival and persistence in cheese and cheese products

Outbreaks of STEC are becoming more frequently reported, as the surveillance laboratories are improving techniques for

etiology agent detection. For instance, most recent data of 2015 stated that 4831 cases of STEC infections were confirmed in United Stated, which is 9% more than in 2014 (CDC 2017). As STEC are considered to be a fecal-oral bacteria, the most common source of infection is contaminated food and water, mainly because of direct contact of food with ruminant's feces (Koudelka, Arnold, and Chakraborty 2018). Moreover, infections are also likely to occur through direct contact with infected people and STEC-hosting animals (Kintz et al. 2017). Regarding cheese products, the risk of STEC contamination starts at the raw material obtention. As evidence, STEC has been isolated in raw milk from different zootechny species, such as cow, ewe, goat and yak (Bandyopadhyay et al. 2012; Gonzales-Barron et al. 2017; Nobili et al. 2016; Otero et al. 2017). While the major related cases of STEC-containing cheese come from raw milk cheese production, a few reports had shown that STEC can be isolated from pasteurized cheese products, probably due to post contamination processes (Callon, Arliguie, and Montel 2016; Cardoso and Marin 2016; Fereydouni and Darbouy 2015).

As mentioned before, cheese has a great variety of formulations, culminating in several different types of products. Currently, there is no official classification on cheese based on its characteristics. However, a classic general classification of Davis (1965) based on moisture property of cheese is still accepted nowadays. For instance, very hard cheeses like Parmigiano Reggiano present the lowest moisture percentage (less than 25%), moderate low pH (5.0–5.5), and lactic acid content between 0.8% and 1.2%, whereas hard cheeses, such as Emmental, show a moisture percentage between 25% and 26% and moderate low pH (5.4–5.5). On the other hand, semihard cheeses, such as Cheddar possess an average of 36% to 40% of moisture, moderate low pH (5.0–5.4), and soft cheeses like Camembert usually have more than 40% of moisture content and lower pH (4.6–5.2) (Batty, Waite-Cusic, and Meunier-Goddik 2019; Deegan et al. 2013; Gobbetti 2004; Lawrence et al. 2004). Moreover, parameters such as oxidation-reduction potential (E_h) and water activity (a_w) also play a role in cheese composition. While milk E_h is normally ranging from +250 mV to +350 mV, cheese is credited to possess a reduced oxidation-reduction potential. In fermented cheeses, the E_h can reach −250 mV, mainly attributed to fermentation of residual lactose by starter cultures and lactic acid bacteria, together with oxygen consumption caused by cell metabolization (Bulat and Topcu 2019). In a similar way, a_w values may vary depending on the type of cheese. Although most cheeses have an a_w above 0.94, this parameter can range from 0.92 to 0.99, and may be used, together with pH values, as a new consensus categorization of cheeses, even allowing alternative procedures to milk pasteurization (Trmčić et al. 2017). In general, the intrinsic characteristics of cheeses, associated with pasteurization process and other technologies, should function as hurdles to prevent bacterial growth (Leistner 2000), although cheese from several varieties and types have been described as a source of STEC contamination or outbreak, as cited previously.

Different developing and developed countries have created standards regarding the presence of commensal or pathogenic *E. coli* in food. For instance, the Hong Kong Centre for Food Safety (2007) establishes *E. coli* as the only indicator organism for ready-to-eat foods, considering the presence of *E. coli* O157 unacceptable for consumption. Further, the Food and Drug Administration (FDA 2013) states that the presence of *E. coli* in cheese and cheese products should not surpass 110 MPN/g, and processed cheese spread must be absent for *E. coli*. Similarly, the European Commission (2005) establishes that the presence of *E. coli* in heat-treated cheese should be lower than 10^3 CFU/g. More recently, the Food Standards Australia New Zealand (2016) attributes the presence of STEC in ready-to-eat food as potentially hazardous. Besides that, some developing countries such as Brazil still lacks an up-to-date legislation where *E. coli* presence in dairy should be considered (Brasil - ANVISA 2001).

Several studies have been shown the fate of STEC strains in cheese and cheese products regarding its manufacturing process. Raw milk cheese consumption is related to more intense sensory aspects compared to pasteurized milk cheese (Yoon, Lee, and Choi 2016). However, raw milk cheese has been reported to harbor STEC after long ripening periods. For instance, STEC strains were isolated from Spanish raw ewe's milk cheese ripened for two and a half months, and also for 12 months in cheese factories, showing STEC is able to survive aging (Caro and García-Armesto 2007). In addition, STEC strains were also isolated from cow's and goat's semihard ripened cheese in Switzerland, where some strains were positive for the presence of hemolysin production, revealing a threat to human consumption (Stephan et al. 2008).

Moreover, in an attempt to challenge previously STEC isolates for ripening steps, Schlesser et al. (2006) inoculated acid-resistant *E. coli* O157 in raw milk designated for Cheddar cheese production and after testing, samples were still positive for STEC after 360 days of ripening. Similarly, milk environment-adapted STEC strains were able to resist to production plus 16 days of ripening in semihard raw milk cheese, with some samples presenting counts higher than 10 CFU/g, which can be a concern because of the relatively low infectious dose of STEC (Peng et al. 2013). This suggests that the FDA (2017) standards previously mentioned of 60 days of maturation should be revised. In fact, controlling the raw material quality seems to be more effective to avoid STEC presence in raw milk cheese.

Miszczycha et al. (2013) evaluated the persistence of different STEC serotypes (O26:H11, O103:H2, O145:H28, and O157:H7) submitted to different manufacturing and ripening conditions, such as blue-type cheese (from sheep milk), uncooked pressed cheese with both long and short ripening periods (cow milk), cooked cheese (cow milk), and lactic cheese (goat milk). First, regarding blue cheese, the persistence of *E. coli* O26:H11, O103:H2, and O157:H7, was tested. All strains decreased their concentrations during ripening, whereas after 240 days, only O26:H11 could be isolated after enrichment, besides showing a significantly higher growth

and persistence than the other tested serotypes. Second, uncooked pressed cheese with seven months ripening was tested to *E. coli* O26:H11, and O157:H7. Until day 60 of ripening, the levels of both strains remained constant. After, by day 210, the concentration levels decreased below detection levels for *E. coli* O157:H7 in both core and rind, and for O26:H11 in the rind. At the 240th day, the concentration of O26:H11 in the core was significantly higher than *E. coli* O157:H7 in both core and rind. Third, regarding uncooked pressed cheese with short ripening of 20 days (plus 20 days wrapping), all types of strains were tested and all strains remained constant until the final day of ripening, which may be a risk for consumers. Next, cooked cheese was tested for *E. coli* O157 and O26:H11. Although no STEC was recovered just after cheesemaking, strains could still be isolated at the end of the ripening period of 120 days after enrichment in the core (*E. coli* O157) and the rind (both). Finally, lactic cheese was tested for all types of strains and only serotype O157 was not able to survive after day 60. This finding suggests that STEC cells can persist to different types of cheese manufacturing, with or without enrichment.

The fermentation step that most cheeses are submitted is a vital preservation method to maintain cheese quality, and this can be made by the indigenous microbiota of raw milk, such as lactobacilli, streptococci and lactococci, or by the addition of starter culture (D'Amico and Donnelly 2017). Although raw milk is not heat-treated prior to cheese manufacturing, it is expected that its inherent microbiota and the starter culture together avoid pathogen survival by means of lowering pH and proper competition (Baylis 2009). Lactic acid bacteria are known for producing organic acids such as lactic, acetic and propionic acids that will decrease the matrix pH, which helps to preserve fermented food, including cheese and cheese products (Özcelik, Kuley, and Özogul 2016). As an example, during the fermentation process, lactic acid bacteria hydrolyze lactose into glucose and galactose by the action of β-galactosidase (found in most species) or phospho-β-galactosidase (lactococci), producing lactate from glucose and exporting it, leading to a pH drop in cheese matrix (Poolman 1993). While this is true, although *E. coli* O157 strains present an ideal growth around pH 7, studies show that some O157 strains are able to survive to very acidic conditions, such as pH 4.0 and 3.0 for long periods (Dineen et al. 1998; Meira et al. 2017). This suggests that the acidification promoted by lactic acid bacteria may not be enough to avoid STEC persistence. Moreover, lactic acid bacteria also produce antimicrobial proteins called bacteriocins, such as nisin, pediocin and lacticin, which act as biopreservatives (Favaro et al. 2015; Portilla-Vázquez et al. 2016). As evidence, the addition of an association of *Lactobacillus plantarum*, *Lactococcus lactis* and *Hafnia alvei* reduced the presence of STEC O26:H11 and O157:H7 in ripened cheese, without changing its sensory characteristics (Callon, Arliguie, and Montel 2016).

The fate of STEC during both ripening and storage has been described based on hurdles STEC can challenge in these manufacturing steps. In fact, STEC counts seems to reduce during cheese ripening in a variable way. Upon inoculation of *E. coli* O157 and non-O157 in white mold cheese, authors observed an increasing of STEC counts by 2 to 3 log CFU g^{-1} in the first 24–36 h. During the ripening step of 14 days, the cheese core presented a pH between 5.7 and 6.1 and a_w was 0.98. Under this scenario, STEC O26:H11 kept a constant concentration, while a slight decrease, of less than 1 log CFU g^{-1}, were observed in STEC O103:H2 and O145:H28. Whereas, *E. coli* O157 presented a decrease of 1.3 log CFU^{-1}. The findings were similar regarding cheese rind, despite presenting a pH of 6.1 and a_w between 0.97 and 0.98. On the other hand, in storage of 56 days, the concentration of all STEC decreased slowly in cheese core, but *E. coli* O157 concentrations reduced even more. In general, the rind of the cheese presented more concentration of STEC compared to the core, probably due to the higher pH in the rind. This result suggests that *E. coli* O157 is less prone to survive to ripening and storage of white cheese mold than non-O157 strains (Miszczycha et al. 2016).

Moreover, the use of antimicrobial active packages, a packaging that provides both physical and antimicrobial barrier effects to food, may be used to reduce STEC counts in cheese (Costa et al. 2018). Otero et al. (2014) investigated the action of two antimicrobial packaging films coated with oregano essential oil (*Origanum vulgare*) and ethyl lauroyl arginate HCI (LAE) against *E. coli* O157 in raw milk sheep cheese. Upon 7 days of cold storage, the use of a polyethylene terephthalate film coated with a concentration of $\geq$6% LAE significantly decreased the STEC counts, without affecting cheese sensory characteristics, suggesting that this cited film may be useful for reducing STEC cells in sheep cheese. In another study, Pérez et al. (2011) added potassium sorbate (PS) into whey protein concentrate edible film and tested against eight non-O157 strains, five of them isolated from soft and cottage cheese. After testing three different concentrations of PS (0.5%, 1%, and 1.5%) under pH 5.0 and 6.0, they observed that increasing the concentration of PS increased bacterial inhibition. Also, inhibition was higher under pH 5.0 than 6.0, which could cause food rejection due to a denser appearance of the film under pH 5.0. Still, the film may be beneficial to avoid STEC post-processing contamination.

Furthermore, in order to infect an organism, STEC has to be able to survive stress conditions in the gastrointestinal tract, where STEC will be in contact with extreme low pH, acids formed by digestive processes or commensal bacterial metabolism, gastrointestinal secretions, adapted microbiota, peristalsis, among other conditions (De Biase and Lund 2015; Miszczycha et al. 2014). In fact, Miszczycha et al. (2014) simulated human digestion of raw milk cheese experimentally contaminated with *E. coli* O157 and O26:H11, using a gastro-intestinal tract system model, to evaluate the survival of these bacteria during such stress conditions. In an environment that simulates the stomach, they observed that the survival of *E. coli* O157 was significantly affected after 60 min. In contrast, O26:H11 concentration was significantly lowered only after 90 min, showing that the survival rate of O26:H11 was significantly higher

than *E. coli* O157. Interestingly, in the duodenum environment, *E. coli* O157 presented a significant decrease after 120 min, while O26:H11 was not affected in the entire process, except for 60 min when the percentage of viable cells significantly increased for an unexpected reason. In the jejunum compartment, *E. coli* O157 showed no significant changes, whereas O26:H11 was able to grow in this environment. Finally, in the ileum environment, no significant changes were observed in *E. coli* O157, but O26:H11 showed much higher recovery percentages. These data suggest that ecological differences between O157 and non-O157 strains may show a superior behavior of non-O157 strains over O157 strains, although more studies still need to be done in order to make this statement an affirmation. In fact, it has been observed an increasing number of human infections caused by non-O157 strains around the world (Castro et al. 2017).

Further, the use of strains isolated from food matrices, with specific phenotypic backgrounds should be incorporated into research. To corroborate, Castro, Rosario, et al. (2019) described a higher survival capacity of a wild-type O26 isolate when exposed to a simulated gastric fluid, compared to a clinical isolate (ATCC) of the same serogroup. This highlights the importance of using wild-type strains in experimental studies in order to achieve more reliable results.

General STEC stress responses and tolerance in cheese matrix

Survival of Bacteria, whether commensal or pathogenic, in any matrix, depends on its intrinsic and extrinsic factors. In regards to cheese, pH, acidity, a_w, processing steps and storage temperature are some of the most critical parameters to related to bacterial persistence (D'Amico and Donnelly 2017). After the contact with the food matrix, the bacterial cell is exposed to several challenges and, in order to survive, it needs to surpass different types of barriers, demanding from the bacteria several mechanisms to overcome these hurdles. The physiological responses of STEC to stress condition are promoted by initiation factors that start a specific or a cascade of reactions that increase the resistance of the cell (Hengge-Aronis 2002). Moreover, the general stress adaptive response is regulated by several genes responsible for maintaining the cell homeostasis (Elhadidy and Álvarez-Ordóñez 2016; Hengge-Aronis 2002).

Furthermore, different types of cheese have different manufacturing steps, which are intimately related to different responses to stress. For instance, hard cheese usually has low moisture content and low pH level, which contributes to an increase in osmotic pressure, osmotic and acid responses, compared to soft cheese. Thus, hard cheese is less likely to host pathogens than soft cheese (Leistner 2000). Pasteurized milk cheese is submitted to higher temperatures compared to raw milk cheese. Here, thermal technology makes it harder or even impossible for non-adapted bacteria to surpass such conditions (Baylis 2009).

The majority of the adaptative response in STEC cells is promoted by sigma factor σ^S (RpoS or σ^{38}), the general stress regulator (Stella et al. 2017). Sigma factors are subunits of RNA polymerase (RNAP) holoenzyme that acts as initiation factors, promoting an attachment of RNA portions to specific initiation subunits (Hengge-Aronis 2002; McKenna et al. 2019). This subunit is responsible for the expression of several genes, regarding metabolism, transport and also general stress response (Hengge-Aronis 2002). In non-stress conditions, *rpoS* mRNA is verified at high levels and has little variation when compared to a stress response. Thus, during a stress condition, *rpoS* mRNA starts to rearrange itself from an inactive form to an active one, which causes an abrupt increase of translations (Peng et al. 2011). Furthermore, there are multiple signals of *rpoS* expression activation. For instance, the nucleotides guanosine 5′-diphospate 3′-diphosphate, known as ppGpp, are credited to positively affect *rpoS* expression in response to amino acid, carbon, nitrogen, phosphate or energy source limitation (Gentry et al. 1993). Two proteins, RelA and SpoT, are known to synthesize and hydrolyze ppGpp in *E. coli*. While the synthesis of ppGpp regulated by RelA is activated upon amino acid starvation, SpoT is activated upon several different stresses (non amino acid starvation related) by ppGpp stabilization after inhibiting the hydrolase activity (Fujita et al. 2002; Stella et al. 2017). Another mechanism closely related to the general stress response is the Pho (phosphate) regulon. *E. coli* can sense the extracellular phosphate concentration by the two-component regulatory system, PhoBR: while PhoR encodes a histidine kinase/phosphatase sensor protein, the PhoB encodes a response regulator aiming to activate or repress gene expression (Crépin et al. 2011; Ruiz and Silhavy 2003). After phosphate starvation, the PhoBR activates the transcription of the Pho regulon that encodes, among other systems, the high-affinity phosphate-specific transporter (Pst) system. The Pst transport system is then responsible for the transport of phosphorus into the cell at the expenditure of ATP hydrolysis (Hsieh and Wanner 2010; Jha, Dafale, and Purohit 2019).

As cheese matrix is induced to many different antimicrobial stress conditions during manufacturing, a specific or a conjugated pathogen response can be triggered. A combined stress may generate a cross-protective response, which triggers different protective effects. For example, the sigma factor σ^S, that could be induced in response to acid stress, can also have effects on heat, cold, high-pressure, UV-C radiation, H_2O_2, and salt presence (Cheville et al. 1996; Li et al. 2018; Mei et al. 2015; Mutz, Rosario, Paschoalin, et al. 2019; Mutz et al. 2020; Robey et al. 2001). The cross-protection phenomenon refers to the acquired adaptation of a bacteria to different subsequent stress after the previous contact with stress found in the environment (Mutz, Rosario, Castro, et al. 2019; Riordan et al. 2000). For instance, acid adapted *E. coli* O157 exposed to pH 5.5 in HCl for 4 to 5 hours increased its resistance to salt presence (10% NaCl) and high temperature of 55 °C (Cheng, Yang, and Chou 2002). Mechanisms are not very clear yet. Moreover, Shadbolt, Ross, and McMeekin (2001) showed that when *E. coli* is

exposed to a very aggressive stress and then it is submitted to a second lethal stress, the energy expenditure to maintain the homeostasis is so high that the cells are inactivated rapidly. However, when both stresses are applied at the same time, the bacteria are easily able to survive. In cheese produced from raw milk, for example, STEC may contaminate milk in any step of manufacturing. If this contamination occurs directly in milk, STEC could face cold stress regarding milk cooling and this may induce general stress response in order to resist to subsequent stress. In this case, the surviving cells could overcome next stress challenges, persisting in the final product, therefore becoming a risk to human health.

Thermal stress in cheese

As stated before, STEC can face temperature up- and downshifts during the cheesemaking process. Although considered a mesophilic bacterium, it can respond to thermal stresses with physiological and structural changes that allows the survive of this pathogen in cheese matrix, becoming a possible risk to human consumption.

Cold stress response

In the dairy industry, the use of low temperatures is applied to regulate the growth/survival of microorganisms in raw milk and some derivatives, which helps to extend quality and shelf life of products (Shashi et al. 2018). As stated before, milk is cooled after milking to prevent bacterial growth. Also, as dairy products are considered as perishable foods, they must be kept under refrigeration during processing steps and storage, with optimal storage temperature ranging from 0 °C to 2 °C (Mercier et al. 2017). Bacterial growth in these low temperature environments demand some physiological mechanisms, such as alterations in cell membrane by production of unsaturated fatty acids, transcriptional and translational changes, and production of cold shock proteins (Kocharunchitt et al. 2012).

When a temperature downshift occurs, the cell membrane, which usually has a liquid form, tends to become more rigid, in a gel form (Yamanaka 1999). In this case, STEC cell needs to transiently change its membrane composition from a nonfluid- to a fluid state (homeoviscous adaptation), altering its fatty acid composition by increasing cis-vaccenic acid (unsaturated) values while decreasing the quantity of palmitic acid (saturated) incorporated into membrane phospholipid (Vidovic, Mangalappalli-Illathu, and Korber 2011; Yamanaka 1999). Thus, β-ketoacyl-ACP synthase II, an enzyme encoded by *fabF*, elongates the palmitoleic acid to cis-vaccenic acid, which results in a greater degree of membrane flexibility due to the increase of unsaturated fatty acid in membrane composition (Garwin and Cronan 1980; Yamanaka 1999). Despite β-ketoacyl-ACP synthase II induction and action at low temperatures, the activity of this enzyme is not related to cold shock response itself. Still, it is intimately related to cell viability under low temperatures, since *E. coli* mutants lacking this enzyme are unable to change their membrane lipid composition, presenting no thermal protection (Garwin and Cronan 1980; Yamanaka 1999).

The cold shock response also plays a role in incorporating unsaturated fatty acids to the lipid membrane. In fact, Li et al. (2018) demonstrated that the palmitoleoyltransferase (LpxP), recognized as a specific acyltransferase responsible for attaching palmitoleate to lipid A under a temperature downshift, is up-regulated when STEC cells are submitted to cold environments. As aforementioned, the incorporation of palmitoleate on cell membrane provides adequate fluidity to maintain cell viability. In fact, there is still little information about *E. coli* induction of specific acyltransferases upon cold shock, which means that the bacteria also utilize unspecific acyl donors available in the acyl-ACP and acyl-coenzyme A pools (Vorachek-Warren et al. 2002).

Following the general stress regulator, the major modulator of cold stress in STEC is the sigma factor RpoS (Li et al. 2018; Vidovic et al. 2011). In order to achieve the desired fluidity in STEC cell membrane under cold adaptation process, the sigma factor RpoS regulates two genes that work conjointly: *fabD* and *cfa* (Vidovic, Mangalappalli-Illathu, and Korber 2011). While the *faD* gene is responsible for encoding malonyl CoA-acyl transacylase carrier protein, which participates in fatty acid synthesis and polyketide synthase, the *cfa* gene encodes cyclopropane-fatty-acyl-phospholipid, which acts by transferring a methylene group to modify the membrane lipid bilayers (Vidovic and Korber 2016). Thus, *fadD* and *cfa* genes are responsible for maintaining STEC membrane fluidity constant at low temperatures under cold shock response.

Another mechanism related to membrane fluidity is associated with sensing the membrane lipid composition. The two-component sensor protein CpxA is believed to create a constant signal for the CpxA membrane sensor each time an alteration in cell envelope structure occurs (Mileykovskaya and Dowhan 1997). Indeed, *cpxa* gene was demonstrated to be upregulated upon cold stress in STEC (Li et al. 2018). Regarding membrane protection, colanic acid is credited to protect *E. coli* surfaces against several environmental stresses, such as cold, osmotic and acid stress, although the role of colanic acid in stress physiology of STEC strains remains unclear (Vidovic and Korber 2016). However, it was already demonstrated a huge up-regulation of Rcs phosphorelay system (responsible for the biosynthesis of colanic acid), upon cold stress of STEC strains (Kocharunchitt et al. 2012).

Regarding temperature variation, two types of cold stress response are studied: the cold shock process, where cells are transferred from an optimum temperature environment to a low/moderately low temperature environment directly; and the cold adaptation process, where cells are "pre-adapted" in a stable low temperature during some time before challenging lower temperatures (Li et al. 2018). Indeed, both situations can be applied to cheesemaking process, since low temperatures can be used during the main processing steps and storage, as aforementioned.

Exposure of STEC cells to low temperatures results in transient induction of effector proteins regulated by Rpos, the so-called cold shock proteins are essential for thermal adaptation (Li et al. 2018). The cold shock proteins are normally divided in two groups, based on their expression patterns: the class I proteins (CspA, CspB, CspG, CspI, CsdA, RbfA, NusA, and PNP), are expressed at a very low rate at 37 °C, but incredibly induced at a low temperature environment; and class II proteins (IF-2, H-NS, RecA, α subunit of DNA gyrase, Hsc66, HscB, trigger factor, dihydrolipoamide acetyltransferase, and pyruvate dehydrogenase), normally expressed at 37 °C, but discreetly induced after temperature downshift (Phadtare, Alsina, and Inouye 1999; Yamanaka 1999). In fact, CspA is considered the major cold shock protein in *E. coli* (Mei et al. 2015).

When *E. coli* cells are submitted to a moderately low temperature of 15 °C, the synthesis of cold shock proteins rapidly increase in the first hours (induction stage), before reducing to basal levels (repression stage) (Yamanaka and Inouye 2001). This period between induction and repression stages is called acclimation phase, and it can be summarized as the period where bacterial growth is delayed due to the occurrence of important cellular events necessary to thermal adaptation (Yamanaka and Inouye 2001).

During the acclimation phase, occurs a block in most of the cellular protein synthesis, related to the initiation of translation step. However, cold shock proteins are capable of avoid this synthesis blocking, which grant its cellular levels increasing during cold temperatures (Yamanaka 1999). This capability of avoiding synthesis blocking is due to the fact that some class I group cold shock proteins possesses a downstream box sequence in its mRNA, that improves translational capacity, and serves as an independent translational signal (Mitta, Fang, and Inouye 1997). To explain this situation, Jones and Inouye (1996) proposed a cold shock adaptation model that states that upon cold shock, there is a transient blocking of translation initiation of non-cold shock proteins, resulting in decreased polysomes and increased 30S, 50S, and 70S ribosomes, all incapable of translation. However, the cold shock proteins (responsible for translation) are able to convert these cold-sensitive/non-translatable ribosomes, to cold adapted/translatable ribosomes. This conversion results in cell retrieval of protein synthesis and, consequently, STEC cold-adaptation (Yamanaka 1999).

However, after cold adaptation is achieved, the bacterial cell needs to degrade the major cold shock proteins as they are not needed to any further extent, as cell homeostasis is finally accomplished (Li et al. 2018). Indeed, Neuhaus et al. (2000) demonstrated that high levels of cellular cold shock proteins may prevent translation of bulk mRNAs, consequently supressing the resumption of cell growth. Thus, an increase in synthesis of polynucleotide phosphorylase (Pnp) is observed (Vidovic, Mangalappalli-Illathu, and Korber 2011). According to the authors, Pnp is the most significantly up-regulated enzyme in cold-adapted STEC strains. In fact, Pnp is responsible for the degradation of CspA, CspB and CspC mRNA molecules at the end of acclimatization phase, which finally enables the growth resumption in cold-adapted cells (Vidovic and Korber 2016).

The accumulation of compatible solutes is also part of the cold shock response. Per definition, compatible solutes are organic osmolytes synthesized by microorganisms, used when cells are under stressful situations (Schulz et al. 2017). In a study conducted by Li et al. (2018) was demonstrated an increase of betaine transporter OpuA and OpuC in STEC strains submitted to cold stress. These transporters are related to betaine intake and synthesis, and are believed to be cryoprotectants (Hoffmann et al. 2018). In addition to betaine genes, authors observed an up-regulation of genes *otsA* and otsB, responsible for trehalose synthesis, another compatible solute believed to participate in resisting to cold shock. The role of compatible solutes in STEC envelope components is further described in the next topics.

Heat stress response

Although *E. coli* is known as a heat sensitive bacterium, some strains are considered to be among the most heat resistant foodborne pathogens (Li and Gänzle 2016). In cheese manufacturing, STEC can be exposed to different sources of heat, for different reasons. First, the pasteurization process applied to raw milk in order to destroy pathogenic bacteria. Second, the different technological processes of cheese manufacturing that heat can be applied, e.g., ricotta and processed cheese are both manufactured at approximately 90 °C (De Giorgi et al. 2018; Johnson 2017; Talbot-Walsh, Kannar, and Selomulya 2018). For instance, Fusco, Riccardi, and Quero (2012) described that in pasta filata cheese (a variety where the curd is stretched and mold at high temperatures and then cooled), *E. coli* O157:H7 can also challenge a temperature as high as 90 °C and still persist in the final product. This occurs due to the temperature in the core of the curd that can stay around 55 °C. In fact, STEC has been reported in pasteurized dairy products, which may be due to the fact that STEC encode the expression of genetic adaptations and may survive to thermal stress, mainly to inefficient pasteurization or sub-pasteurization processes (D'Aoust et al. 1988; Farrokh et al. 2013; Peng et al. 2011; Schlesser et al. 2006). It is essential to mention that, once produced by STEC strains, Stx2 does not reduce its biological activity upon contact with pasteurization temperatures, which can only be achieved after heat treatment at 100 for 5 °C min (Rasooly and Do 2010).

The main response of *E. coli* upon heat stress can be separated in induction period and adaptation period. Briefly, the induction period occurs just after the heat upshift, increasing heat shock proteins (HSPs) synthesis, reaching the highest after 5–10 minutes (Bukau 1993). At the same time, two chaperones, Hsp70 (DnaK) and Hsp60 (GroEL), increase their synthesis at the rate of eight and 13-fold, respectively (Bukau 1993). Further, the adaptation period occurs when the synthesis of HSP starts to decrease until it stabilizes. Consequently, an increase of cellular HSP levels is observed (Bukau 1993). The HSPs are able to mediate refolding, transporting, repairing and degradation of

proteins that were denatured by heat stress, thus guaranteeing the cell survival and persistence (Raivio and Silhavy 2001).

In *E. coli*, the sigma factor σ^{32} is responsible for regulating the cytoplasmic general heat shock response (Yura 2019). The first mechanism regarding the heat shock response is *rpoH* transcription. Four promoters are known to be responsible for *rpoH* transcription: P1, P4, and P5 recognized by σ^{70}, and P3 recognized by σ^{E}. The most important promoters in heat shock response are P1 and P4, while the activity of P5 acts under specific conditions, requiring an additional cAMP plus its receptor to function (Yura 1996). The transcription of the cited promoters is influenced by the amount of heat stress applied to *E. coli*. For instance, when the cell is in a stable temperature of 30 °C, P1 is the major promoter of expression, together with P4. However, when the temperature increases to 42 °C, a little elevation in P1 and P4 transcription is observed, although P3 intensively increases its expression. Finally, when the cell is in contact with a temperature of 50 °C, expression of P1 and P4 starts to decrease until inactivation whereas P3 transcription continues to increase. (Bukau 1993; Gross et al. 1990). The control of *rpoH* at basal expression levels are mainly due to σ^{70} and σ^{E}, while an increased σ^{32} synthesis relies on translational control (Yura 1996).

The following mechanism of heat shock response is *rpoH* translation. The importance of σ^{32} translational control can be explained because upon heat shock, an increase of σ^{32} synthesis is observed, but not of *rpoH*. Besides that, *rpoH* mRNA levels increase its synthesis after the increase of σ^{32} synthesis (Nagai, Yuzawa, and Yura 1991; Yura, Nagai, and Mori 1993). When the bacterial cell is at a normal temperature, *rpoH* translation is repressed. However, upon temperature upshift, there is a transient depression of translation, reaching a 12-fold rate. The control of *rpoH* translation is mediated by mechanisms that include three cis-acting *rpoH* mRNA regions, namely A, B and C regions (Arsène, Tomoyasu, and Bukau 2000; Nagai et al. 1991). The region A is known as a positive regulatory region on the 5′ portion of *rpoH* mRNA. The also called "downstream box" is located close to the initiation codon and has 15 nucleotides. The region is a complementary part of 16S RNA, possessing a translation enhancing function (Arsène, Tomoyasu, and Bukau 2000; Morita et al. 1999). The region B, a negative regulatory region, is located within nucleotides 100 and 247, and seems to be involved in repressing translation under non-stress conditions (Arsène, Tomoyasu, and Bukau 2000; Morita et al. 1999). Base pairing between region A and part of region B seems to negatively modulate *rpoH* translation at steady-state conditions, by preventing translation initiation due to the formation of an internal loop that makes the Shine-Dalgarno (SD) sequence and initiation codon inaccessible (Arsène et al. 2000; Morita et al. 1999; Peng et al. 2011). The induction of *rpoH* translation comes from the increasing of temperature that partially melts the mRNA structure, increasing the entry of ribosomes, so that translation can occur (Morita et al. 1999). Moreover, the region C is also a negative regulatory region, that is located within nucleotides 364–433 of *rpoH*. It represses *rpoH* translation during the shut-off phase of the heat shock response. Also, region C seems to act in a protein level by mediating translational repression of *rpoH* (Arsène et al. 2000; Nagai et al. 1994).

When stability to heat is achieved, there is a decreasing of σ^{32} level as well as a repression of its activity. This include the modulation of HSPs, DnaK, DnaJ, and GrpE, and also degradation of σ^{32} by the AAA-protease, FstH. FstH is a cytoplasmatic membrane protein that is believed to be the responsible for σ^{32} degradation, upon a destabilization caused by DnaK and DnaJ (Tatsuta et al. 1998; Arsène, Tomoyasu and Bukau 2000; Rodriguez et al. 2008). DnaK chaperone system facilitates the inactivation of heat shock stress response, and seems to indirectly participate of σ^{32} degradation and repression of *rpoH* translation during the ending phase of the heat shock response (Arsène et al. 2000; Tatsuta et al. 1998). When there is high availability of DnaK substrates, they compete with σ^{32}, leading to a stabilization of σ^{32}. Further, the concentration of Dnark substrates start to decrease, whereas the degradation of σ^{32} increases, causing the ending of heat shock response (Peng et al. 2011). This occur due the fact that DnaJ acts by binding to a specific location of σ^{32}, destabilizing a region of σ^{32} that sits close the DnaK binding site. After that, DnaK destabilizes a region in N terminus, which seems to be the main target for FtsH, leading to degradation of σ^{32} (Rodriguez et al. 2008).

Regarding chaperone actions, two chaperone systems play a major role in *E. coli* heat shock response: DnaKJ/GprE and GroEL/ES systems. DnaK chaperones are known for refolding proteins and preventing aggregation (Georgopoulos and Welch 1993). These actions are only possible because DnaK are associated with short hydrophobic segments of polypeptides substrates, avoiding the aggregation or folding of these substrates (Bukau and Horwich 1998). DnaK system activity presents two conformations to DnaK that are related to ATP and ADP, respectively. In fact, DnaK presents a N-terminal ATPase domain related to the nucleotide and another related to the substrate. When ATP binds to the nucleotide biding domain, it provokes a conformational change in the substrate biding domain, resulting in an open substrate cavity with high association (k_{on}) and dissociation (k_{off}) rate, leading to a faster exchange rate. However, if ADP attaches to the nucleotide biding domain, the substrate biding cavity closes, resulting in low k_{on} and k_{off}. Further, the DnaJ chaperone induces ATP hydrolyzes, leading to a shifting to the closed conformation, confining the substrate. GrpE replaces ADP with ATP, which will turn DnaK back to the open conformation, thus releasing the substrate (Mayer et al. 2000; Peng et al. 2011).

Moreover, if a protein has more than one hydrophobic segment, DnaJ can bind to it forming a complex made of Dnak-substrate-DnaJ. Also, upon high temperature, GrpE suffers reversible conformational changes, thus decreasing its nucleotide exchange activity. Both of these mechanisms can lead to the closed conformation of DnaK under heat stress, which will result in many bound substrates (Grimshaw et al. 2001; Peng et al. 2011).

Equally important, the GroEL/GroES chaperonin system is important for basal temperature conditions as well as for heat shock response. The GroEL structure is a double-ring structure, that forms a cylindrical-like structure with a central cavity for folding proteins (Braig et al. 1994). Also, the GroES structure is a heptamer that resembles a dome, which is able to bind to GroEL central cavity, closing it (Hunt et al. 1996). In the GroEL/GroES reaction cycle, only one GroES ring is able to bind GroEL, thus forming the folding chamber. Seven ATP molecules are needed to provoke a conformational change, allowing GroES to bind to GroEL ring. This will cause another conformational change that will expand the folding chamber, shifting the cavity surface. Further, the cavity becomes hydrophilic, which leads to the liberation of the substrate to the *cis* chamber. Now that the cavity is hydrophilic, the non-native substrates start the folding process until the association of GroEL/GroES is broken, caused by ATP hydrolysis on the *cis* side. The detachment of GroES, the substrate, and ADP from the *cis* GroEL is activated by the biding of ATP, plus the new substrate on the *trans* side. The other side becomes the new *cis* side, so the two GroEL rings shift between binding and folding, proceeding the continuation of the reaction cycle (Peng et al. 2011; Rye et al. 1997).

In a similar fashion, the sigma factor σ^E also plays an important role in heat stress response because it is responsible for maintaining the cell envelope integrity. Sigma factor σ^E is responsible for every reaction regarding cell envelope. The activation of σ^E in heat shock response is mediated upon protein unfolding in both cytoplasm and cell envelope, and also upon an accumulation of unfolded proteins in cell envelope. This last activation is modulated by specific inducers, like overexpression of porins (Ades et al. 1999; Mecsas et al. 1993).

Upon activation, σ^E transcribes genes intimately related to the cell envelope that acts by encoding chaperones and proteases that will remove or refold damaged or misfolded proteins. Because σ^E sites in the cytoplasm, it needs a mediator to sense the damage in the envelope. Signal of stress response is then transmitted from the envelope to σ^E through regulated proteolysis of the inner membrane anti sigma factor RseA. Prior to initiation phase, σ^E is inhibited by RseA because RseA blocks σ^E association with RNA polymerase (RNAP). According to Campbell et al. (2003), this association is about 300-fold greater than σ^E affinity to RNAP. Because RNA cannot compete with the σ^E:RseA complex, the complex needs to be dissociated in order to σ^E interact with RNAP, activating the genes responsible for promoting the heat shock response. Thus, degradation of RseA is the main mechanism for this complex disruption (Ades, Grigorova, and Gross 2003). The proteolysis of RseA is associated with two proteases: DegS and YaeL. The process starts when DegS cleaves RseA in the periplasmic domain. Next, when the periplasmic domain is already removed, YaeL is able to degrade RseA (Alba et al. 2001; Kanehara, Ito, and Akiyama 2002). This entire process is induced by a signal from the disruption of outer membrane porins, a significant inducer of σ^E (Walsh et al. 2003). The cited reactions are autoregulatory because they are mediated by σ^E itself, which allows the cell to transcribe activation and inhibition in a same way. After RneA degradation, σ^E is free to bind to RNAP and start the transcription of genes of heat shock response, thus guaranteeing *E. coli* survival and persistence in food (Ades 2004; Ades, Grigorova, and Gross 2003). The role of σ^E in stress response is not fully explored yet.

Recent bioinformatic studies correlate the presence of the genomic island called *locus of heat resistance* (LHR) to heat resistance in *E. coli* and other pathogens (Bojer et al. 2010; Gajdosova et al. 2011; Mercer et al. 2017). For instance, *E. coli* AW1.7, a nonpathogenic food isolate is capable of resisting to an elevated temperature of 71 °C (Dlusskaya, McMullen, and Gänzle 2011). If pathogenic strains, such as any STEC strain could acquire this attribute, they may become a threat to food safety and public health. The heat resistance of this isolate is not related to σ^{32} as previous stated, but to LHR. This region is responsible for regulating heat chock response, whereas the activation of LHR gives *E. coli* AW1.7 an extreme heat resistance behavior. LHR is composed of 16 open reading frames, where moderately resistant and sensitive strains possess 10 out of 16 orthologs. A full length LHR provides a highly heat resistance behavior and a shorten LHR provides a moderate heat resistance. Thus, the heat resistance depends directly of the whole genomic island (Mercer et al. 2017). Yet, Liu et al. (2015) screened 100 STEC strains, but did not find any highly heat resistant pathogen. While this is true, it is believed that 2% of all *E. coli* genome contains the LHR, making lateral gene transfer a possible danger do human health (Mercer et al. 2017).

Osmotic stress in cheese

The sodium chloride (NaCl) addition in cheese is intimately related to food preservation. Besides its role in nutritional and flavor aspects, NaCl is important to control microbial growth because of its direct interaction with osmoregulation and also its direct effect in decreasing the moisture content of the final product, thus reducing water activity (Fox et al. 2017f). In cheese production, NaCl can be added to cheese through direct addition or utilizing a brine (Velázquez-Varela et al. 2018). This increase of salt content will create an osmotic upshift that will require STEC to adapt to the new cited conditions. Upon direct addition of NaCl in the curd (less commonly in milk), STEC is exposed to salt as it diffuses in the cheese matrix. However, the utilization of brine exposes only the surface microbiota in a first place, and then diffuse to the core (Peng et al. 2011). It is important to mention that *E. coli* O157 has been described as able to resist for several weeks in cheese brines (Ingham, Su, and Spangenberg 2000), thus representing a potential source of infection to cheese consumers.

E. coli protective surface structures

Exopolysaccharides are extracellular polysaccharides produced by bacteria, with importance related to pathogenicity and biofilm formation (Schmid, Sieber, and Rehm 2015). In

fact, such polymers seem to play a role in cell desiccation. For instance, the aforementioned colanic acid (CA), a viscous capsular polysaccharide produced by several *E. coli* variants, is credited to protect bacterial cell from desiccation. Ophir and Gutnick (1994) found out that *E. coli* strains that were able to produce CA, showed higher resistance against water loss, protecting the colonies against desiccation in a better way than those colonies that were not able to produce CA. In addition, Chen, Lee, and Mao (2004) observed that CA producer *E. coli* O157 had a significant higher survival rate in the presence of 1.5 M and 2.5 M of NaCl compared to O157-strains that were unable to produce the saccharide, showing that CA protects the cell from osmotic stress. Another exopolysaccharide involved in protection is cellulose. A study presented that the population of STEC producing cellulose were significantly higher than their non-producing counterparts at 24- to 48-h intervals for 1 M NaCl treatment and at 9- to 48-h intervals for 2 M NaCl, evidencing the role of cellulose in cell protection against osmotic stress (Yoo and Chen 2010).

Moreover, in Mattick, Rowbury, and Humphrey (2003) study, commensal *E. coli* and *E. coli* O157 strains were able to form protein elongated filaments upon exposure to a_w of 0.96, which may be involved in osmotic stress response somehow. In fact, both commensal and pathogenic *E. coli* strains also demonstrated to form such filaments under different stress conditions, such as low and high temperature and low and high pH. The authors also stated that plate counting techniques for the tested strains exhibited wrong results, implicating that, since the infectious dose of STEC strains are very low, these very used techniques may underestimate the precise number of pathogens in a food outbreak.

Osmotic stress response following cheese salted with sodium chloride or brine

Upon exposure to osmotic stress, bacterial cell responds in two different ways: as an immediate response of seconds to minutes, on protein activity level by increasing or decreasing enzymes and transport systems; and as an extended response of minutes to hours, on gene transcription level, acting by means of *de novo* synthesis of enzymes, transport systems and wall components (Wood 1999). Because the long-term response usually occurs as a result of a prolonged exposure to high external osmolarity, this response can recover the steady-state of the cell by restoring the intracellular concentration and solute composition, the energy for metabolism and also helps with growth and cell division (Krämer 2010; Wood 1999).

The immediate *E. coli* response to an osmotic upshift is the uptaking of potassium ions via K^+ uptake systems while synthesizing glutamate (which acts as a counter ion for K^+, avoiding depolarization) (Burgess et al. 2016; Dinnbier et al. 1988). *E. coli* possesses two potassium uptake systems: Kdp and Trk. The Kdp-ATPase system has a high affinity to K^+, being composed of four subunits: KdpA (K^+ binding and translocating), KdpB (energy supply), KdpC (complex gathering) and KdpF (stability). They are expressed under low potassium concentration and osmotic stress by the regulation of the KdpD-KdpE protein system (Altendorf, Voelkner, and Puppe 1994). In contrast, the Trk system has a low affinity to K^+, but possesses a higher rate of transport. The system is composed of a translocating subunit, TrkH and TrkG (this last not found in all *E. coli* species), and a cytoplasmic regulatory subunit, TrkA and TrKE (SapD), that codes an ATP-binding cassette. The Trk system is presumed to symport $1\,H^+$ and $1\,K^+$ to the cytoplasm, somehow involving ATP (Corratgé-Faillie et al. 2010).

Regarding long-term adaptation to osmotic stress, the cell replaces K^+ for compatible solutes because the high concentration of potassium affects metabolic functions overtime (Dinnbier et al. 1988). These selected solutes are mainly trehalose, proline and glycine-betaine that also act as protein stabilizers (Peng et al. 2011). Three different cytoplasmic transport systems are responsible for this process in *E. coli*, ProP, ProU and BetT (Burgess et al. 2016; Krämer 2010). First, ProP is an osmoregulatory compatible solute-H^+ symporter activated upon an increase of extracellular osmolarity. Its activation is mediated by two promoters, P1 and P2, that are controlled by σ^S (Mellies, Wise, and Villarejo 1995). Second, ProU is a biding protein dependent system that, like ProP, possesses a high affinity to import solutes such as glycine-betaine, but a low, although functional, affinity to import proline, ectoine and others organic compounds (Lucht and Bremer 1994).

Similar to cold stress response, *E. coli* is also able to synthetize or convert compatible solutes in order to maintain homeostasis in an osmotic stress situation. For instance, the *bet* genes have high affinity to import choline upon osmotic stress, which is realized by the osmosensing BetT transporter. This way, the cell has substrate to convert choline to glycine-betaine, a potent osmoprotectant. This pathway is induced upon osmotic stress or addition of choline to the medium, happening under aerobic conditions (Eshoo 1988). Similarly, *E. coli* is also able to synthetize and accumulate trehalose from glucose in an attempt to maintain cellular osmolarity. The pathway is dependent of two genes, *otsA* and *otsB* being induced by osmotic stress and by σ^S, when cells ate entering in stationary phase (Hengge-Aronis et al. 1991). This accumulation of trehalose is vital for the growth and survival of cells, mainly in a moderate osmolarity environment (Giaever et al. 1988).

Moreover, *E. coli* can utilize other membrane proteins to deal with osmotic stress. As evidence, OmpC and OmpF are porins that are able to form channels in the external membrane, facilitating the passive diffusion of small hydrophilic molecules, which will help the cell to recover osmolyte level. Their expression is mediated by the signaling system EnvZ-OmpR (Cai and Inouye 2002). OmpC and OmpF differ regarding structure and function: while OmpC possesses a smaller pore and slower flux, OmpF has a larger pore and consequently a faster flux. While in low osmolarity levels OmpF is the major porin, in a high osmolarity environment the high concentration of OmpR-P causes an up-regulation of *ompC*, repressing *ompF* transcription by biding and avoiding transcription from an upstream promoter of *ompF* (Peng et al. 2011), which will turn OmpC the predominant porin (Feng, Oropeza, and Kenney 2003).

Acid stress and pH homeostasis response in cheese

Acid resistant bacteria was first designated as those that were able to resist to pH 2.5 for 2 h with a survival of at least 10% of the challenged inoculum (Gorden and Small 1993). For instance, several studies had attested the acid resistance behavior of *E. coli* and STEC (Arnold and Kaspar 1995; Gorden and Small 1993; Leyer, Wang, and Johnson 1995). The acid resistance (AR) enables *E. coli* to survive harsh conditions like the presence of gastric acid. In fact, the low infectious dose related to STEC O157:H7 strains comes from its acid resistant behavior (Castanie-Cornet et al. 1999).

In cheese production, STEC can be exposed to acid stress depending on the employed technology. For example, in semihard Gouda cheese, the final product pH should be between 4.9 and 5.6. This happens mainly due to the fermentation processes of lactic acid bacteria used as starter culture (van den Berg et al. 2004). Also, in soft ripened cheese the pH can be as low as 4.6, as a result of fermentation by lactic acid bacteria (Batty, Waite-Cusic, and Meunier-Goddik 2019; Shaw 1981). In addition, *E. coli* O157:H7 has been described to persist for 12 days in yogurt (pH 4.0) and 28 days in sour cream (pH 4.3) (Dineen et al. 1998).

In an acidic condition, STEC needs to adapt its internal pH in order to stay alive. If the intracellular pH drops too much, the cell is likely to die due to loss of function or denaturation of enzymes. To overcome these conditions, *E. coli* possesses distinct acid tolerance mechanisms related to its log and stationary phases. Such mechanisms involve σ^S and cAMP functions as well as decarboxylase enzymes that requires specific amino acids like glutamate, arginine, lysine and ornithine (Foster 1999, 2004).

Acid tolerance response in stationary phase cells and log-phase cells

Currently, five major mechanisms are accepted to be part of *E. coli* acid tolerance response in stationary phase cells. This include four enzyme-based responses and an oxidative response. The oxidative system, also known as AR1, requires both the alternative σ^S and CRP (cAMP receptor protein) to mediate acid tolerance. It is repressed by glucose thus often cited as glucose-repressed acid resistance system. AR1 functions when cells grow in complex media at pH 5.5 and then are challenged with a pH as low as 2.5, in a minimal media, with no amino acids supply. However, when the cells grow on same complex media but at pH 8 and are shifted to low pH, they are inactivated rapidly. This occurs because pH 8-grown cells are likely to synthesize an inhibitor of AR1. Nevertheless, the role of AR1 components and mechanisms are still not fully elucidated (Castanie-Cornet et al. 1999; Foster 2004).

The other four decarboxylase-based pathways are based on a decarboxylase enzyme induced by low pH, a specific amino acid and antiporters. They work by exchanging α-carboxyl groups from the substrate for a proton from the cytoplasm, which will result in CO_2 and the end product, some of them known as biogenic amine (Benkerroum 2016). The antiporters will then transport the end product out of the cell and import another substrate at the same time. This will lead to an increasing of intracellular pH because of intracellular proton consumption (Peng et al. 2011). These enzyme-based pathways work upon specific conditions, where: AR2 (glutamate-dependent system) and AR3 (arginine-dependent system) allow *E. coli* to survive at extreme acidic conditions, like pH 2.5; AR4 (lysine-dependent system) and AR5 (ornithine-dependent system) enable *E. coli* to survive in less acidic condition, such as pH 4.5 (Zhao and Houry 2010). These processes are summarized in Figure 2.

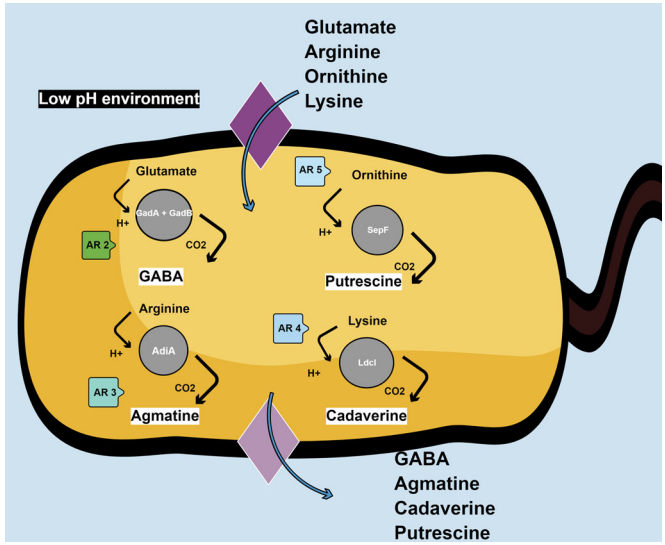

Figure 2. General scheme of the amino acid AR systems.

The AR2 is the most studied and efficient acid response system in *E. coli*. In this system, the decarboxylase enzymes GadA and GadB convert glutamate to gamma-amino butyric acid (GABA) and CO_2, thus consuming an intracellular proton. GABA is then transported out of the cell by antiporter GadC while more glutamate enters the cell. Similarly, in AR3, the decarboxylase enzyme AdiA converts arginine into agmatine and CO_2, consuming one proton. Agmatine in then taken out of the cell by antiporter AdiC in exchange for more arginine (Zhao and Houry 2010). Because these systems work better at extreme pH (2.5), they are less likely to function in cheese manufacturing.

Furthermore, AR4 and AR5 work in a mild pH condition of 4.5–5, which is more likely to be found in cheese products. In AR4, the lysine decarboxylase Ldcl (CadA) converts lysine into cadaverine and CO_2, consuming one proton. Similar to the others, the antiporter CadB takes out cadaverine in exchange for lysine. The same occurs to AR5, where the ornithine decarboxylase is SpeF and the ornithine-putrescine antiporter is PotE. Just like AR1 system, AR5 mechanisms are still unclear (Zhao and Houry 2010).

Interestingly, consumption of food with high quantities of putrescine and cadaverine have already been related to histamine toxicity enhancement, carcinogenic nitrosamines formation and, together with agmatine, inhibition of detoxification enzymes (Costa et al. 2018).

Besides that, chloride and potassium also play a role in acid resistance response. When facing an extreme acidic condition e.g., pH 2.0, the transmembrane potential of *E. coli* is disrupted. *E. coli* can then revert the electrical membrane potential from −90 mV to +30 mV (in the presence of glutamate) or +80 mV (in the presence of arginine) (Zhao and Houry 2010). This may be due to the accumulation of protons in the cytoplasm and also due to the result of AR2 and AR3 pathways (Zhao and Houry 2010). Thus, when exposed to elevated acid stress, *E. coli* ClC chloride channel imports Cl^- to cytoplasm, bringing negative charge while removes excess protons, which helps to restore the membrane potential (Foster 2004). Furthermore, the influx of potassium also regulates *E. coli* intracellular pH (pH_i). Symporters and K + channels acts maintaining the membrane potential and also maintaining pH_i homeostasis (Epstein 2003).

Another system involved in acid stress is the chaperone-based acid stress response. This system is related to the protection of periplasmic proteins. The HdeA and HdeB are the chaperones responsible for the response in periplasm. In an acidic situation, they function by maintaining the substrates in a soluble state. Also, the chaperone system is able to aggregate themselves to proteins that failed to be solubilized. Their optimal pH is below 3, so they function better under extreme acidic conditions (Zhao and Houry 2010).

The *E. coli* acid tolerance response in log-phase is called acid adaptation (AA) (Lianou, Nychas, and Koutsoumanis 2017). Although only a few studies had been done, it is known that AA occurs when *E. coli* is grown in nutrient broth at pH 5.0 and can survive the challenge of pH 3.5 or 3.0. The mechanisms are still unclear, but it seems to involve protein synthesis steps and mainly repair of DNA damage. It is proposed that PhoE, a phosphate-specific porin, works as a channel for the influx of H^+, which culminates in periplasmic acidification and stimulation of a transmembrane sensory protein that could induce AA (Bearson, Bearson, and Foster 1997).

Acid tolerance response in acid coagulation, ripened cheese and cheese storage

As cited before, lactic acid bacteria are involved in many fermentation processes of dairy products, especially cheese. The fermentation process results in a drop of pH due to the production of different organic acids, such as lactic, acetic and propionic acids (Özcelik, Kuley, and Özogul 2016). Then, besides a drop in pH, STEC may also challenge the presence of weak organic acids in cheese. A weak organic acid acts by acidifying the internal pH by entering the cell in protonated form, which will then deprotonate based on the internal pH depending on the pK_a (acid dissociation constant) of the acid. Besides the acidification of the cytoplasm, this will allow the accumulation of the acid as an internal anion, increasing the cell turgor, which will affect the cell growth (Foster 1999; Peng et al. 2011).

Acidification of ripened cheese such as camembert, parmesan, and cheddar are mainly caused due to the accumulation of organic acids produced by starter cultures. This is also true regarding cheese storage (Batty, Waite-Cusic, and Meunier-Goddik 2019; Todaro et al. 2017). However, in acid coagulated cheese such as petit suisse, cream cheese or cottage cheese, there is an acidification step. The pH drop is achieved by direct addition of acids such as citric, acetic or lactic (Lucey 2011). In pathogenic bacteria, the presence of such organic acids can cause a disruption of membrane integrity due to the lipophilic behavior of weak organic acids. This accumulation can also cause osmotic stress, as well as interaction with enzymes and proteins, impairing some metabolic functions (Peng et al. 2011; Roe et al. 1998).

Cross-protection stress

The cheesemaking process and the cheese matrix itself constitute several antimicrobial hurdles that helps preventing bacterial growth by acting in different and sometimes simultaneous ways. In this case, when STEC are exposed to more than one stress condition, up- or downregulation of different stress responses might occur, originating what is called the cross-protection effect. In fact, Lee et al. (2012) already documented a tendency where *E. coli* O157 strains with better resistance to a particular stress tended to have greater resistance to other types of stressors.

Trying to demonstrate the cross-protection effect between acid stress adaptation with following thermal inactivation of *E. coli*, (Haberbeck et al. 2017) selected 48 *E. coli* strains that presented higher acid resistance than *E. coli* O157, and adapted the strains to acid resistance by letting them grow overnight in pH 5.5. Strains were submitted to thermal inactivation at both pH 7.0 and 6.2. Authors found out that acid adaptation to pH 5.5 improved acid resistance of all 48 the strains, compared to the treatment control (pH 7.0). For instance, the average D-value (time required for one logarithm reduction of the population) at 58 °C (D_{58}) for acid adapted cells was 6 min, whereas the control group was 4 min. Authors also described that strains presented more thermal resistance when inactivated at pH 6.2, rather than 7.0. Interestingly, they also described three highly heat resistant strains (not food related) with D_{58}-values ranging from 17.6 to 69 min. In comparison, Gabriel and Nakano (2011) demonstrated that upon incubation in slowly acidified nutrient broth supplemented with 1% glucose, *E. coli* O157 presented an increasing in thermal resistance, by enhancing its D_{55}-values from 51.39 s (control group) to 250 s (glucose supplemented). This later scenario can be applied to cheese matrixes containing glucose. Indeed, these results demonstrates that acid adaptation promoted a cross-protection behavior regarding thermal inactivation. More recently, Castro, Rosario, et al. (2019) reported the cross-protection effect of O26 strains thermally treated with subsequent acid tolerance response. They observed that O26 strains pre-exposed to heat treatment (65 °C) were less susceptible to be inactivated in a simulated gastric fluid (pH 2.0), than non pre-exposed strains, showing that heat stress induced the persistence of the tested strains in an acidic environment. While this is true, more studies are needed, specially those testing higher temperatures, such as used in the pasteurization processes.

Regarding the cross-protection effect between salt and thermal resistance, Lee et al. (2016) adapted three STEC strains, *E. coli* O157, *E. coli* O111 and *E. coli* O26 to salty environments containing 0%, 2% and 4% NaCl. After that, authors challenged the strains at 50 °C for a heating time ranging from 0 to 120 minutes. As a result, *E. coli* O157 adapted to 4% NaCl presented significant less reduction than those adapted to 2% NaCl, or non-adapted (0%), showing an increasing of thermal resistance. In contrast, *E. coli* O111 and *E. coli* O26 did not show an increasing of thermal resistance upon salt adaptation. This suggests that thermal resistance after salt adaptation is strain-dependent.

The effect of salt addition in STEC acid stress response was described by Bae and Lee (2017), where they submitted *E. coli* O157 strains to a combination of concentrations of acetic acid (0%–2%) and NaCl (0% and 3%). Authors found out that although the levels of *E. coli* O157 decreased as the concentration of acetic acid increased, the survival of *E. coli* O157 in the presence of 3% NaCl was significantly higher than the survival in the absence of NaCl. In fact, this pattern occurred equally for all concentrations of acetic acid added, revealing that salt promotes a synergistic effect when combined to acetic acid. In a like manner, Lee and Kang (2016) submitted five *E. coli* O157 strains to a medium containing 1.5% acetic acid combined with concentrations of NaCl, ranging from 0 to 18%. As a result, four *E. coli* O157 strains were capable of increasing recover in a significantly way, upon exposure of up to 15% NaCl, compared to treatment control (1.5% acetic acid and 0% NaCl). However, only one *E. coli* O157 strain was able to survive in an environment of up to 9% NaCl. Yet, this data suggests that the previous exposure to acid enhanced the survival capacity in the presence of salt. One more time, results demonstrated that NaCl promotes increased resistance to the presence of acid, which can be applied to cheese matrix if we take in consideration that salt is added during cheesemaking as well as weak organic acids are produced during fermentation and ripening steps.

Leenanon, Elhanafi, and Drake (2003) demonstrated the combined effect of pre-adaptation of *E. coli* O157 to cold (4 °C) and cold-acid (4 °C/pH 5.5) environment for 4 weeks, on acid (pH 1.5 and 2), freeze-thaw (−20 °C/−21 °C) and heat tolerance (56 °C). After the 4-week incubation in each of the cited conditions, authors observed that although cold and cold-acid stress significantly decreased acid tolerance of STEC strains, cold stress alone decreased more dramatically compared to cold-acid. This implicates in a possible compensation effect due to the previous contact with an acidic environment. In addition, cold stress increased freeze-thaw survival of the strains, while cold-acid had no effect upon this challenge. Interestingly, both cold and cold-acid stress enhanced heat tolerance of one strain, demonstrating a possible cross protective effect. In a similar way, Elhanafi et al. (2004) reported significantly lower D values for *E. coli* O157 strains submitted to cold and cold-acid stress followed by incubation in pH 2.0 trypticase soy broth and pH 1.5 simulated gastric fluid, compared to control cells. Besides observing a decrease in acid tolerance in all treatments, authors found out that cold stressed cells reduced their acid tolerance more, compared to cold-acid stressed cells. Again, this finding suggests a compensatory effect due to prior contact with an acidic environment. In addition, authors also reported enhancement of heat tolerance after cold and cold-acid stress for one out of three tested strains, while the other two strains had similar heat tolerance behavior than control cells. Also, cold stress increased freeze-thaw resistance in all three strains, while cold-acid stress results were statistically similar to control group, showing no co-effects. Furthermore, Leenanon and Drake (2001) described a decrease in heat resistance of cold-adapted *E. coli* O157, whereas they also reported freeze-thaw resistance enhancement after cold adaptation. As most dairy products, including some cheese varieties, are kept under cold storage after manufacturing, these results suggest that STEC cells are very prone to survive to freeze-thaw once they were already cold stressed previously.

Moreover, Akhtar et al. (2016) evaluated the correlation between antibiotic resistant strains and stress situations, such as exposure to lactic acid (2.5%, 3.5%, 5%), sodium hypochlorite (0.2, 0.5, 1 ppm free chlorine), and heat treatment (60, 61, 62.5 °C). Authors selected six strains belonging to the serotypes O26 and O103, being two high antibiotic resistant strains, two low resistant strains, and two susceptible trains. In fact, reduced levels of all bacteria were achieved with increased levels of lactic acid, sodium hypochlorite and temperature, as expected. All strains were reduced in 5 log CFU/mL after contact with 5% lactic acid for 10 minutes. Similar results were described for sodium hypochlorite assay. D values for all O26 and 103 strains ranged from 0.37 to 2.09 and 0.37 to 1.71 minutes, respectively. In conclusion, authors could not find a strong relation between antibiotic resistance and enhanced stress response mechanisms, although more studies are needed to surely confirm this statement. This result is very important regarding cheese production because multidrug resistant STEC, including *E. coli* O157, have been isolated from milk and cheese, as well as dairy cattle (Ahmed and Shimamoto 2015; Iweriebor et al. 2015).

Relation between stress and virulence

Stress response genes activated when STEC is in contact with cheese matrix may be involved with virulence potential. As Mutz, Rosario, Paschoalin, et al. (2019) suggested, the adaptation to certain type of stress during food processing is a concern since it can lead bacteria to become adapted, and to produce virulent traits, as they are able to overcome such conditions.

Virulence determinants: an overview

The pathogenesis of STEC is primarily associated with Shiga toxin, encoded by *stx* genes within the genome of a prophage (Stx phage), present on the pathogenicity island of LEE (*locus of enterocyte effacement*) (Castro et al. 2017). Two toxin variants are known: Stx1, first described in *Shigella dysenteriae*, but also produced by STEC strains; and Stx2, which is immunologically distinct yet very similar (Byrd et al. 2017). Stx toxin is highly associated with the capacity of the bacteria to produce HUS (Exeni et al. 2018).

After establishing Upon the contact with DNA-damaging agents such as antibiotics or stressful agents, STEC strains induce Stx phages, triggering the SOS response, which leads to cell lysis and release of Stx products (Bonanno et al. 2017). This occurs due to the molecular mechanism of RecA-mediated cleavage of phage repressor (Imamovic and Muniesa 2012). In fact, the toxin acts by destroying the 28S rRNA, stopping protein synthesis, which leads to cell death upon exposure to the toxin (Melton-Celsa et al. 2011).

Apart from shiga-toxin production, other reported virulence mechanisms of STEC are genes located in LEE pathogenic island: *eae*, *tir*, *espA*, *espB*, *espC*, and *espD*, related with adherence, initiation of host signal transduction, and attaching and effacing lesions; *ure* gene cluster (*ureA*, *ureB*, *ureC*, *ureD*, *ureE*, *ureF*, and *ureG*), associated with colonization and pathogenesis of *E. coli* O157; the type 3 secretory system (T3SS), a filamentous secretory system that is credited to inject STEC own receptor Tir into host cell, facilitating invasion; *E. coli* common pilus (ECP), associated with adherence and colonization; hemorrhagic coli pili (HCP), a pylus produced by *E. coli* O157 related to motility, invasion and biofilm formation; Efa1 and Cah, adhesins related to grave diseases, among others (Bolton 2011; Tran et al. 2018). Also, STEC produces many non-LEE encoded effectors (*nle* genes) that plays a role in invasion, cytotoxicity and attachment, as well as others (Bolton 2011).

Virulence status of STEC cells exposed to sublethal stress in cheese

As stated before, the contact of STEC with sublethal conditions in cheese or other food matrixes may enhance STEC capability to survive stress conditions inside the human body, thus promoting infection by enhancing its virulence characteristics. Further, Table 1 summarizes the relation between virulence promoters and different abiotic stress conditions, such as heat, cold, osmotic, and acid.

In order to characterize heat shock response of STEC using a transcriptomic approach, Carruthers and Minion (2009) submitted an *E. coli* O157 strain to 50 °C for 15 min. Among others results, authors found out that there was no increase in *stx1* or *stx2* transcription, suggesting heat shock may not have a relation with virulence enhancement in broth growth. More recently, Singh and Jiang (2015) determined the expression of virulence genes in *E. coli* O157 exposed to heat shock at 47.5 °C in tryptic soy broth. As a result, virulence genes such as *stx1*, *stx2*, *hlyA*, and *fliC* were down-regulated. On the other hand, the *eaeA* gene were up-regulated, although the result was non-significant. Similarly, Elhanafi et al. (2004) reported the virulence expression of three *E. coli* O157 strains submitted to cold stress (4 °C) and cold-acid stress (4 °C and pH 5.5) in trypticase soy broth for four weeks. Authors reported no effect of cold nor cold-acid stress on *stx2* expression, despite observing enhancement of *eaeA* and *hlyA* expressions after bacterial growth in acidic media (pH 5.5). More studies are needed to understand the role of heat shock and cold stress in virulence genes transcription.

Table 1. Prophage induction, sigma factor, regulatory system and genes reported to be involved in resistance and virulence of STEC strains exposed to stress environments.

System	Heat	Cold	Osmotic	Acid	Virulence	References
Prophage induction	No	Yes	Yes	No	Yes	Shkilnyj and Koudelka (2007); Allen et al. (2008); Harris et al. (2012); Bonanno et al. (2017)
σ^S	Yes	Yes	Yes	Yes	Yes	Kazmierczak, Wiedmann, and Boor (2005); Bergholz et al. (2009); Mei et al. (2015); Li et al. (2018); Gayán et al. (2019)
Cpx	No	No	Yes	No	Yes	Raivio and Silhavy (2001)
tir	Yes	Yes	Yes	Yes	Yes	Olesen and Jespersen (2010); Elhadidy and Álvarez-Órdoñez (2016)
eae	Yes	No	Yes	Yes	Yes	Olesen and Jespersen (2010); Wang, Gill, and Yang (2014); Singh and Jiang (2015)

Trying to understand the fate of acid stressed STEC strains in gastric passage, House et al. (2009) challenged three *E. coli O157* strains to pH 3.0 for 15 and 30 min, with and without an adaptation period in pH 5.0, also for 15 and 30 min. As expected, the adapted cells submitted to pH 5.0 prior to inoculation in pH 3.0 had a better survival rate, independent of the exposure time. In addition, authors also tested the adhesion of several acid stressed *E. coli* O157 strains to epithelial cell lines, HEp-2 and CaCo-2. The adhesion of such acid-adapted strains highly increased in both cell lines, compared to the unstressed control strains. As an example, adhesion of one STEC strain in CaCo-2 cell line increased in 486% after 6 h of adhesion. Similarly, other two STEC strains increased adhesion in 257% and 322% after 3 h in HEp-2 cells. This is intimately related to another finding of this study, where it was observed that infected epithelial cells with acid-adapted strains increased apoptosis levels in 200%–400%, compared to the infected cells by unstressed strains. Moreover, authors tested the relation between the production of Shiga toxin and acid stress in Vero cells. None of the treatment resulted in significant results regarding the amount of toxin production, either from periplasmic or secreted extracts, which makes toxin production unchanged regardless acid stress. Similarly, Leenanon et al. (2003) described an increasing of Shiga-toxin mRNA levels, whereas the production of the toxin did not increase subsequently in acid stress adapted *E. coli* O157. These results show that enhancement of acid stressed STEC cells virulence is not related to Shiga-toxin production, but to other virulence mechanisms.

Regarding salt addition, Harris et al. (2012) demonstrated that salt addition plays a role in virulence by increasing or decreasing Stx2 production and Stx2 prophage induction in *E. coli* O157 strains, according to salt concentration. In their study, the addition of 2% salt in Luria-Bertani broth caused an osmotic stress in *E. coli* O157, which resulted in significantly decreased population, while significantly increased Stx2 production, compared to control group (1% salt). To confirm, authors demonstrated a significantly increase in

stx2 mRNA expression following 2% salt addiction to culture media. As expected, the 2% salt treatment presented the highest induction of Stx2 prophage, compared to the other treatments. However, after increasing the media salt concentration to 3%, both *E. coli* O157 population and Stx2 production decreased. In comparison to these results, Bonanno et al. (2017) reported one STEC strain capable of inducing Stx1 phage upon 3% salt addition, whereas other two strains were not capable of inducing Stx2 and Stx1-Stx2 phages in the same environment. As salt content of different types of cheese can vary from 0.5% to 6% (Guinee 2004), the concentration of 2%–3% salt may enhance STEC virulence by promoting increased Stx production in some strains, followed by an osmotic stress response.

In an attempt to evaluate gene expression in *E. coli* O157 isolated from both clinical and animal source foods, Bergholz, Vanaja, and Whittam (2009) submitted three strains to osmotic and acidic environments. As expected, stationary-phase cells showed a significantly higher survival rate than log-phase cells under pH 3.5, and 37 °C. After 10 minutes in this acidic environment, authors observed the significant expression of genes known to be related to acid (*asr*, *osmY*, *gInK*, *adiY*) and osmotic (*bdm*, *proV*, *proW*, *osmC*, and *osmY*) responses, which can probably suggest a cross-protecting effect between these two responses. Also, several genes related to the envelope stress response were activated, suggesting damage in cell membrane. Interestingly, genes related to the pathogenic island LEE such as *ler*, *orf4*, and *orf5* were also induced, as well as those related to cell invasion encoded by T3SS, such as *espJ*, *espB*, *espM2*, *espL3*, and *espZ*. Besides, a gene present on the adherence island, *terZ*, was induced and highly expressed in exponential phase. This finding suggests invasion, adherence and virulence enhancement may be induced by acidic stress conditions, such as found in lactic cheese, acid coagulated cheese and some ripened cheese.

Olesen and Jespersen (2010) investigated the fate of *E. coli* O157 virulence genes transcription upon long-term acid adaptation of 24 h to pH 5.5, and exposure to salt (4.5% NaCl). After 24 h in acidic pH, *stx2A* transcription was significantly induced in one strain, whereas significantly repressed in the other two tested strains. The same pattern was verified upon 24 h under salt exposure. Besides that, only one strain encoded Stx1, and *stx1A* transcription was significantly induced in salty environment. Regarding *eae* and *tir*, intimin and translocated intimin receptor respectively, only a modest yet significant fold change occurred in both salt and acid environment, with exception of *tir* under salt stress where there was a significant transcription induction of one strain and a significant reduction of another one. Further, no significant changes were noted in *IpfA*, long polar fimbriae, in any tested strain under both challenges. Finally, all salt-adapted STEC strains presented an increasing of adherence in CaCo-2 cells. However, no significant changes were noted between acid adapted cells and control cells, in contrast to the previous cited study. As stated before, these results suggest that the addition of salt or brine to cheese may not avoid STEC persistence in this product, besides enhancing its virulence.

Concluding remarks

In general, cheese properly produced, rather than made by pasteurized or raw milk, is often considered as a safe food due to several hurdles its manufacturing processes present. However, the presence of pathogenic strains of *E. coli*, such as STEC, besides indicating direct or indirect fecal contamination, are a risk to consumption due to the production of well-known toxins. STEC is able to infect humans upon oral intake of contaminated food, which is in agreement with several reports of occurrence and outbreaks caused by STEC in cheese. Although the pasteurization process is thought to completely inactivate STEC strains in milk and cheese, consumption of raw milk cheese and post-pasteurization contamination are still a concern that can lead to the occurrence of the pathogen in such products. Undoubtedly, the certification of a good quality raw material free of pathogens, and together with the adoption of good manufacturing practices is the best way to achieve a final product that is safe for consumption.

STEC is challenged by several stress situations during cheese production, including high temperatures due to pasteurization and different cooking processes, osmotic upshifts due to the addition of salt or brine, leading to a decreasing of a_w, and the decrease of pH due to the addition of organic acids in acid-coagulated cheese and the metabolism of lactic acid bacteria added as starter cultures. However, STEC developed stress response mechanisms to overcome these situations, such as the activation of sigma factors, upregulation of genes related to stress responses, chaperone and chaperonin system mechanisms, membrane sensors and protective surface structures, enzymes and transport systems, among others. In addition, researches have reported the interaction between stress responses and enhanced virulence, which indicates the importance of the development of new instruments to avoid bacterial presence in food. Thus, a better understanding of STEC adaptation to stress conditions and the role of virulence enhancement of persistent STEC strains are crucial for the achievement of a safe product production.

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