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Recent advances in understanding the virulence of enterohemorrhagic *Escherichia coli* in food*

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A b s t r a c t: Most of the genes recently found in pathogenic E. coli encode various factors which directly determine their virulence and pathotype. Two main virulence factors characteristic for enterohemorrhagicE. coli(EHEC) are attaching/effacing lesions and Stx1/Stx2 toxins. Genes responsible for expression of aforementioned virulence factors are heavily regulated by environmental conditions. Low iron concentration induces massive expression of stx1 gene and subsequent toxin synthesis. Stress response of EHEC to starvation, acid challenge, cold shock and osmotic changes which damage DNA, induce"SOS" response. This response mediated by RecA protein not only repairs damaged DNA fragments but also induces conversion of lysogenic bacteriophage lifecycle to lytic phase followed by intensive expression of stx2 genes. Bacterial stress adaptation of E. coli to novel technologies and the potential for stress-associated enhanced virulence need to be addressed in more detail to prevent potential risk of disease. An increased understanding of expression of virulence-associated genes will provide information for control of pathogens and increase microbial safety of foods.

***Key words**: EHEC, virulence, gene expression, food.*

Introduction

The scientific history of *Escherichia coli* started with its first description in 1885 by Theodor Escherich. He identified a bacterium he called *Bacterium colicommune* as the cause of infantile diarrhea. Its present name *Escherichia coli*was officially accepted in 1958 in honor of its originator. Although in research on basic genetics and molecular biology *E. coli* was generally known as a non-pathogenic bacterium, in medicine *E. coli* is known as an important pathogen infecting worldwide millions of humans each year both in industrialized and in developing countries. Therefore, during the last few decades molecular biologists have started to work on the mechanisms of bacterial pathogenicity of *E. coli*(*Neidhardt,* 1996; *Gyles,* 1994; *Nataro*, 1998,; *Kaper,* 1998). The huge amount of new knowledge and genetic data on pathogenic *E. coli* indicates that up to 10–20% of the genomic information found in highly pathogenic *E. coli* is not present in *E. coli* K–12. Most of the additional genes found in pathogenic *E. coli* encode various virulence factors which directly determine their virulence and pathotype. Diagnostic methods used nowadays focus on the detection of either specific toxins and their virulence attributes or specific target genes which permit he identification of the corresponding pathotype.

E. coli is a main component of the normal intestinal flora of humans and other mammals. A great diversity of commensal non-pathogenic *E. coli* strains belonging to many different serotypes can be isolated from the feces of healthy persons. These strains are massively shed in the environment and may contaminate food of animal origin or other foods like vegetables, fruits and their derivatives. They may also contaminate surface and underground water, generally without any adverse effects on human health.

E. coli from the normal intestinal flora are usually harmless to the host and represent opportunistic pathogens. Only in very rare cases can they become a threat to healthypersons. This is mainly the case in patients with impaired immune defenses not able to

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contain these commensals in their natural habitat or after a traumatic break in the natural barriers between the gut and other normally sterile sites of the body or after surgical interventions. They can also be part of mixed infections when primary pathogens break down the local defenses of a host.

Enteric pathogenic *E. coli* (*Nataro,* 1998; *Levine,* 1987) have been broadly divided into enterotoxigenic*E. coli* (ETEC), enteropathogenic*E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive*E. coli* (EIEC), enteroaggregative*E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC).

STEC (previously called Shiga-like toxin-producing *E. coli* or verotoxin-producing *E. coli*) cause a broad range of symptoms in humans (*Griffin,* 1999) including uncomplicated diarrhea, but also more severe diseases like hemorrhagic colitis and the often deadly hemorrhagic uremic syndrome. STEC infections represent a typical disease of industrialized countries and severe forms of infection are observed mainly in young children and the elderly. Cattle form the main reservoir of STEC and fecal contamination of food represents the usual source of infection for humans but due to an apparently low infectious dose, human to human transmission has also been observed in outbreaks. STEC have also been shown to be responsible for diarrhea in calves (*Butler,* 1994) and some specific serotypes are responsible for edema disease in pigs (*Bertschinger,* 1994) STEC belong to a very large variety of serotypes, but the majority of clinical infections registered in humans and particularly in food-borne outbreaks are associated with the serotypes and serogroups O157:H7, O157:H-, O26, O103, O111, O113, and O145. Those serotypes and strains associated with severe disease and outbreaks are also called enterohemorrhagic*E. coli* (EHEC). STEC colonize the colon where they cause necrosis of villus tips but they do not invade the intestinal mucosa. The majority of EHEC have been shown to present the typical pattern of localized adherence on cell cultures and to be able to cause attaching and effacing lesions also associated with the latter pathogens (*Nataro,* 1998).

Escherichia coli O157:H7, the prototype and most virulent enterohemorrhagic*E. coli* (EHEC), was isolated in 1982 from outbreaks of hemorrhagic colitis associated with eating undercooked meat in fast-food restaurants (*Riley et al,.* 1983). EHEC O157 was also isolated from sporadic cases of hemorrhagic colitis (*Uyeyama et al.,* 1982).

The detection of toxin production by EHEC O157 led to the discovery of its causative role in the development of a previously idiopathic condition known as hemolytic uremic syndrome (HUS), a clinical pathological triad consisting of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure (*Johnson et al.,* 1983; *Karmali et al.,* 1985; *O'Brien et al*., 1983). Although EHEC O157 is the most common serotype isolated from humans, over 100 other serotypes, characterized collectively as non-O157 EHEC, are recognized by the World Health Organization as zoonotic emerging pathogens (*Nastasijevic,* 2009). Non-O157 EHEC have pathogenic and outbreak potential and are associated with diarrhea, hemorrhagic colitis, and HUS in humans (*Brooks et al*. 2005; *Hedican et al*. 2009). Genomic comparison of EHEC O157 and three clinically important non-O157 EHEC (O26, O111, and O103) revealed that all share very similar virulence gene sets, providing insight into EHEC parallel evolution (*Ogura et al*., 2009).

Main virulence factors

A/E Lessions

The key figure of EHEC pathogenicity is the formation of attaching and effacing (A/E) lesions on intestinal epithelial cells (*Nataro,* 1998). A/E lesions are characterized by the localized destruction of brush border microvilli and the intimate attachment of bacteria to the membrane of host cells. The formation of A/E lesions is mediated by the type III secretion system (T3SS), which translocates virulence factors called effectors into host cells, and the outer-membrane protein intimin, which is necessary for intimate attachment. These genes are encoded in the LEE pathogenicity island, a chromosomal locus in enteropathogenic strains of *E. coli.* The LEE genes consist of five operons and several cistrons, and their expression is coordinately regulated at the level of transcription (*Mellies et al.,* 2007). The expression of the LEE genes is regulated by a variety of environmental factors through a cascade controlled by two O157-specific virulence regulators. The LEE-encoded protein Ler activates the transcription of the LEE genes, except for the LEE1 operon genes, including the ler gene, whose transcription is activated by the Pch regulators, which are encoded by extra-LEE chromosomal loci (*Iyoda*, 2004). The combined actions of the Ler and Pch regulators control the expression of many virulence-associated genes, along with the LEE genes (*Abe et al.,* 2008).

Toxins

Toxins are the most obvious virulence factors found in almost all pathogenic *E. coli*. Some toxins show a strong association with specific pathotypes (Table 1). The Shiga toxins (formerly designated

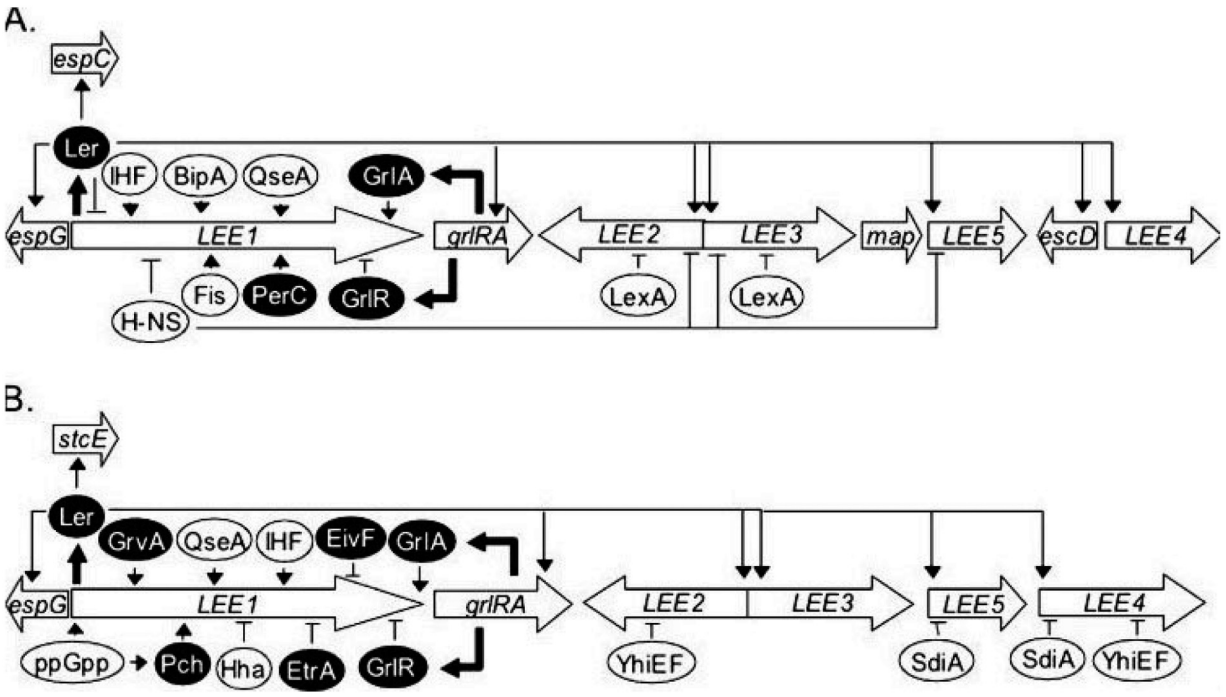

Figure 1. Regulation of the LEE pathogenicity island in EPEC (A) and EHEC (B) (by *Iyoda,* 2004).
Slika 1. Regulacija LEE ostrvapatogenostikod EPEC (A) i EHEC (B), (prema *Iyoda,* 2004)

Shiga-like toxins or verotoxins) Stx1 and Stx2, encoded by the genes *stx1* and *stx2*, respectively, are noticeable toxins and gave the name to the Shiga toxin-producing *E. coli* pathotype STEC. The stream of pathogenesis shows that after internalization (*Melton-Celsa et al.,* 1998) Stx1 and Stx2 depurinate specific residues of the host cell ribosomes (*Endo et al,*. 1988, *Saxena et al.,*1989) thereby blocking the binding of aminoacyl-tRNA to the ribosomes and inhibiting the protein synthesis.Stx, produced by EHEC during colonization of the intestinal tract, gains entry to the host through epithelial cells and acts on submucosal immune cells that release cytokines; these in turn induce inflammation and increase the expression of the Stx receptor globotriaosylceramide (Gb3) (*O'Loughlin,* 2001).Stx then targets the endothelium of organs in which the Gb3 receptor is expressed (e.g., the intestine, kidneys, and brain; *Boyd,* 1989). Because the Gb3 receptor is a glycosphingolipid, variations in the lipid moieties of its structure may influence Stx binding (*Kiarash et al.,* 1994). Stx-mediated endothelial injury activates coagulation, and inhibition of fibrinolysis leads to accumulation of fibrin and thrombosis (*Tarr et al.,* 2005). The combination of Stx and O157 lipopolysaccharide (LPS) induces platelet-leukocyte aggregates and tissue factor release and thus contributes to a prothrombotic state (*Stahl et al,*. 2009). Upon induction, Stx-encoding bacteriophages increase toxin production and play a role in horizontal transfer of stx genes by infecting other bacteria, as demonstrated in in vivo and in vitro experiments (*Acheson et al.,* 1998; *Herold et al.,* 2004; *Wagner et al.,* 2001)

Gene expression and regulation

Pathogenic bacteria must respond properly to their surrounding environment to coordinate virulence gene expression and to survive within a specific niche. Clarification of the kinetics of AE lesion formation demonstrated that this complex phenotype is tightly regulated in response to temperature and growth phase.

Activation of EHEC at 37°C in tissue culture medium enhanced the formation of AE lesions on human epithelial cells in culture (*Rosenshine,* 1996). AE lesions do not form if the bacteria are incubated at 28° C prior to infecting host cells at 37° C, and they are formed more readily by cultures in the early exponential phase of growth. Consistent with the temperature control of AE lesion formation, protein secretion via the EPEC TTSS occurs maximally at host body temperature in tissue culture medium such as Dulbecco's modified Eagle's medium (DMEM), at pH 7, and at physiological osmolarity (*Kenny,* 1995; *Kenny,* 1997). Secretion of EspA, EspB, EspC, and Tir proteins is also stimulated in the presence of iron and sodium bicarbonate, whereas it was inhibited by ammonium chloride or by omission of calcium from the growth medium (*Ide,* 2003).

Table 1. Some *E. coli* virulence factors
Tabela 1. Neki faktori virulencije kod *E. coli*

FACTOR	PATHOTYPE	TOXIN CLASS	TARGET MOLECULE	ACTIVITY/EFFECT
Thermolabile enterotoxin (LT)	ETEC	AB subunit, effector type II	G_s	Activation of adenylatecyclase, ion secretion
Shiga toxin (Stx)	EHEC	AB subunit	rRNA	Depurination of rRNA, no protein synthesis, inducing apoptosis
Cytolethal distending toxin (CDT)	Different	ABC subunit	DNA	DNA-se I activity, mitosis blocked in G2/M phase
*Shigella*enterotoxin 1 (ShET1)	EAEC, EIEC	AB subunit	-	Ion secretion
Urease	EHEC	ABC subunit	?	Urea decay NH_3 i CO_2
EspC	EPEC	Autotransporter	?	Serine protease, Ion secretion
EspP	EHEC	Autotransporter	?	Serine protease
Haemoglobine-binding protease (Tsh)	EPEC	Autotransporter	Hem	Haemoglobin decay, release ofiron
Pet	EAEC	Autotransporter	Spectrin	Serine protease, Ion secretion
Pic	EAEC, EIEC	Autotransporter	?	Protease, mucinase
Sat	EPEC	Autotransporter	?	Vacuolization
SepA	EIEC	Autotransporter	?	Serine protease
SigA	EIEC	Autotransporter	?	Ion secretion
Cycle inhibiting factor (Cif)	EPEC, EHEC	Effector type III	?	Mitosis blocked in G2/M phase, Cdk1 inactivation
EspF	EPEC, EHEC	Effector type III	?	Opens tight junctions, induces apoptosis
EspH	EPEC, EHEC	Effector type III	?	Phyllopodia modulation
Map	EPEC, EHEC	Effector type III	Mitochondria	Breaks membrane potential of mitochondria
Tir	EPEC, EHEC	Effector type III	Nck	Loss of microvilli
IpaA	EIEC	Effector type III	Vinculin	Actin depolymerization
IpaB	EIEC	Effector type III	Caspase 1	Apoptosis, release of IL-1
IpaC	EIEC	Effector type III	Actin	Actin polymerization, activation of Cdc42 i Rac
IpaH	EIEC	Effector type III	Nucleus	Modulation of inflammation

An additional two-component system controlling the EHEC AE phenotype has been recently described. The QseEF proteins are a sensor kinase and response regulator, respectively, and are transcribed from a single operon QseF activates transcription of the EspFu effector protein secreted into the host cell by the TTSS, and a *qseF* deletion mutant fails to form AE lesions. The QseEF system is activated by epinephrine through the QseC sensor kinase. The precise mechanism of the sensor kinase/response regulator control of quorum-sensing signaling in EHEC continues to be under intense investigation (*Sircili,* 2004).

An elegant study by *Deng et al.,* 2004 revealed that SepD and SepL, encoded by the *LEE2* and *LEE4* operons, respectively, constitute a molecular switch controlling secretion of translocators and effector molecules, and they began to clarify the role of calcium in these processes. Beginning with *C. rodentium*, but expanding their studies to EHEC and EPEC, they found that low-calcium conditions inhibit the secretion of translocators, such as EspA, EspD, and EspB, but enhance the secretion of effectors, such as Tir and NleA.

Genes responsible for synthesis of shiga-like toxins (stx1 and stx2) are not encoded by bacterial chromosome rather they are encoded by λ-like bacteriophage. It has been adopted that during the evolution this lambdoid bacteriophage participated in horizontal transfer of stx genes from *Shigelladysenteriae* to *E. coli.* This also explains high degree of homology between toxins produced by these bacteria. Bacteriophage can exist in one out of two possible forms: lysogenic or lytic phase. Being in lysogenic phase, bacteriophage has been incorporated into bacterial circular chromosome and it synchronously replicates along with bacterial DNA. When it comes to lytic phase, bacteriophage inserts its genetic material into a bacterium, replicates independently, reproduces and rearranges its ori-

ginal structure. At some moment, host cell bursts and release a new progeny of phages.

Considering that genes encoding Stx subunits in the genome of prophages are located next to the lytic genes in lambdoid family of bacteriophages there are two main signals from environment which induce switching from lysogenic to lytic lifecycle: **low iron concentration** and **DNA damaging** caused by acid stress, cold stress, presence of antibacterial substances etc. (*Velebit,* 2010).

Iron is the most important micronutrient used by bacteria. This metal is essential for cellular metabolism, since it is required as a cofactor for a large number of enzymes, except for *Lactobacillae*. However, this element is not easily available to microorganisms in aerobic environments. While in anaerobic conditions Fe^{2+} is soluble at physiological pH and cells easily obtain iron from the external medium, Fe^{2+} becomes quickly converted to Fe^{3+} upon exposure to oxygen and forms insoluble hydroxides at neutral pH, making the available metal very scarce. In order to acquire iron from the extracellular medium, virtually all aerobic bacteria produce and secrete low-molecular-weight compounds termed siderophores (siderosphoros, iron carriers). These compounds chelate Fe^{3+} with high affinity and specificity. Subsequently, the cell recovers the ferrisiderophore complexes through specific outer membrane receptors. Some of these high-affinity systems of iron uptake are important virulence factors in bacteria infecting animal fluids and tissues because they can chelate the metal bound to host proteins. Furthermore, because iron availability is generally growth limiting for bacteria thriving in an animal milieu, the lack of the metal is a major environmental signal to trigger expression of virulence determinants. However, an excess of iron is toxic because of its ability to catalyze Fenton reactions and formation of active forms of oxygen. Iron uptake has to be, therefore, exquisitely regulated to maintain the intracellular concentration of the metal between desirable limits. Considering that excretion mechanisms for iron are not known in bacteria, microorganisms appear to control iron homeostasis, regulating its transport through the membrane (*Escolar et al.,* 1999).

The Fur protein of *E. coli* is a 17-kDa polypeptide which acts as a transcriptional repressor of iron-regulated promoters by virtue of its Fe^{2+} dependent DNA binding activity.Under iron-rich conditions Fur binds the divalent ion, acquires a configuration able to bind target DNA sequences (generally known as Fur boxes or iron boxes), and inhibits transcription from virtually all the genes and operons repressed by the metal. On the contrary, when iron is scarce, the equilibrium is displaced to release Fe^{2+}, the RNA polymerase accesses cognate promoters, and the genes for the biosynthesis of siderophores and other iron-related functions are expressed. In reality functional promoter pStx1, up streams from stx1 regulated by iron concentration through the iron-dependent Fur transcriptional repressor. Subsequently iron binding confers Fur metalloprotein a conformational change, and then high iron concentration Fur-regulated promoters become repressed. If the iron concentration is low, Fur does not bind close to pStx1 and toxin transcription can occur. Since bacteria need iron to grow could this be the reason why bacteria express and release toxins?

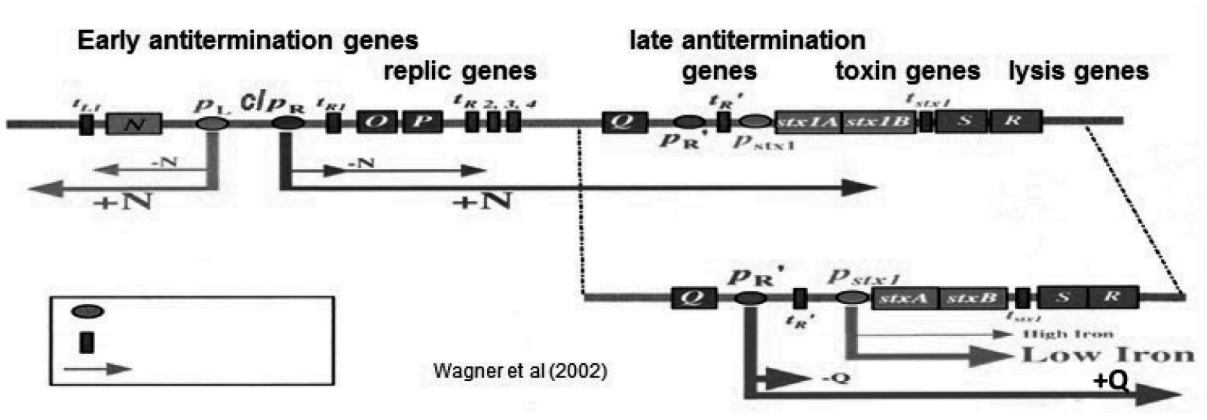

Figure 2. „Visual prints“ of pork, beef, lamb and venison made with the „E-nose“
Figura 2. „Vizuelni otisak“ svinjskog, junećeg, jagnjećeg mesa i divljači pomoću „Elektronskog nosa“

Damaged DNA sensors induce DNA repair mechanism known as “SOS” response. There are two SOS-induced genes:Lex A transcriptional re-

pressor protein and Rec A co-protease. This protease has several roles, of which most important are participation in re combinational DNA repair, assembly ofss DNA gains a co-protease activity Rec A, it is being involved with the self-cleavage of Lex A.de-repressionof SOS-regulated genes and finallyand the most important, cleaves the cI repressor of λ lambda phage, allowing the conversion of lysogenic form to a lytic form (*Wagner et al.,* 2001).

Stress response and changes of virulence

Under stress conditions microorganisms have developed signal transduction systems (movement of signals from outside the cell to inside) to sense environmental stresses and to controlthe coordinated expression of genes involved in cellular defense mechanisms (*Kennelly,*1996). These evolved protective or adaptive networks assist microorganisms to modify their environments and/or to survive the stress condition. A common regulatory mechanism involves sigma factors. Sigma factors are small proteins that bind to RNA polymerase (RNAP). The core RNAP has 5 components or subunits (α1, α2, β, β, and ω). In orderto bind promoter-specific regions, the core enzyme requires another subunit, a sigma factor (σ). The sigma factor greatly reduces the affinity of RNAP for nonspecific DNA, while increasing specificity for certain promoter regions, depending on the sigma factor. A sigma factor is normally present as part of the RNA polymeraseholoenzyme complex and the complete holoenzyme, therefore, has 6 subunits. The presence of the sigma factor allows theRNAP enzyme to bind at specific promoter sequences on thechromosome, and initiate transcription of particular genes (*Abee,* 1999). In this manner, genetic expression is controlled at the transcription level. In E. coli, the housekeeping σfactor, σ70, is responsible for transcription from many of the genepromoters under normal nonstress conditions.Under stress conditions, alternative σ factors to σ70 with different promoter specificities are induced, resulting in the expressionof specialty regulons (a system in which two or more structuralgenes are subject to coordinated regulation by a common regulator molecule) in response to a variety of stresses. In this manner, gene expression is modified by different sigma factors. In E. coliand other enteric bacteria, σS(RpoS) is the master regulator of thegeneral stress response.

In a recent study, cytotoxic genes (stx1, stx2, eaeA) expression of *Escherichia coli* O157:H7 in foodstuffs of animal origin was studied (*Velebit,* 2010). Experiment was set up in a way it simulated the most common storage conditions. Minced beef, UHT milk and soft cheese were inoculated by an overnight broth culture of reference strain ATCC 35150 E. coli O157:H7. Samples were incubated at the temperatures of 4°C and 12°C during 24 hours and 48 hours, respectively.Genetic expression profile was frozen at determined time points. In order to measure relative expression of cytotoxic genes, prokaryotic mRNA has been purified and converted to cDNA to be used in a RealTime PCR. Simultaneously, changes in growth kinetics were recorded.

Results indicated that growth of *E. coli* O157:H7 in experimentally contaminated minced beef kept at the temperature of 12°C during 48 hours and expression of stx1 and stx2 genes were considerably higher rather than in foodstuffs subjected to other experimental conditions.Regarding UHT milk growth kinetics and gene expression remained as similar as in minced beef, however at a somewhat lower intensity.Growth kinetics in soft cheese appeared to be not affected. Interestingly, stx1 gene was down--regulated during initial phase of incubation; while later up-regulation to a base level occurred by the end of experiment.Expression of eaeA gene in case of experimentally contaminated beef, milk and cheese and expression of stx2 and eaeA genes in milk and cheese was negligible i.e. experimental conditions didn't provoke up-regulation of respective genes. It seemed that foodstuffs with higher fat content down-regulated stx2 genes. A strong correlation has been established between growth kinetics and gene expression in minced beef and partially UHT milk while no correlation has been established between growth kinetics and gene expression in soft cheese.

Conclusion

Food is composite environment in which varieties of microorganisms are present and compete strongly for uptake of limiting nutrients to initiate and maintain growth. Survival of pathogens in foods takes place under stress conditions such as limited nutrient availability, adverse pH osmolarity, oxidation, temperature, chemical residues, as well as competition by other microorganisms. Spoilage and pathogenic bacteria coexist and compete in foods. In this respect, prediction of *E. coli* response to stress should take into account all microecological factors involved in the processing of a specific food.

The primary objective of food scientist is to control occurrence and evolution of stress-resistant pathogens in real foods and, consequently, to impro-

ve food safety. The stress response of *E. coli*is complex, robust, and versatile. Bacterial stress adaptation of *E. coli* to novel technologies and the potential for stress-associated enhanced virulence need to be addressedin more detail to prevent potential risk of disease. An increased understanding of expression of virulence-associated genes will provide information for control of pathogens and increase microbial safety of foods. It will also encourage development of new efficient methods for reducing the virulence of this pathogen in contaminated foods.

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Nova istraživanja virulentnosti enterohemoragičnih *Esherichia coli* u hrani

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R e z i m e: Većina gena koji su poslednjih godina utvrđeni kod patogenih E. coli kodiraju različite faktore koji direktno utiču na njihovu virulentnost i pripadnost određenom patotipu. Dva glavna faktora virulencije kod enterohemoragičnih E. coli (EHEC)su attaching/effacing lezije Stx1/Stx2 toksini. Geni odgovorni za ekspresiju pomenutih faktora virulencije pod jakim su uticajem uslova sredine. Niska koncentracija gvožđa indukuje jaku ekspresiju stx1 gena i posledičnu sintezu velike količine Stx1 toksina. Stresni odgovor EHEC na manjak hranljivih supstanci, povećanje kiselosti sredine, izloženost hladnoći kao i na promene osmolarnosti, koji oštećuju DNK, indukuje „SOS" odgovor. „SOS" reakcija posredovana RecA proteinom ne samo da popravlja oštećene fragmente DNK molekula, već i pokreće mehanizam konverzije bakteriofaga integrisanog u hromozom E. coli iz lizogene faze u litički ciklus tokom koga dolazi do intenzivne ekspresije stx2 gena. Da bi se prevenirao potencijani rizik nastanka bolesti, neophodno je detaljno proučavati prilagođavanje E. coli novim tehnologijama pripreme i konzervacije hrane i potencijal za stres-indukovanu virulenciju. Dobro poznavanje ekspresije gena odgovornih za virulenciju obezbediće informacije neophodne za kontrolu patogena i povećaće mikrobiološku bezbednost hrane.

Ključne reči:*EHEC, virulentnost, genskaekpresija, hrana.*

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