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QUANTITATIVE REAL-TIME PCR DETECTION OF PUTRESCINE-PRODUCING GRAM-NEGATIVE BACTERIA

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ABSTRACT

Biogenic amines are indispensable components of living cells; nevertheless these compounds could be toxic for human health in higher concentrations. Putrescine is supposed to be the major biogenic amine associated with microbial food spoilage. Development of reliable, fast and culture-independent molecular methods to detect bacteria producing biogenic amines deserves the attention, especially of the food industry in purpose to protect health. The objective of this study was to verify the newly designed primer sets for detection of two inducible genes *adiA* and *speF* together in *Salmonella enterica* and *Escherichia coli* genome by Real-time PCR. These forenamed genes encode enzymes in the metabolic pathway which leads to production of putrescine in Gram-negative bacteria. Moreover, relative expression of these genes was studied in *E. coli* CCM 3954 strain using Real-time PCR. In this study, sets of new primers for the detection two inducible genes (*speF* and *adiA*) in *Salmonella enterica* and *E. coli* by Real-time PCR were designed and tested. Amplification efficiency of a Real-time PCR was calculated from the slope of the standard curves (*adiA*, *speF*, *gapA*). An efficiency in a range from 95 to 105 % for all tested reactions was achieved. The gene expression (R) of *adiA* and *speF* genes in *E. coli* was varied depending on culture conditions. The highest gene expression of *adiA* and *speF* was observed at 6, 24 and 36 h (R_{adiA} ~ 3, 5, 9; R_{speF} ~11, 10, 9; respectively) after initiation of growth of this bacteria in nutrient broth medium enchired with amino acids. The results show that these primers could be used for relative quantification analysis of *E. coli*.

Keywords: putrescine; Gram-negative bacteria**;** *speF*; *adiA*; Real-time PCR

INTRODUCTION

Biogenic amines (BAs) are low molecular weight nitrogen compounds formed by several bacterial species in food and beverages during fermentation. These amines are indispensable components of living cells, but they could exert toxic effect in humans in higher concentrations **(Santos, 1996)**.

However, in food, such as fermented products, BAs are also produced by genera of the family *Enterobacteriaceae*, such as *Escherichia*, *Salmonella Citrobacter*, *Klebsiella*, *Proteus* and *Shigella* **(Suzzi and Garnini, 2003)**. The main biogenic amines encountered in foods and drinks include histamine, tyramine, putrescine, and cadaverine **(Landete et al., 2005)**. Moreover, the ingestion of contaminated food containing high amounts of BAs can be toxic. For example, histamine induces headache, diarrhea, edema, respiratory difficulties, allergy; tyramine can causes hypertension, migraine and neurological disorders, while putrescine and cadaverine could possess carcinogenic effects **(Ladero et al., 2010; Shalaby, 1996)**.

Nevertheless, removing of produced BAs from material is very complicated because of their persistence. They resist high temperature, even autoclaving **(Zaman et al., 2010)**. Therefore, attention is focused on the prevention of their formation, such as using fresh raw material, keeping hygienic precautions and technological procedure **(Avarez and Moreno-Arribas, 2014)**.

Nowadays, the prevention of BAs accumulation in food industry has become one of the major priorities. Thus, the development of tools for research on BAs accumulation appears to be extraordinarily important. Putrescine is supposed to be the major biogenic amine associated with microbial food spoilage. Putrescine can be produced by the starter cultures and contaminating microbiota in food. Contaminating microorganisms from the family *Enterobacteriaceae* and *Pseudomonadaceae* among many cases are thought to be the most frequent producers of putrescine **(Curiel et al., 2011; Lavizzari et al., 2010; Pons-Sánchez-Cascado et al., 2005)**.

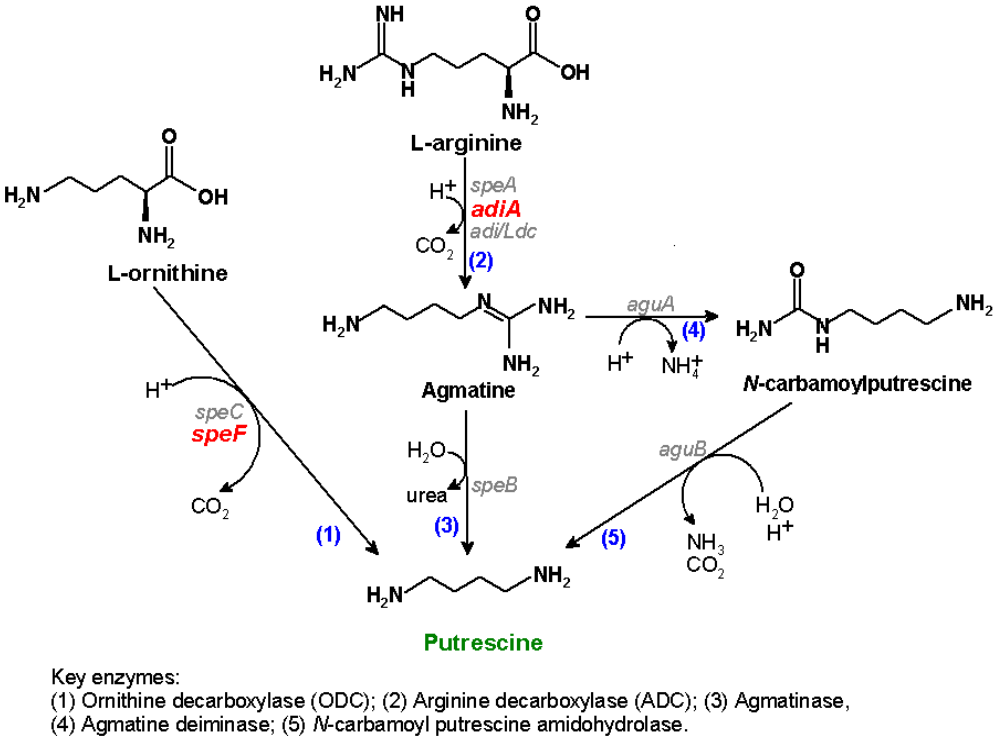

Figure 1 Putrescine metabolism in Gram-negative bacteria **(Wunderlichova et al., 2014)**.

In contrast to other BAs formed by straight decarboxylation of appropriate amino acids, Gram-negative bacteria can produce putrescine by three different metabolic ways, in which certain enzymes can even exist in two different forms (in biosynthetic or biodegradative form) as shown in Figure 1.

Putrescine might be formed directly by decarboxylation of ornithine by the ornithine decarboxylase enzyme (ODC) in the first pathway. The gene *speC* codes for biosynthetic form of this enzyme. The catabolic form of ODC is encoded by the *speF* gene included in genome of only few *Enterobacteriaceae* family members, such as *Escherichia coli* and *Salmonella enterica*. Both, the second and third pathway of putrescine formation begin with decarboxylation of arginine by arginine decarboxylase (ADC) which leads to production of agmatine. This enzyme may also be present as a biosynthetic form, encoded by the *speA* gene that occurs in a wide range of Gram-negative bacteria or it might be present in its catabolic form, inducible ADC, which is encoded by the *adiA* gene. The genome of several Gram-negative bacteria such as *E. coli* and *Salmonella enterica* strains includes the *adiA* gene. The third pathway typically occurs in plants. Within the third pathway, agmatine is converted to putrescine via formation of intermediate *N*- carbamoyl putrescine and the overall pathway is catalyzed by two enzymes. The first step is catalyzed by agmatine deiminase (the *aguA* gene product) and the second one by *N*-carbamoyl putrescine amidohydrolase (the *aguB* gene product) **(Wunderlichova et al., 2014)**.

Methods for detection of bacteria which produce biogenic amines in higher, potentially dangerous concentrations to humans have been developed during the last three decades. Several detection methods are based on use of differential growth media where the increase of the pH signalling BAs formation **(Maila, 1993)**. The most useful method for BAs detection in food is chromatography, especially high performance liquid chromatography **(Lorencova et al., 2012; Costa et al., 2015)**. However, detection of biogenic amines producing bacteria by conventional culture techniques is often unreliable, tedious with certain disadvantages, such as low throughput score, frequent appearance of false positive or negative results, unsufficient sensitivity, and high demands for expensive and sophisticated equipment **(Actis et al., 1999)**. Molecular biology methods for detection and identification of food-borne bacteria have become an alternative tools in contrast to traditional culture microbiological methods. The PCR technique has become one of the most important methods and offers the advantages of simplicity, sensitivity, specific detection of targeted genes and high throughput outcome. Moreover, molecular methods can detect potential risk score even before BA is produced. Though standard PCR and Real-time PCR follow similar principles and rules, Real-time PCR has many advantages over basic PCR technique. Real-time PCR measures the product formation (gene amplification) during the exponential phase while standard PCR measures the amount of the product during the plateau phase. It is more effective to perform the measurement during the exponential phase because results obtained during the plateau phase do not always reliable indicate the quantity of starting material. For example, with the Real-time PCR results can be acquired in an hour but with traditional PCR the whole procedure usually lasts 3 - 4 h to obtain the final results. Standard PCR is followed by post-PCR analysis, usually with agarose gel electrophoresis; the product of our interest is identified either by size or sequence. Although gel electrophoresis is relatively cheap technique, it falls in the group of low

throughput techniques since it is time-consuming and non-automated. Moreover, it also exerts low specificity, since the molecules with the same or similar molecular weight cannot be easily differentiated. In comparison, technique beats down traditional PCR in terms of highthroughput outcome, selectivity and sensitivity. Furthermore, major disadvantage of traditional PCR is the detection of non-viable cells. In contrast, Real-time PCR method is capable of detecting of the viable cells **(Postollec et al., 2011)**.

In this study, sets of new primers for the detection of two inducible genes (*speF* and *adiA*) involved in the putrescine metabolism in Gram-negative bacteria were designed and tested. Real-time PCR method for the direct detection and relative quantification of bacterial gene expression in nutrient broth medium was proposed and finally optimized.

The aim of further experimental studies will be the monitoring of putrescine producers (*E. coli* and *Salmonella enterica*) by Real-time PCR in real food samples.

MATERIAL AND METHODOLOGY

Design of PCR primers

The optimal length of primers is generally accepted as 18 - 24 bp. For better optimization of Real-time PCR the finding of primers of minimal length which have melting temperatures (Tm) that are between 59 and 68 °C, with an optimal Tm of 63 – 64 °C is essential **(Thornton and Basu, 2011)**. Following the study of **Wunderlichova et al. (2014)**, the specific oligonucleotide primers for detection and relative quantification of *speF* by Real-time PCR in *E. coli* a *Salmonella enterica* were designed and tested.

Primers for endogen (housekeeping gene; *gapA*) in *E. coli* were adopted from Fitzmaurice´s study **(Fitzmaurice et al., 2014)**. The last sets of primers, those for *adiA*, enabling analysis of gene expression by Real-time PCR were developed especially for this study. Sequences for the gene encoding the arginine decarboxylase (ADC) in *Salmonella enterica* and *E.coli* were obtained using database GenBank. Sequences were aligned using Jalview Editor and a consensus sequence was generated. Two conserved regions were identified and primers were designed in these regions. The size of amplified PCR product was anticipated to be 192 bp. The acquired sequences of desired genes were compared using BLAST at the NCBI site (http://www.ncbi.nlm.nih.gov./blast). Primers designed and used in this study are listed in Table 1.

Bacterial strain

Escherichia coli CCM 3954 used in this study, was purchased from the Czech collection of Microorganism (CCM).

Escherichia coli was cultivated in nutrient broth (NB) medium for 24 hours at the temperature of 37 °C, in the next stop, strain was incubated in a mineral broth medium enriched with glucose and vitamins for 48 hours at 30 °C (decarboxylases genes were inactivated in this step). Bacteria with inactivated decarboxylase activity were subsequently transferred to the NB medium with 0.2% amino acids (L-ornithine, L-arginine) and cultured for 48 hours at 37 °C. Samples were collected during incubation in a time mode, *Escherichia coli* strain was tested separately at time intervals of 0, 6, 9, 12, 24, 30, 36 and 48 hours.

Reverse transcriptase

Collected samples were centrifuged at 5,000 RPM for 5 min at 4 °C. After centrifugation, isolation of total RNA using High pure RNA isolation kit (ROCHE, Germany) was performed following the ROCHE´s protocol (ROCHE Web site, High Pure RNA Isolation Kit). Isolated RNA was immediately translated into cDNA form by Transcriptor first strand cDNA synthesis kit (ROCHE, Germany) following the ROCHE´s manuscript (ROCHE Web site, Transcriptor First Strand cDNA Synthesis Kit).

The mix of template and primers (the final volume of 13 μL included 60 μmol.L^{-1} of Random Hexamer Primer and maximum volume of RNA) was incubated for 10 min at 65 °C. Then 4 μL of Transcriptor reverse transctriptase reaction buffer, 0.5 μL of Protector RNase inhibitor (40 U.μL^{-1}), 1 mmol.L^{-1} of deoxynucleotidemix, 0.5 μL of Transcriptor reverse transcriptase (40 U.μL^{-1}) were added to the final volume of 20 μL.

Real-time PCR

Relative expression of *speF* and *adi*A genes was determined using the Real-time PCR technique. Each cDNA sample was amplified using SYBR Green (Fast Start Universal SYBR Green Master (Rox ROCHE, Germany) using the Thermocycler CFX 96 Real-Time (BIO RAD). Briefly, the reaction mixture consisted of 12.5 μL of ROCHE mix, 300 nmol.L^{-1} of forward primer, 300 nmol.L^{-1} of reverse primer and 2.5 μL of cDNA in the final volume of 25 μL of supermix. Each cycle included initial denaturation step at 95 °C for 3min, denaturation step at 95 °C for 30 s, annealing at 57.9 °C for 30 s, extension at 72 °C for 60 s, melting curve in range of 55 – 95 °C and final extension step at 72 °C held for 5 min. The gene *GapA*, which encodes for expression of a member of the glyceraldehyde-3-phosphate dehydrogenase protein family, was used as an endogenous control to normalize

Table 1 PCR primers used in the quantitative Real-time PCR assays.

Target gene	Primer name	Sequence 5´ → 3 ´	Amplicon size (bp)
speF	2F	5´-TCGCCRCTGYTGCTG-3´*	196
	4R	5´-GATAGAAYGGGCTGGTGG-3´*	
adiA	adiAU F	5´-CTGGTTGAAGCGGGAGAART-3´*	192
	adiAU R	5´-TGGTACGGCTATGCRCGYTT-3´*	
gapA	gapA F	5´-ACTTCGACAAATATGCTGGC-3´	200
	gapA R	5´-CGGGATGATGTTCTGGGAA-3´	

Note: *Y=C or T: R= A or G.

each sample for evaluation of real quantification of desired genes. The experimental study was designed as three independent experiments with each sample as triplicate.

The baseline and cycle threshold (Ct) were automatically calculated by CFX 96 Touch™ System Software, version 2.1. The melting curve analysis was done on the same device (CFX 96 Real-Time) after the completion of Real-time PCR analyses. Amplification efficiency, E, was calculated from the slope of the standard curves. It is expressed as a percentage that is the percent of template that was amplified in each cycle. For calculation the following formula (1) **(Bio-Rad Laboratories, 2006)** was applied:

$$(1) \qquad \%E = \left(10^{1/slope} - 1\right) \cdot 100\%$$

The relative expression ratio (R) was subsequently calculated for each gene of interest by using an equation (2) described by **Pfaffl (2001)**:

$$(2) \qquad R = \frac{E_{target}^{\Delta Ct_{target}(control-sample)}}{E_{reference}^{\Delta Ct_{reference}(control-sample)}}$$

Where: E_{target} is Real-time PCR efficiency of the target gene transcript (*speF* or *adiA*); $E_{reference}$ is Real-time PCR efficiency of a reference gene transcript (*gapA*); ΔCt_{target} is the Ct deviation of control (0 h) – sample (6, 9, 12, 24, 30 36 and 48 h) of the target gene transcript; $\Delta Ct_{reference}$ is the Ct deviation of control (0 h) – sample (6, 9, 12, 24, 30 36 and 48 h) of the reference gene transcript.

The geometric mean of the reference gene was applied as a normalization factor in the analysis. Measurement of gene expression was conducted in triplicate and the geometric mean of these values was used for the analysis.

RESULTS AND DISCUSSION

In this study, sets of primers were designed, tested and used for the detection and relative quantification of two inducible genes involved in putrescine metabolism (the ornithine decarboxylase (ODC) is encoded by the *speF* gene and the arginine decarboxylase (ADC) by the *adiA* gene) occurring in *E. coli* and *Salmonella enterica*.

In the first step, designed primers were tested with use of the classical PCR technique. Agarose gel electrophoresis was used for the separation of DNA fragments. Subsequently, the optimal temperatures for primer annealing were selected (based on the Tm primers) followed by optimalization of Real-time PCR.

Optimization Real-time PCR

Efficiency, reproducibility, and dynamic range of Real-time PCR assays for *adiA* and *speF* were determined by constructing standard curves using serial dilutions of template with a known amount.

Slopes for standard curves achieved the values from -3.18 to -3.40 for *adiA*, *gapA* and *speF*, as shown in Figure 2. The slope of the curve with an ideal efficiency of the PCR reaction is around the value -3.32 **(Mackay, 2007)**. Real-time PCR efficiencies (E) calculated from the standard curve slope were 106%, 103% and 97% for *adiA*, *speF and gapA*, respectively. Ideal efficiency value of Real-time PCR reaction should be 90 − 110% **(Mackay, 2007)**. The coefficient of determination (R^2) was >0.99 for all the *adiA*, *gapA* and *speF* Real-time PCR assays.

Specificity of the primers and thus verification that nonspecific products were not amplified during PCR cycles was determined by melting curve analysis. Primer-dimers formation can limit the dynamic range of the desired standard curve due to competition for reaction

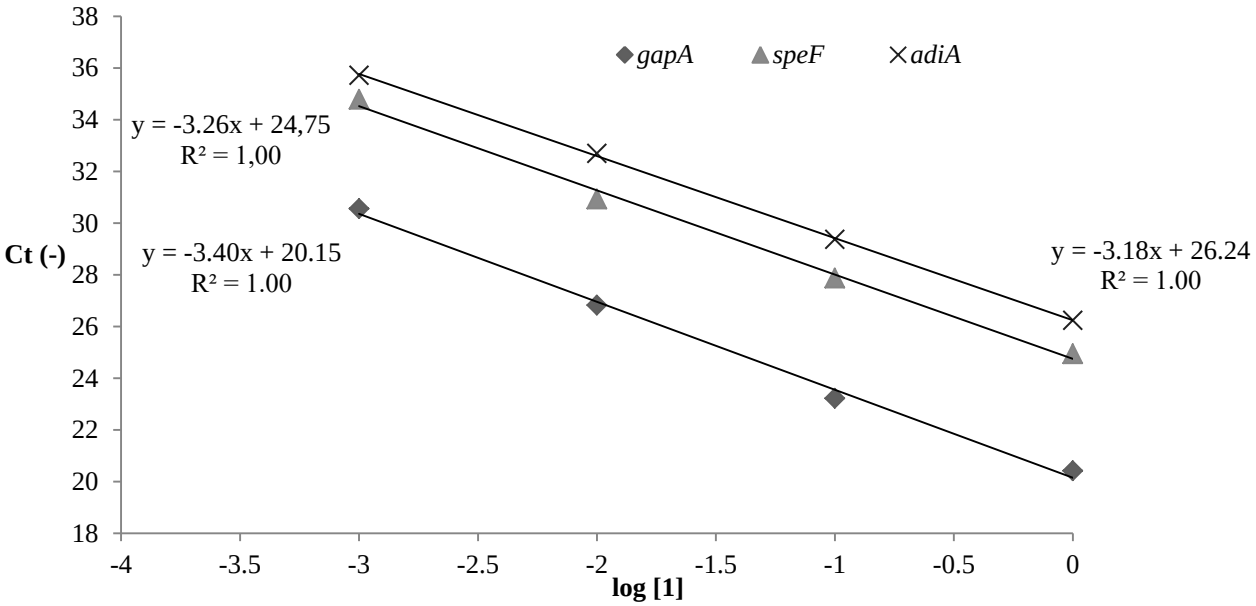

Figure 2 Real-time PCR standard curves of *speF* and *adiA* gene with endogenous control *gapA*.

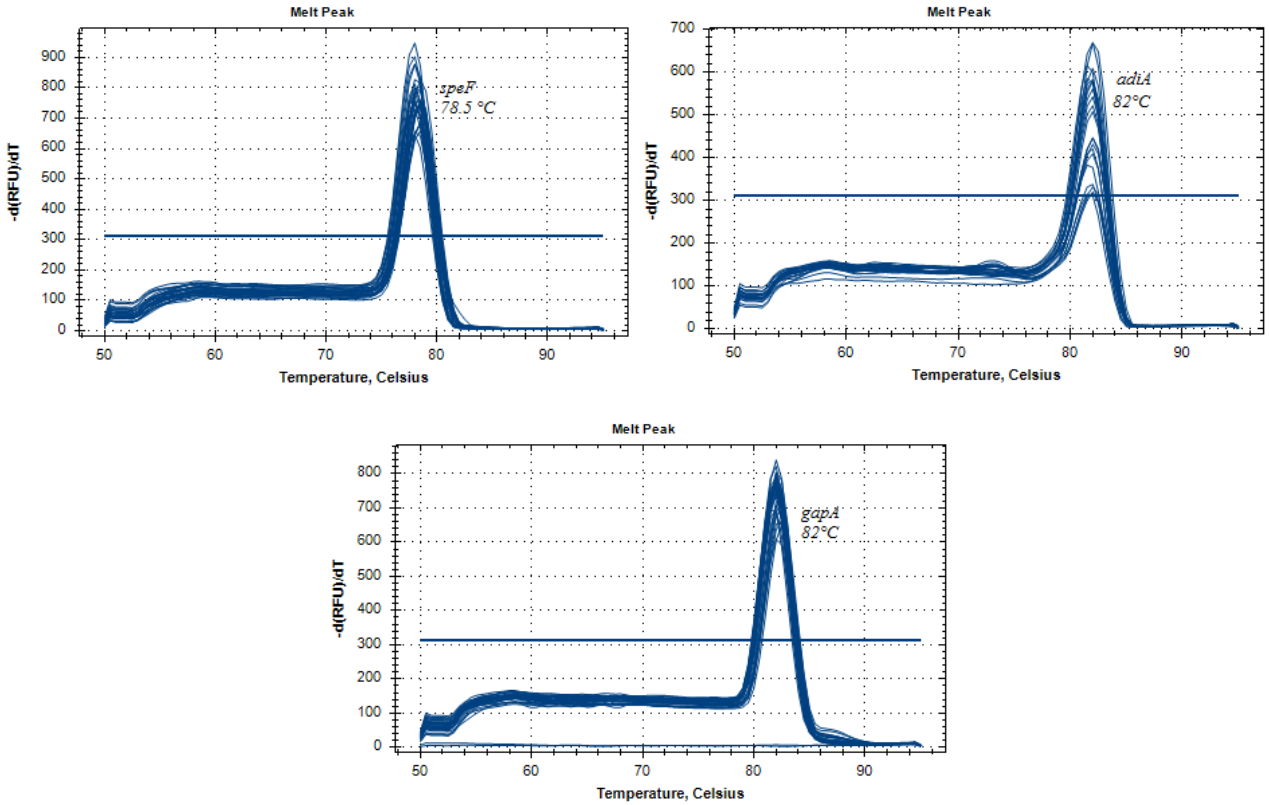

Figure 3 Melting curves for *speF*, *adiA* and *gapA*, (melting temperature *speF* 78.5°C, *adiA* 82 °C and *gapA* 82 °C).

components during amplification. Therefore, melt-curve analysis is essential in designing an efficient and specific quantitative PCR assay **(Wilhelm and Pingoud, 2003)**. Only one peak appeared in the melt curve, as can be seen in Figure 3, indicating that only one product was amplified. In other words, primer dimmers were not generated with any of the primer sets used.

Relative genes expressions

Quantitative PCR is the method of choice for precise quantification of gene expression. Analysis of expression of target gene compared to expression of an endogenous control gene is commonly used method for quantification of gene expression (Relative Quantification). As an important aspect in gene expression studies, the housekeeping gene (endogenous control gene) must be properly selected for the normalization of cDNA content.

In this study, the *E. coli* CCM 3954 was the tested strain. Based on the literature review, two housekeeping genes were chosen to be tested. First endogen was *gyrA* that encoded protein DNA gyrase subunit A **(Weigel, 1998; Yin, 2008)**. Results indicated (data not shown) that this gene was not suitable as endogene control for this study.

For this reason, the other gene, *gapA*, was chosen and tested. The *gapA* encoded protein glyceraldehyde-3-phosphate dehydrogenase which catalyzes the oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate using the cofactor NAD **(Carey, 2009; Fitzmaurice, 2004)**. Based on the results obtained in preliminary tests, the last mentioned housekeeping gen was evaluated as suitable control for the purpose of our study.

The gene expression (R) of *adiA* and *speF* genes was varied depending on culture conditions. At time intervals of 0, 6, 9, 12, 24, 30, 36 and 48 hours after initiation of incubation samples were collected. Isolated total RNA obtained from individual samples was immediately reverse transcripted into cDNA. Relative expression of *speF* and *adiA* was determined using Real-time PCR technique. Expressions of *E.coli* decarboxylases genes were determined using *gapA* as the endogenous control selected for the normalization.

The results have shown the change of gene expression of tested genes (*speF* and *adiA*) in different time points from the start of cultivation. In other words, after six hours of cultivation of *E. coli* CCM 3954 in the NB medium enriched with L-ornithine, gene expression of *speF* was 11 x higher than it was detected at the time point 0 h. Likewise, if this bacteria was cultivated in NB medium with L-arginin, gene expression of the *adiA* was 3 times higher than at the beginning (time 0 h). Changes in gene expression could be monitored for both genes at all time points (Figure 4).

The highest gene expression of *speF* and *adiA* was observed at 6, 24 and 36 h (R_{speF} ~11, 10, 9; R_{adiA} ~ 3, 5, 9; respectively) during growth of *E. coli* CCM 3954 in nutrient broth (NB) medium with addition of the respective amino acids. These results were consistent with the growth curve of bacteria (data not shown). The phenomenon of variations in expression of both genes could be explained by autolysis of the bacteria with the subsequent use of the dead biomass. Amino acids might have been released and again converted to putrescine.

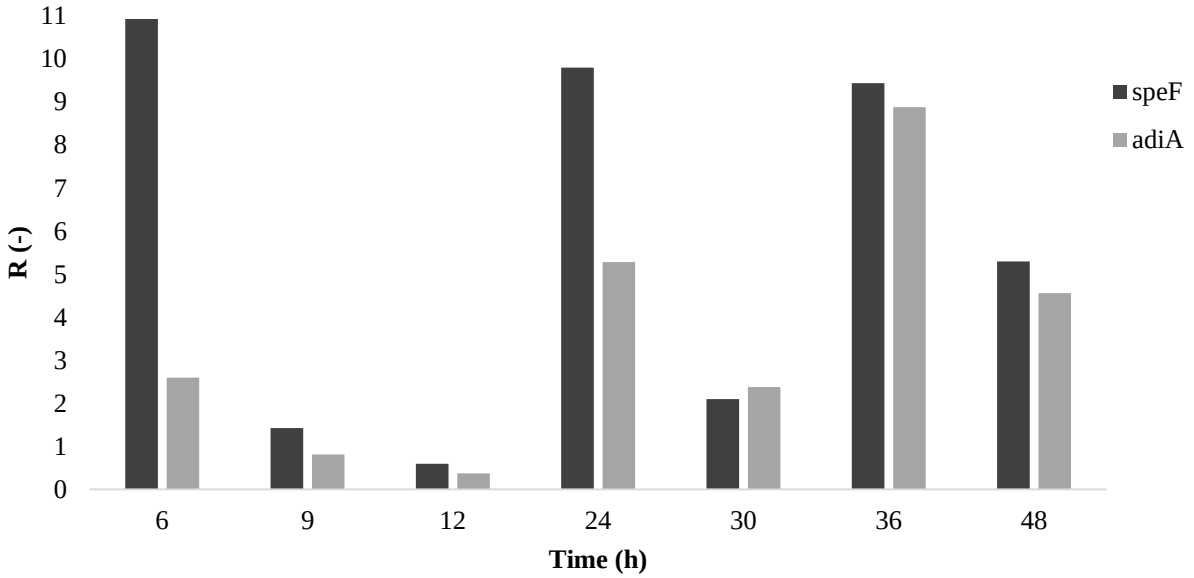

Figure 4 Relative expression of *speF* and *adiA* genes in different culture conditions (*gapA* was used as a housekeeping gene).
Note: The results show the change of gene expression of both enzymes in different time points of cultivation of *E. coli* in NB medium with appropriate amino acids (relative to time 0 h point).

In this study, the development of Real-time PCR method for detection of Gram-negative putrescine-producing bacteria (*E. coli* and *Salmonella enterica*) was described.

In the last few years, a large number of methods have been developed to determine the content of BAs or their producers in food but the complexity of putrescine metabolism is probably the main reason for a relatively small number of studies focussing on the detection and investigation of putrescine metabolism in Gram-negative bacteria.

Only a few PCR methods have been developed to determine the producers of putrescine in a food material. Assays for the detection of a single gene need careful choice of primers, target sequence, and suitable method for detection of the amplified DNA product. Most of the developed primers are designed to detect a mixture of genes encoding ODC in Gram-negative bacteria **(de las Rivas et al., 2005, 2006, 2007)**. Several of these primers were also used in multiplex PCR for simultaneous detection of more decarboxylase genes **(Nannelli et al., 2008; Coton et al., 2010)**.

For typical cycling conditions (Real-time PCR), ideal amplicon size is between 70 and 200 bp. Therefore, a number of primers that can be used for conventional PCR or touchdown-PCR are not suitable for Real-time PCR. However, several Real-time PCR methods for the quantification of putrescine producers (Gram-positive bacteria) were published. **Nannelli et al. (2008) and Ladero et al. (2011)** used *agdif*/*agdir* and *odcf*/*odcr* primers for the quantification of lactic acid bacteria (LAB)-producing putrescine.

Molecular methods, primarily PCR-based ones, have been previously developed for the detection of Gram-negative putrescine-producing bacteria **(Wunderlichova, 2014)**. However, our method constitutes one of the first reported efforts to develop a Real-time PCR assay for Gram-negative putrescine-producing bacteria detection.

CONCLUSION

In this study, the development of an unique Real-time PCR method for rapid and sensitive detection of high putrescine-producing Gram-negative bacteria (*Escherichia coli* and *Salmonella enterica*) were established.

Sets of new primers for the detection of two inducible genes (*speF* and *adiA*) involved in the putrescine metabolism in Gram-negative bacteria were designed and tested. The results have shown that these sets are suitable for Real-time PCR analysis (Tm of primers: 56 – 62 °C; length of amplicons: approximately 200 bp; efficiency of amplification: 95 – 105%).

Based on the results of our experimental study, newly designed sets of primers could be useful tool for relative quantification of *E. coli* in food. Moreover, our team has been working on monitoring of gene expression of *speF* and *adiA* genes in *Salmonella enterica*.

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