Clin. Lab. 2017;63:XXX-XXX

ORIGINAL ARTICLE

The Effects of Insulin and Glucose on Different Characteristics of a UPEC: Alterations in Growth Rate and Expression Levels of some Virulence Genes

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SUMMARY

Background: **Host factors are known to modulate virulence, antibiotic susceptibility, and growth rate of bacteria. The effect of human insulin and glucose on growth rate and expression of virulence genes (*usp*, *sfa/foc*, *cnf1*) of a uropathogenic *E. coli* (UPEC) strain were investigated in this study.**
Methods: ***E. coli*** **C7 was grown in tryptic soy broth (TSB-control) and TSB containing 20 µU/mL insulin, 200 µU/mL insulin, 0.1% glucose, and 200 µU/mL insulin + 0.1% glucose. Growth rates were determined via optical density measurement in a spectrophotometer. Real-time polymerase chain reaction was used to determine the gene expression levels. Statistical analyses were performed via Tukey's post hoc-test.**
Results: **Differences were found to be not statistically significant for bacterial growth rate in TSB and TSB with insulin and/or glucose. The expression levels of all three virulence genes were shown to be reduced significantly in the presence of insulin and/or glucose. The highest degree of repression was observed in 200 µU/mL insulin added to TSB. Also, the repression level of the gene expression was revealed to be reduced in 0.1% glucose supplemented TSB.**
Conclusions: **In the present study, it was shown that insulin and glucose can modulate UPEC's gene expression while the growth rate was not affected.**
(Clin. Lab. 2017;63:xx-xx. DOI: 10.7754/Clin.Lab.2017.170313)

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Manuscript accepted April 26, 2017

KEY WORDS

glucose, insulin, UPEC, gene expression, inter-kingdom communication

INTRODUCTION

During infection, bacteria must withstand stress conditions induced by diverse environmental factors in their host, such as hormones and many other chemicals (sugars, vitamins, ions, pH, bile salts, and microbial metabolites such as indole) which directly affect microbial survival and other biologically important processes, such as growth and gene expression [1-10].
Some microorganisms are known to produce mammalian hormones; they are also able to recognize and re-

spond to eukaryotic hormones (an inter-kingdom communication) [11] which indicates the long coexistence and coevolution of bacteria with humans, animals, and plants [12-14]. As an example, insulin, corticotrophin, somatostatin, progesterone are produced by *E. coli* K12 strain, *Tetrahymena pyriformis, Bacillus subtilis,* and *Trichophyton mentagrophytes*, respectively [11,15,16]. Nowadays, microbial endocrinology, representing the intersection of microbiology, endocrinology, and neurophysiology, provides a basis of a new paradigm to understand the interaction of microbes and their hosts [1]. In several studies, it has been shown that mammalian hormones such as epinephrine (E), norepinephrine (NE), dopamine, dopa, estrogen, progesterone, serotonin, testosterone, and insulin can affect bacterial growth rate, gene expression, pathogenicity (including biofilm formation) and antibiotic susceptibility [4,6,17-24]. On the other hand, not only hormones, but also nutritional factors such as sugars in the bacterial habitat could also affect some biological processes, such as the expression of virulence genes [25,26] and alteration of metabolic pathways [26].

In our study, we aimed to investigate the possible influence of insulin and glucose, two factors of the host, on the growth rate and the expression levels of selected virulence genes [*sfa/foc* (encode fimbrial adhesions SF1C), *cnf 1* (encodes cytotoxic necrotizing factor 1), and *usp* (encodes a uropathogenic-specific protein)] in a uropathogenic *Escherichia coli* (UPEC) strain [27-37].

MATERIALS AND METHODS

Strain

E. coli C7 (positive control for *pap*, *sfa/foc*, *cnf1*, *hly*, and *usp*) strain, kindly provided by Prof. Dr. Shingo Yamamoto from Hyogo College of Medicine, Japan, was used as the standard UPEC strain. Organism was kept at -20°C for Real Time PCR analysis.

Media and solutions

Tryptic soy broth (TSB) with and without insulin (20 µU/mL and 200 µU/mL) and/or glucose (0.1% v/v) were used in the experiments. Insulin provides the storage and cellular uptake of glucose and regulates carbohydrate and lipid metabolism. Moreover, insulin is a highly conserved protein among different taxonomic kingdoms [27-30] and its blood level is approximate 10 µU/mL in a healthy individual. The reabsorption only occurs for 98% of the total insulin while the 2% of it is excreted in urine [31-33]. Two insulin concentrations used in the present study have been determined as 2 and 20 times more than the normal blood levels. The glucose concentration was determined according to its physiological blood level concentration (100 mg/dL) [5, 31-33].

Effects of insulin and glucose on growth rate

E. coli C7 was inoculated into TSB medium alone (as control) or supplemented with 20 µU/mL insulin, 200 µU/mL insulin, 0.1% glucose, and 200 µU/mL insulin + 0.1% glucose to an initial turbidity of ~0.05 OD at 600 nm. Organisms were incubated at 37°C. Growth rate was determined by the measurement of changes in absorbance at 600 nm over time. The measurements of the turbidities were calculated every hour during a 24-hour period. The samples were tested in triplicate and each experiment was performed twice.

Effects of insulin and glucose on gene expressions

Total RNA extraction and cDNA synthesis

E. coli was grown in TSB with or without insulin and/or glucose and incubated for 16 - 24 hours and then used in total RNA extraction. Total RNAs were isolated from 1.5 mL broth culture by using a total RNA isolation kit (Hibrigen, Turkey). The manufacturer's protocol was followed in the extraction process. Qualitative and quantitative analysis of isolated total RNAs were carried out via 1% agarose gel electrophoresis, with a spectrophotometer (Shimadzu, Japan), and using Gel Pro 3.2 software (Exon-Intron, USA). One µg/µL amount of total RNA molecules were used in cDNA synthesis reactions. cDNA synthesis reaction was maintained in a reaction volume of 20 µL using a commercial first strand cDNA synthesis kit (Applied BioSystems, USA). Reaction mix was conducted according to manufacturer's recommendations. High quality (Δ260/280 = 1.9 - 2.0) and quantity (2 - 3 µg/µl) of total RNAs were used in order to obtain cDNAs. Total RNAs with pure characteristics were diluted to 200 ng/µL and then they were used in cDNA synthesis.

Real Time PCR (Q-PCR) and Reverse Transcriptase PCR (RT-PCR) Assays

Real Time PCR Assays

The Eva Green (Bio-Rad, France) based assay was used in gene expression analysis. The expression level of the 16S rRNA gene was used as internal control, and *sfa/foc, cnf,* and *usp* genes associated with virulence of *E. coli* were target genes. Experiments were replicated at least three times, and standard curves were formed by cDNA dilution series of four logs, and E values were calculated. x̄Cp and normalization values were recorded from Light Cycler 480 II software (Roche, Switzerland). Normalization ($2^{-\Delta\Delta CT}$) values were calculated according to formula developed by Livak and Schmittgen (2001). PCRs were conducted in a reaction volume of 24 µL including 1 x Sybr Green I master mix, 5 pmol of each primer (Table 1), cDNA amount corresponding to 1 µg RNA. Cycling conditions were followed as 95°C for 5 minutes, 45 cycles of 95°C for 15 seconds, 58°C for 15 seconds, 72°C for 20 seconds, and final extension at 72°C for 10 minutes. Melting curve analysis was carried out in order to analyze the accuracy of experiments.

RT-PCR Assays

RT-PCR assays were used for qualitative verification of gene expression analysis. RT-PCR was conducted in a 25 µL volume including cDNA amount corresponding to 1 µg of total RNA, 1 x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTPs, 5 pmol of primer, and 1 U of *Taq* DNA polymerase (Thermo, U.S.A.). PCR cycling conditions were performed at 94°C for 5 minutes for predenaturation, 40 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 45 seconds, and at 72°C for 5 minutes for final extension. PCR bands were analyzed via 1.5% agarose gel electrophoresis and gel imagination system Gel Pro Analyzer 3.2 software.

Statistical analysis

Each assay was performed in triplicate, and the results were expressed as the mean of three independent experiments. Statistical analyses of growth rate alterations were determined using one-way ANOVA test. Significant differences of gene expressions were obtained via Tukey's post hoc-test. Variance analysis via ANOVA was carried out using Graph Pad Prism 5.0 software (San Diego, CA, USA).

RESULTS

Effect of insulin and glucose on growth rate

E. coli C7 was grown in supplemented TSB and TSB alone for 24 hours, and the turbidity at 600 nm was measured compared to the control culture. The differences between the growth rates in supplemented TSB and TSB without insulin and/or glucose were found to be statistically not significant ($p = 0.076$, $p > 0.05$). (control: 3.0 ± 0.01; 0.1% glucose: 3.1 ± 0.03; 0.1% glucose + 200 µU/mL insulin: 2.87 ± 0.04; 20 µU/mL insulin: 3.0 ± 0.02, and 200 µU/mL insulin: 3.1 ± 0.03) (Figure1).

Effects of insulin and glucose on gene expressions Real Time PCR assays

In qPCR analysis, the $\bar{x}E$ value was recorded as 1.87 ± 0.07. Mean melting curve scores were 0.98 ± 0.01, 0.95 0 ± 0.04, 0.96 ± 0.04, and 0.93 ± 0.05 for 16S rRNA, *sfa/foc, cnf* and *usp* genes, respectively. These scores showed that qPCR analysis was efficiently carried out. Mean cross point values $\bar{x}Cp$, together with their standard errors, belong to housekeeping, and target genes were recorded in five sample groups (Table 2). While the Cp values for *sfa/foc, cnf,* and *usp* genes ranged from 19.90 ± 0.027 to 24.74 ± 0.12, 20.03 ± 0.004 to 25.27 ± 0.037, and 20.68 ± 0.075 to 26.21 ± 0.093, the $\bar{x}Cp$'s for the internal control gene was found as 12.22 ± 0.933 - 14.43 ± 0.087.

$\bar{x}2^{-\Delta\Delta CT}$ values belonging to the experiment and control groups were listed in Table 3. After normalization, $\bar{x}2^{-\Delta\Delta CT}$ values, which correspond to relative gene expression levels in five samples, ranged from 0.031 ± 0,002 to 0.395 ± 0,055 for target genes, meaning that at least a 40% decrease in gene expression levels were recorded in four experiment groups (bacteria isolated in supplemented TSB) (Table 3).

Analysis of *sfa/foc, cnf,* and *usp* gene expression was carried out with normalization via relative quantification. Fold changes in target genes showed that there was a clear and significant decrease in relative target gene expression in each condition. Increased concentration of insulin resulted in more down regulation of all three target genes (Figure 2). However, co-incubation with insulin and glucose leads to a smaller decrease in gene expression compared to the remaining three experimental conditions. Tukey's multiple comparisons test showed that there were statistically significant differences between TSB (control) and TSB with insulin or glucose for each gene tested ($p < 0.001$).

RT PCR assays

In addition to qPCR analysis, results were confirmed via RT-PCR analysis. The presence of gene expression was confirmed via agarose electrophoresis. One internal and three target gene expressions were verified from experiment and control groups. The 512 bp, 498 bp, 410 bp, and 615 bp long fragments - corresponding to 16S rRNA (data not shown), of *sfa/foc, cnf,* and *usp,* respectively - were amplified from *E. coli* isolate.

DISCUSSION

As mammalian cells coordinate by synthesizing hormones to regulate and maintain own homeostasis, bacteria coordinate community behavior via chemical signaling which are known as quorum sensing (QS) to optimize their resources, defense, survival, virulence, and antibiotic resistance. Gram-negative bacteria use acylated homoserine lactones (Acyl-HSL), while Gram-positive bacteria use processed oligo-peptides as auto inducing peptide (AIP) for communication via the QS signaling pathway. This signaling process not only occurs between bacterial cell to cell but also occurs between bacteria and their hosts which shows inter-kingdom signaling [38-40]. There are various studies showing that mammalian hormones are also responsible for this inter-kingdom signaling [40-42].

Hormones and various nutritional factors, such as glucose, are environmental factors of bacteria in a host. There are many studies showing the role of mammalian hormones on bacterial biological processes such as growth rate and gene expression [4,7,21,41-44]. Effects of catecholamines on bacterial growth could be mediated by accessing sequestered iron. It was shown that the same catathecol moiety found in many siderophores is also present in catecholamines, NE, and E etc., all sharing the ability to facilitate bacterial acquisition of normally inaccessible transferring and lactoferrin bound iron [1]. On the other hand, this is not the only way to influence the growth of bacteria. It was found that catecholamines also induce production of novel autoinduc-

Table 1. Primer sets used in the gene expression analysis and their amplification product sizes.

Gene	Primer Set	Oligonucleotide sequences (5´ - 3´)	Band size (bp)
16S rRNA	*16S rRNA*-f	CCA GGA TTT GAT YMT GGC	532 bp
	16S rRNA-r	GAA GGA GGT GWT CCA DCC	
cnf	*cnf1*-f	AAG ATG GAG TTT CCT ATG CAG GAG	498 bp
	cnf1-r	CAT TCA GAG TCC TGC CCT CAT TAT T	
sfa/foc	*sfa/foc*-f	CTC CGG AGA ACT GGG TGC ATC TCA C	410 bp
	sfa/fo -r	CGG AGG AGT AAT TAC AAA CCT GGC A	
usp	*usp*-f	CGG CTC TTA CAT CGG TGC GTT G	615 bp
	usp-r	GAC ATA TCC AGC CAG CGA GTT C	

Table 2. Crossing point values belonging to four genes of five broth cultures with different content.

x̄Cp values for four genes in four experiment and control set					
Gene	Control	20 µU Insulin	200 µU Insulin	0.1% Glucose	200 µU Insulin + 0.1% Glucose
16S rRNA	14.43 ± 0.087	13.38 ± 0.074	14.29 ± 0.052	13.04 ± 0.047	12.22 ± 0.933
cnf	20.29 ± 0.121	20.45 ± 0.168	24.74 ± 0.12	20.65 ± 0.048	19.90 ± 0.027
sfa/foc	20.36 ± 0.153	20.03 ± 0.004	25.27 ± 0.037	21.34 ± 0.016	20.39 ± 0.042
usp	21.43 ± 0.118	20.80 ± 0.138	26.21 ± 0.093	22.12 ± 0.030	20.68 ± 0.075

Table 3. $^{-\Delta\Delta CT}$ values of fold changes in expressions of three different virulence genes.

Fold Changes in *cnf* expression (%)					
	Control	20 µU Insulin	200 µU Insulin	0.1% Glucose	200 µU Insulin + 0.1% Glucose
$2^{-\Delta\Delta CT}$ values	100.00 ± 0.0	-60.5 ± 0.055 ***	-95.8 ± 0.001 ***	-95.18 ± 0.011 ***	-73.1 ± 0.05 ***
Fold Changes in *sfa/foc* expression (%)					
	Control	20 µU Insulin	200 µU Insulin	0.1% Glucose	200 µU Insulin + 0.1% Glucose
$2^{-\Delta\Delta CT}$ values	100.00 ± 0.0	-92.1 ± 0.003 ***	-96.9 ± 0.002 ***	-97.4 ± 0.005 ***	-70.9 ± 0.022 ***
Fold Changes in *usp* expression (%)					
	Control	20 µU Insulin	200 µU Insulin	0.1% Glucose	200 µU Insulin + 0.1% Glucose
$2^{-\Delta\Delta CT}$ values	100.00 ± 0.0	-89.9 ± 0.002 ***	-96.7 ± 0.009 ***	-95.4 ± 0.011 ***	-66.7 ± 0.053 ***

*** - means significant differences among control and experiment groups as $p < 0.001$.
- - means downregulation in gene expression.

ers, such as norepinephrin-induced autoinducer (NE-AI) and AI-3, which are able to enhance the bacterial growth. The activity of NE-AI functions independently from transferrin and lactoferrin [1,6,7,45,46].

It has been also shown that mammalian/bacterial insulin produced by *E. coli* K12, another group of hormones, can function as a QS compound (homoserine lactones, autoinducer-2, autoinducer-3, and indole etc.) as catecholamines do [11]. As an example, AI-3/ E/NE signaling system is shown to regulate the virulence mechanisms of Enterohemorrhagic *E. coli* (EHEC) strain [39, 47].

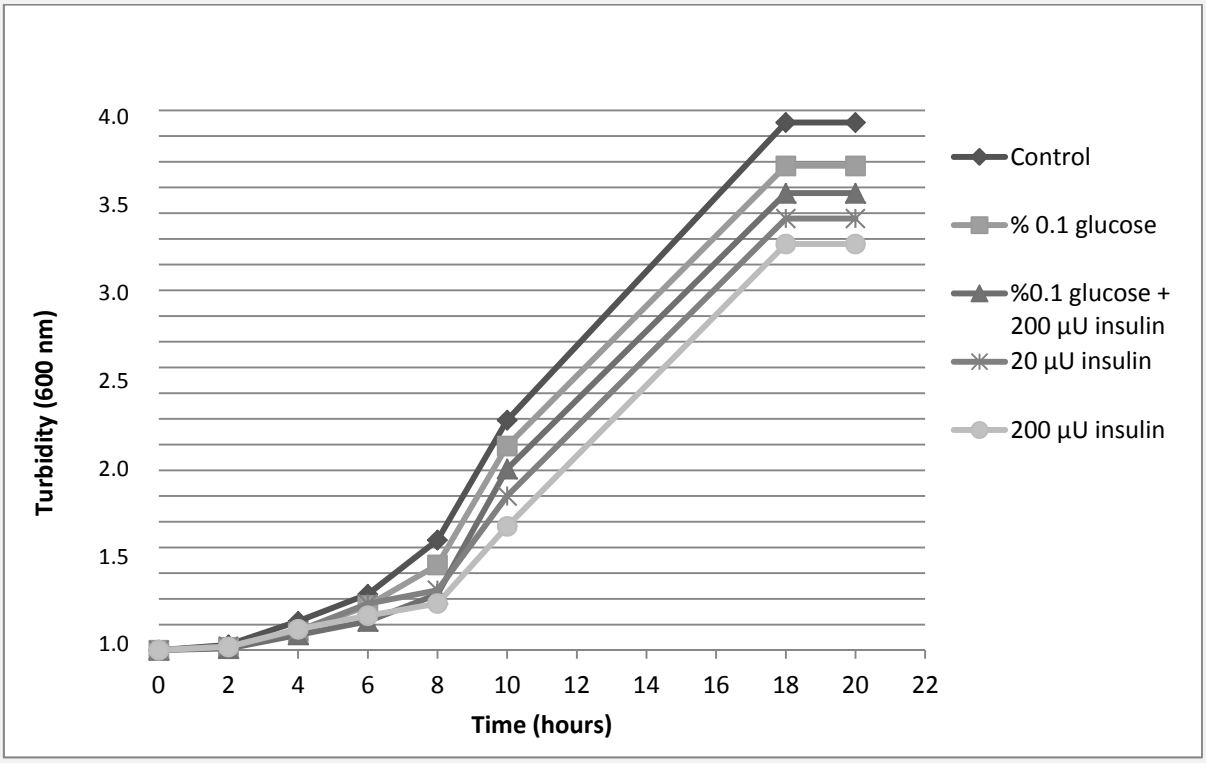

Figure 1. Effect of different insulin concentration and glucose on growth.

***E. coli* C7 was grown in TSB medium at 37 °C for 24 hours in the presence of 20 µU/mL insulin, 200 µU/mL insulin, 0.1% glucose and 200 µU/mL insulin + 0.1% glucose. Data shown mean turbidity ± standard deviation.**

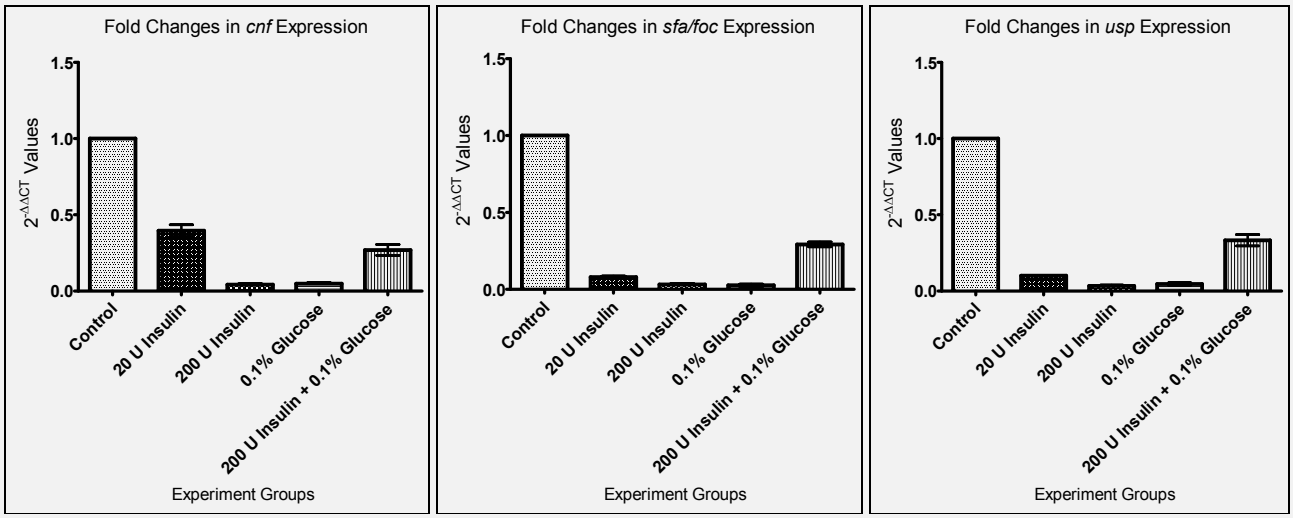

Figure 2. Fold changes in gene expression graphics of three target genes for four experiment groups and the control group.

Numerous studies have reported that the growth rate of bacteria, such as *Pseudomonas aeruginosa* [45], *Bacteroides melaninogenicus* [6], *Campylobacter rectus* [20], *E. coli*, *Staphylococcus aureus*, *Enterococcus faecalis* [4], and *Burkholderia pseudomailei* [48] was altered when exposed to catecholamine, gender hormones, and insulin. Coulanges et al. have suggested that effects of catecholamines on the growth of *Listeria* may be related to its function as a siderophore [49]. This may be related to some mechanisms which steal iron from their host in iron limited conditions. Freestone et al. also showed that catecholamines could regulate the releasing of iron from Tf in a concentration dependent manner [50,51]. It has been reported that gender hormones could also serve as substitutes for vitamin K, and some bacteria (such as *Prevotella intermedius*) uptake progesterone and estradiol which increase their growth rate in the absence of vitamin K. Kornman and Loesche informed that the growth of *Bacteroides melaninogenicus* was shown to be inhibited when exposed to estradiol, in contrast with progesterone, which stimulated the ability of bacterial growth in a concentration dependent manner [6]. Therefore, they emphasized that, according to alterations of endogenous steroid levels, the normal microflora could be affected during pregnancy. There are also studies examining the possible effect of hormones and other molecules found in the human body on the gene expression of bacteria. Nakano et al. have shown that norepinephrine induced the cytotoxic activity and changed expression of TTSS1-related genes of the *Vibrio parahaemolyticus* strain [52]. Anderson and Armstrong have shown that catecholamines can alter *bfeA* gene transcription and increase the growth of *Bordetella* species under inadequate iron conditions [53]. Bansal et al. have reported that E/NE up-regulated EHEC O157:H7 strain colonization and virulence genes expression [54]. Hedge et al. have shown that pyocyanin and elastase genes of *P. aeruginosa* PA14 strain were upregulated when exposed to NE [55]. Sandrini et al. have demonstrated that NE can modify the biofilm formation of *S. pneumoniae* [21]. Intarak et al. have indicated that E can induce the growth, motility, and virulence genes expressions of *Burkholderia pseudomallei* [48]. Conceição et al. have demonstrated that E increased the expressions of motility related genes in *Salmonella Typhimurium* [43]. Some researchers also suggested that human gender hormones could mediate the regulation of bacterial genes via quorum sensing as well as catecholamines. Beury-Cirou et al. have proven that human gender hormones (estrone, estriol, and estradiol) reduced the quorum sensing molecules (Acyl-HSL) and expressions of QS genes (*lasI*, *lasR*, *lasB*, *rhlI, rhlR* and *rhlA*) in *P. aeruginosa* strain [56]. Amirshahi et al. have observed that estradiol and progesterone treatments induced a significant alteration on the gene expressions which are related to stress response in a *Chlamydia trachomatis* strain [57].

A study conducted by Plotkin and Viselli showed that the growth kinetics of *E. coli*, *P. aeruginosa*, *S. aureus,* and *E. faecalis* were affected by the addition of insulin (2, 20, 200, and 400 µU/mL insulin were used), and glucose (1%, 5%) in Mueller-Hinton Broth [4]. Another study performed by Klosowska and Plotkin has also proven that the presence of glucose appears to be a signal for colonization and long-term survival by increasing the expression of adherence mechanisms [5]. Woods et al. reported that growth rate of *B. pseudomailei* in minimal medium containing human recombinant insulin was significantly lower when compared to control cultures containing no insulin [58].

The enhancement of the *E. coli* K12 adherence by the effect of insulin in the presence of glucose (as an energy source) has been shown [4,5]. On the other hand, gene expression data obtained from the present study contradicted with the results of previous studies [4,5]. For instance, investigations focusing on locus of enterocyte effacement (LEE) in an EHEC strain showed that expression of this gene locus was found to be down regulated within the presence of sugars (lactose and glucose) and bile salts, which is consistent with our findings [2,7, 8]. In the present study, the expression of the *sfa/foc* gene was shown to be reduced in the presence of insulin and glucose. However, the adhesion mechanisms of a UPEC strain are multifactorial processes and could be mediated by the presence of different pili (P pilus, Dr adhesions, S pilus, Type 1 pilus, F1C pilus) and hydrophobic interactions. Therefore, it is not possible to expound on the whole virulence mechanism with the expression of a simple virulence gene. Another study focusing on *E. coli* drug exporter genes has shown that indole induces the expression of *acrD*, *acre*, *cusB*, *emrK*, *yceL,* and *mdtE* genes in a dose-dependent manner [3], which confers multidrug resistance.

According to our results, the expression levels of all three virulence genes of C7 UPEC strain grown in TSB with insulin and glucose were shown to be reduced significantly when compared to control media (TSB) without insulin and glucose. The highest degree of repression was observed in the 200 µU/mL insulin (20 times more than the physiological concentration) added medium. Also, repression level of the gene expression was shown to be reduced by the addition of 0.1% glucose in the medium for all three genes investigated. It has been estimated that the alteration up to 22 - 30% in down-regulation of gene expression can be due to the glycosylation of the insulin which leads to the change in its biological activity [59].

It should not be forgotten that bacterial behavior *in vivo* can be different from that *in vitro*. Environmental conditions in a host are different and more complex from those *in vitro* and also change during infectious processses because of many factors such as inflammatory influxes, tissue breakdown, and spread from one anatomic site to another [60,61]. Nowadays, it is well known that hormones are one of the host's factors which determine the environmental conditions of a pathogen. In this context, "Microbial Endocrinology", established by Lyte in 1992, provides insights for understanding the power of

these factors that determine bacteria-host interaction [1, 17].

Acknowledgement:
We would like to thank graduate students of Istanbul Yeni Yuzyil University Department of Molecular Biology and Genetics Neslinur Ozcelik, Ilkay Civelek, Sevde Hasanoglu, Yunus Emre Elma, and Sabahattin Ekin for technical support and we also thank Umit Ture Pekel (MA.), Sevilay Yavuz Cesmeci (MA.) and Kathleen Sullian Erul (BA.) for critical reading.
This research was supported by TUBITAK (The Scientific and Technological Research Council of Turkey). (Project No. 2209-a/2012)

Declaration of Interest:
The authors have nothing to declare.

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