Impact of Diuretics on Metabolic Activity of Urogenital Tract Microbiota in Women

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Abstract: ***Limited knowledge exists about the effects of commonly used diuretic medications on the human normal flora. Thus, we investigated potential stimulatory effects of diuretic drug furosemide on urogenital tract microbiota in women. Three strains of E. coli and C. albicans with different biofilm forming capacities were obtained from female patients diagnosed with urinary tract infections. All tested strains were treated with two different concentrations of furosemide drug, in comparison to non-treated strains as the negative control. At specific time intervals, samples were obtained from growing culture and analyzed for their proliferation rate, aspartyl proteinase excretion and biofilm formation ability. E. coli and C. albicans strains significantly increased their aspartyl proteinase excretion under furosemide treatment. This effect was frequently observed after 16 hours of incubation at 37°C. This drug has also increased the biofilm forming capacities of E. coli and C. albicans strains. Interestingly, both E. coli and C. albicans non-biofilm former strains, gained the capacity of biofilm formation when treated with furosemide at certain concentrations. E. coli control became a weak biofilm former after 48 hours of incubation, while non-biofilm former C. albicans strain became a weak biofilm former in dose-dependent fashion, after 48 hours incubation with furosemide in concentration of 0.1 mg/mL, and after 16 hours of incubation with furosemide in concentration of 0.5 mg/mL. Loop diuretic drug furosemide is able to increase the microbial virulence and turn commensal microbes into opportunistic pathogens. Additionally, the results suggest that enzyme aspartyl proteinase might act as a signal molecule for the biofilm formation, leading to the increased microbial pathogenicity.***

Keywords*: *aspartyl proteinase, biofilm, C. albicans, E. coli, furosemide.

I. INTRODUCTION

Loop diuretics are frequent drugs in the treatment of different conditions which can cause the buildup of fluid in the body, and are the choice of therapy in heart failure, kidney or liver diseases [1]. Types of loop diuretics are furosemide, bumetanide, and torsemide, and they are quickly absorbed after oral administration, within 0.5-2 hours (2). Diuretic drugs have different pharmacokinetics and pharmacodynamics and can even cause anaphylactic reactions in some patients. However, that is not an obstacle for many clinicians to use them in some stereotyped manner [2,3]. There are several studies which indicate a tight connection between diuretics use and their effects on gastrointestinal microbiota, including their contribution to the spontaneous bacterial peritonitis occurrence in cirrhotic patients [4]. However, there are no published data about potential effects of diuretics on microbiota of urogenital tract. Since the administration of diuretics can cause gender-related differences by which women need lower doses of diuretics [5,6], we focused our study on microbial strains derived from female patients affected with urinary tract infections (UTIs) as more dominant type of infections in this population [7,8]. *Escherichia coli* (*E. coli*) is one of the leading cause of UTIs, which does not only colonize vaginal epithelium but has the ability to reside inside the vaginal cells as well [9]. In some studies it was reported that *E. coli* under influence of certain drugs can change its metabolic activities and start producing virulent factors in form of enzymes or extracellular proteins such as α-hemolysin [10,11]. On the other side, also *Candida* species, including *Candida albicans*, are uropathogenes causing serious infections of urinary tract [12]. Considering emerging studies that demonstrate indirect roles of ordinarily used medications in clinical settings on normal flora, we intended to explore the potential effect of furosemide on urogenital tract microbiota in women. Additional aim was to describe the potential microbial metabolic and structural adaptations.

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II. METHODS

A. Microbial strains

Three strains of *Escherichia coli* (*E. coli 8, E. coli 9, E. coli control*) and three strains of *Candida albicans* (*C. albicans 3 and C. albicans 4, C. albicans 6*) were obtained from samples of hospitalized female patients diagnosed with UTIs using standard microbiological isolation and identification approached based on colony morphology on blood and MacConkey agar (Sigma-Aldrich, Vienna, Austria).

E. coli 8, E. coli 9 and C. albicans 3, C. albicans 4 had a strong biofilm forming capacity. *E. coli control* and *C. albicans 6* are strains without biofilm forming capacity. Additionally, all strains of these microorganisms without furosemide supplementation were used as negative control. *E. coli* strains were grown in trypticase soy broth (TSB) medium (Sigma-Aldrich, Vienna, Austria), while *C. albicans* strains were grown in Sabouraud 2%-glucose broth (Sigma-Aldrich, Vienna, Austria). The microbes were cultured at standard aerobic conditions at 37ºC.

B. Generation of growth curves

10 µL of over-night culture of every *E. coli* and *C. albicans* strain, were inoculated in sterile plastic 15 mL tubes containing 3 mL of respective growth media. The cultures were growing at 37ºC for different incubation times: 0h, 8h, 16h, 24h and 48h. Prior to incubation, furosemide of two different concentrations, 0.1 mg/mL and 0.5 mg/mL, was added into each test tube containing specific microorganisms. Optical density for each test sample and each incubation time was measured at 600 nm. The obtained values were used for generation of growth curves.

C. Aspartyl proteinase assay

Following the incubation, 100 µL of microbial culture was pooled out and mixed with 400 µL of 1% bovine serum albumin (BSA; Sigma-Aldrich, Vienna, Austria) and incubated at 37ºC for 30 minutes. The reaction was stopped by adding 1 mL of 10% trifluoroacetic acid (Sigma-Aldrich, Vienna, Austria). Both, 1% BSA and 10% trifluoroacetic acid, were previously diluted in 0.1 M citrate buffer (pH 3.5).

Afterwards, the samples were centrifugated for 10 minutes at 1000 rpm. The resulting supernatant containing aspartyl proteinase was isolated.

D. Determination of aspartyl proteinase concentration

The concentration of aspartyl proteinase was evaluated with spectrophotometer (Multiskan GO, Thermo Scientific, Waltham, Massachusetts, USA).

Absorbance of test samples was measured at 260 nm and 280 nm, according to Warburg Christian. The final calculations were carried out according to the following equation: mg of protein/mL = 1.55 x A280 – 0.76 x A260.

E. Biofilm forming capacity test

Test tubes containing the microorganisms whose growth has been stopped at different incubation times were pooled out from expanding cultures and washed with phosphate buffered saline (PBS), pH 7.5.

Afterwards, the tubes were inverted for about 20 minutes to air-dry. 1 mL of 0.1% crystal violet (Sigma-Aldrich, Vienna, Austria) was then added. The tubes were cautiously rotated to assure crystal violet reaches all cells attached to the test tube walls.

Following the 5 minutes incubation, the crystal violet was removed and tubes were washed with dH2O. Test tubes were finally analyzed for the biofilm formation. Samples were classified as weak, moderate and strong biofilm formers based on the shade of the film and sum of the cells.

F. Statistical analysis

Depicted data are disclosed as mean ± standard deviation (SD). Datasets are analyzed using student's t-test and one-way ANOVA corrected with Bonferroni post hoc test. A p-value less than 0.05 was considered statistically significant. Results were depicted in tables and graphs created by Microsoft Office (2019, Microsoft, Redmond, Washington, USA).

III. RESULTS

A. Effects of furosemide administration of microbial proliferation

Amongst the most crucial features of microorganisms is their competence to grow and replicate. They do that in various environments. Increased proliferation is possible only if environment is supportive, rich in food and lacks inhibitory substances.

Logarithmic phase of growth for all three strains of *E. coli* was clearly established after 8 hours of incubation at 37ºC. In the following incubation periods, proliferation trend remained similar for the negative control and treated samples (Figure 1). Similar pattern was observed in two *C. albicans* strains. *C. albicans* 3 exhibited the slowest growth of all tested strains. There were no differences in the growth pattern among the tested groups.

Likewise, growing *C. albicans* 4 strain reached the logarithmic phase after 8 hours of incubation and continued to proliferate similarly throughout the later incubation periods. Interestingly, *C. albicans* 6 without treatment showed a higher proliferation in the earlier time points, after 8 and 16 hours of incubation.

However, *C. albicans* 6 under furosemide of both concentrations started to proliferate more at 24 hours, while that difference was significantly higher at 48 hours of incubation (Figure 2).

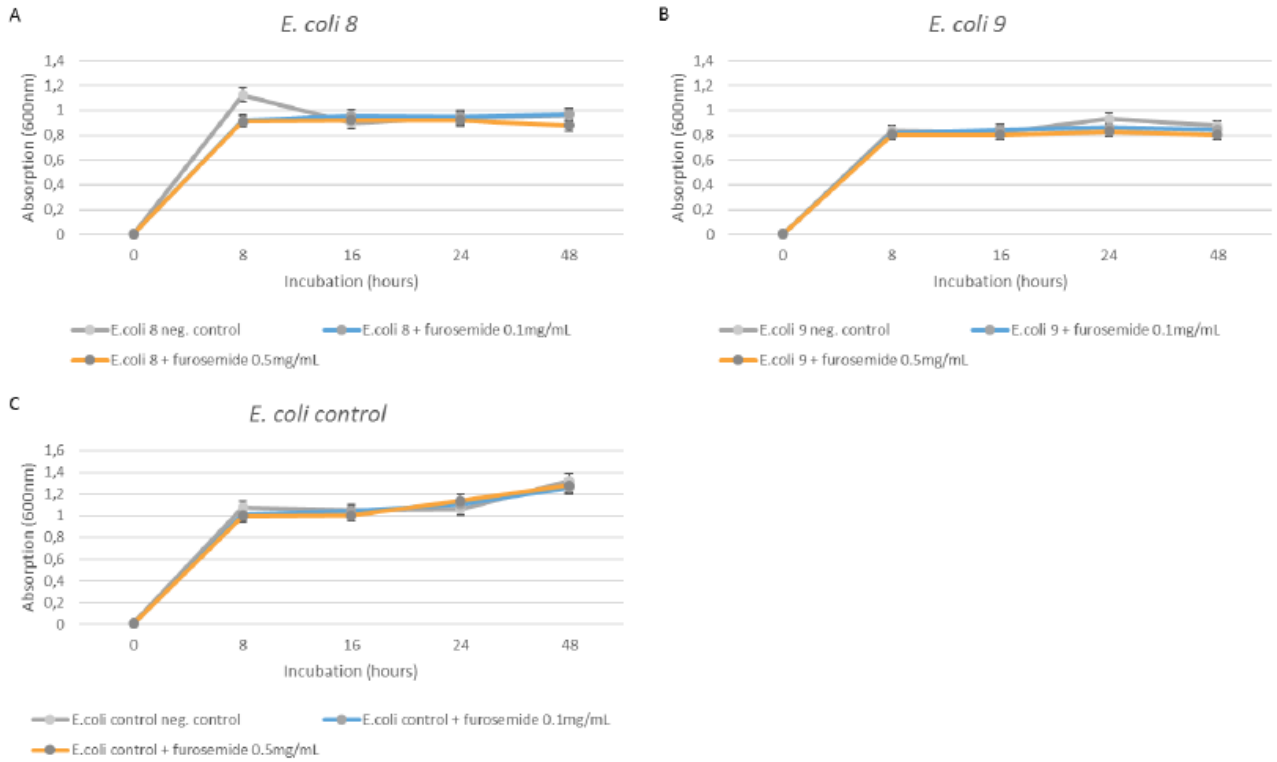

Figure 1 (A, B, C). Effect of furosemide on *E. coli* proliferation. There were no differences in the growth pattern among the tested groups. Logarithmic phase of growth was established after 8 hours. In the following incubation periods, proliferation rate remained similar in all groups.

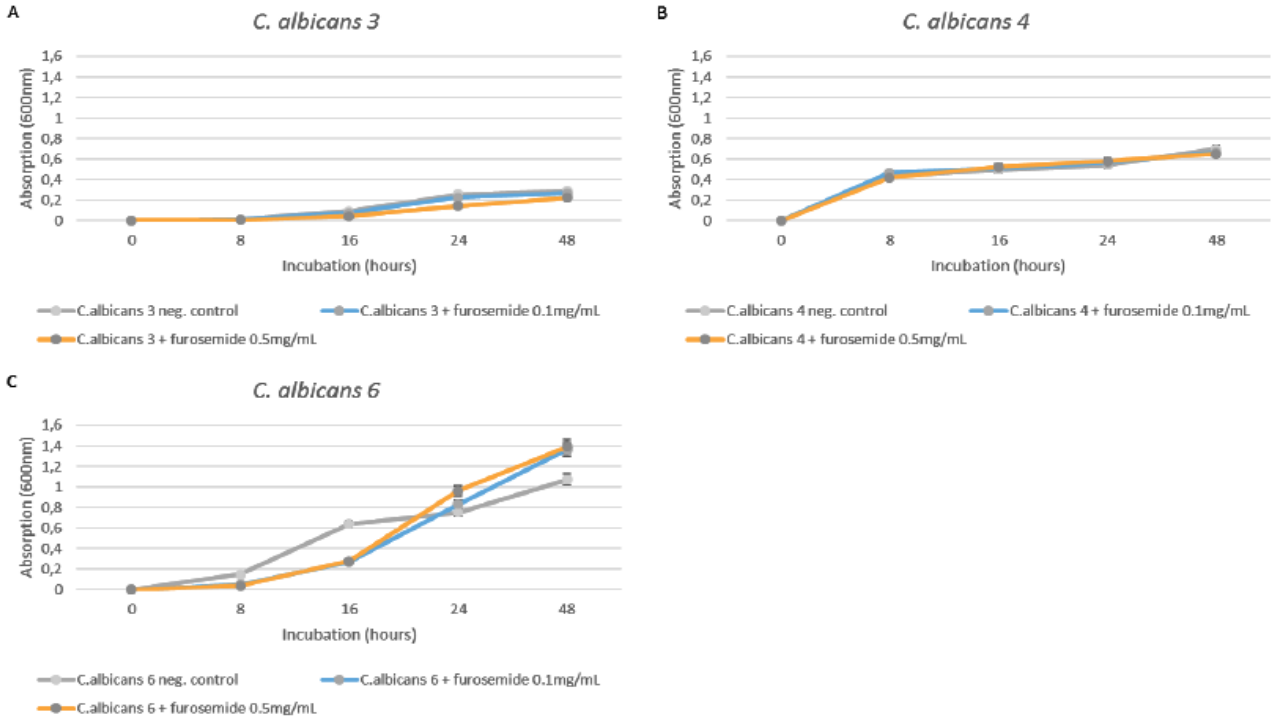

Figure 2 (A, B, C). Effect of furosemide on *C. albicans* proliferation. A: *C. albicans* 3 exhibited the slowest growth, with no differences in three tested groups. B: *C. albicans* 4 strain reached the logarithmic phase after 8 hours of incubation and continued to proliferate similarly throughout the later incubation periods. C: Non-treated *C. albicans* 6 exhibited higher proliferation in earlier time points. However, furosemide increased the proliferation of *C. albicans* 6 after 24 hours and 48 hours of incubation.

B. Effects of furosemide administration on microbial aspartyl proteinase excretion

Administration of furosemide in two dosing concentrations of 0.1 mg/mL and 0.5 mg/mL enhanced metabolic activity of some *E. coli* and *C. albicans* strains. Increased aspartyl proteinase excretion was observed frequently after 16 hours of incubation, while some differences may be explained with intrinsic characteristics of the tested strains. Furosemide had a stimulatory effect on *E. coli* 8 metabolic activity at two incubation times. Significant excretion was observed after 16 and 48 hours of incubation with concentration of 0.5 mg/mL and 0.1 mg/mL, respectively. The similar results were observed in *E. coli* 9 strain. Dose-dependent effect on aspartyl proteinase excretion was observed after 16 hours of incubation. Metabolic activity of *E. coli control* was significantly altered by furosemide after 8 and 16 hours of incubation (Figure 3). However, furosemide exhibited more striking metabolic changes on *C. albicans* strains. Furosemide increased the aspartyl proteinase excretion in *C. albicans 4* throughout the entire incubation periods in dose-dependent fashion. Nonetheless, significant effect was observed after 48 hours of incubation in *C. albicans 3* strain. Interestingly, the metabolism of non-biofilm former *C. albicans* 6 was upregulated under furosemide treatment after 16 hours of incubation and persisted during the entire experiment. This stimulatory effect was also noted in dose-depended fashion (Figure 4).

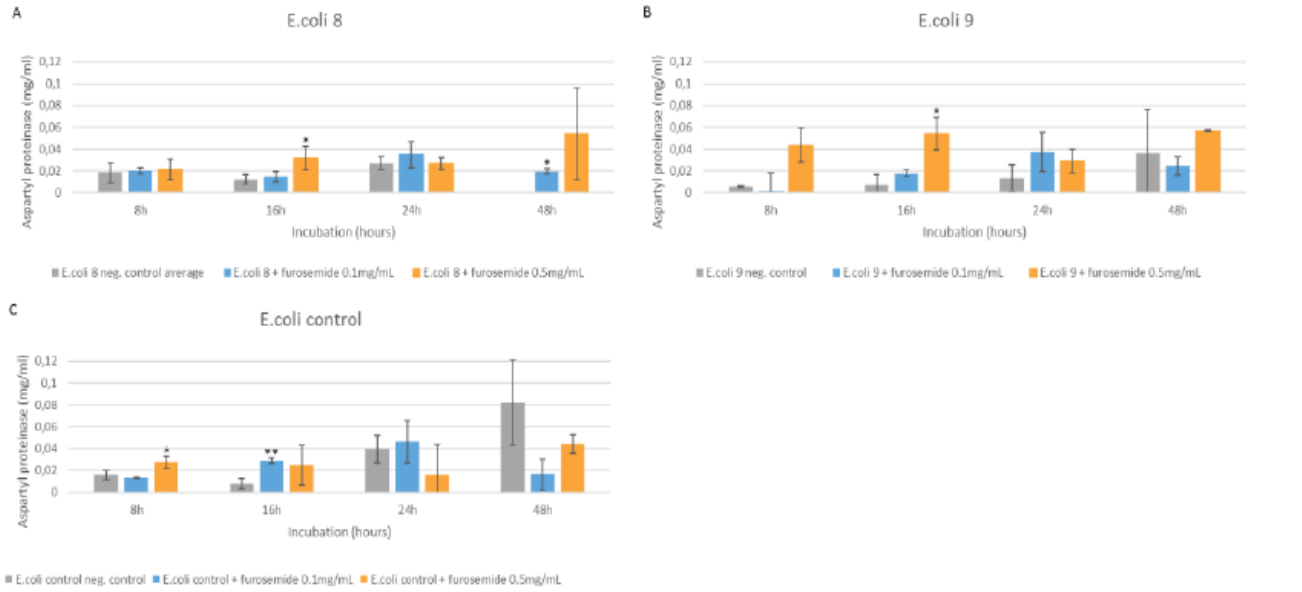

Figure 3 (A, B, C). Excretion of aspartyl proteinase was increased in *E. coli* strains at different incubation times under treatment of furosemide.

A: Furosemide (0.5 mg/mL) increased aspartyl proteinase excretion in *E. coli* 8 after 16 hours of incubation. B: The similar results were observed for *E. coli* 9 strain. C: Metabolic activity of *E. coli control* under furosemide treatment increased after 8h (0.5 mg/mL) and 16h (0.1 mg/mL). Experiments were performed in triplicates. Mean ± SD are plotted in the bar charts, with asterisks that indicate levels of statistical significance (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

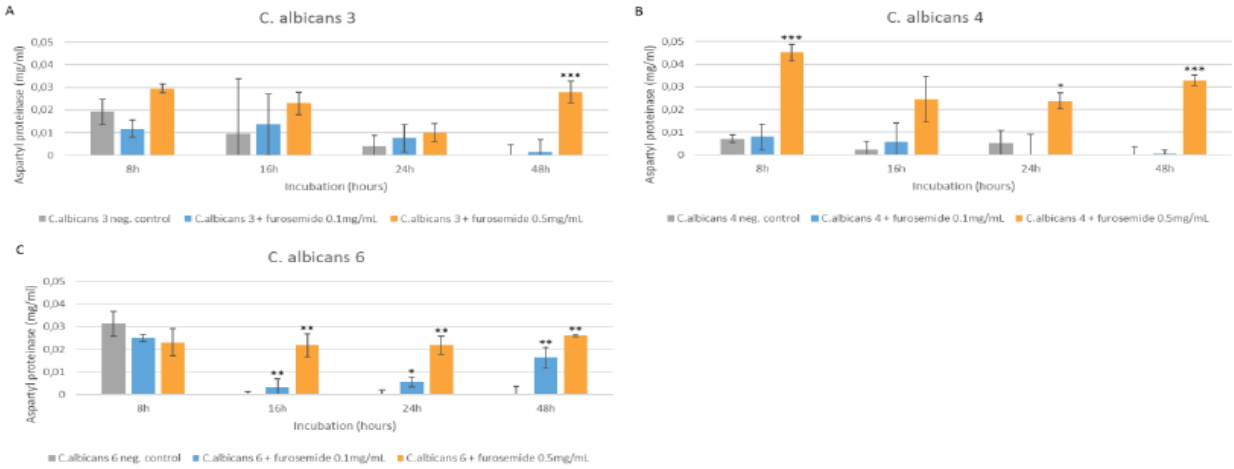

Figure 4 (A, B, C). Excretion of aspartyl proteinase was increased in *C. albicans* strains at different incubation times under treatment of furosemide.

A: Significant increase of aspartyl proteinase excretion in *C. albicans* 3 observed after 48 hours of incubation with furosemide. B: Enhanced excretion in *C. albicans 4* throughout the entire
incubation in dose-dependent fashion. C: Furosemide-induced metabolic upregulation of *C. albicans* 6 started after 16 hours and persisted throughout the remaining incubation. Experiments were performed in triplicates. Mean ± SD are plotted in the bar charts, with asterisks that indicate levels of statistical significance (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

C. Effects of furosemide of biofilm formation

Through metabolic upregulation, drug furosemide may also increase the biofilm forming capacities of *E. coli* and *C. albicans* strains. For *C. albicans* 4 strain, a weak biofilm formation was observed after 16 hours of incubation. However, this strain formed the same biofilm earlier with furosemide, after 8 hours of incubation. In addition, this strain became a moderate biofilm former after 24 and 48 hours of incubation with furosemide of 0,5 mg/mL. Non-biofilm former, *C. albicans* 6 gained that ability after 48 hours with furosemide of lower concentration, and after 16 hours of incubation with furosemide of the higher concentration (Table 1). Similar dose-dependent effect was observed for *E. coli* strains. *E. coli* 9 and *E. coli* 8 formed weak biofilm after 8 and 16 hours of incubation, respectively. The moderate biofilm formation of *E. coli* 8 was observed after 48 hours with furosemide of 0.1 mg/mL concentration, and after 24 hours of incubation with furosemide of higher, 0.5 mg/mL, concentration. For *E. coli* 9 strain treated with furosemide of higher concentration, the moderate biofilm formation was evident after 48 hours of incubation compared to non-treatment and furosemide of lower concentration groups that remained to produce weak biofilm. Non-biofilm former, *E. coli control* became weak biofilm former after 48 hours of incubation at 37°C (Table 2).

Table 1. Biofilm forming capacity of *C. albicans* strains

Biofilm formation	8h	16h	24h	48h
C. albicans 3	-	-	+	+
C. albicans 3 + furosemide 0.1 mg/mL	-	-	+	+
C. albicans 3 + furosemide 0.5 mg/mL	-	-	+	-
C. albicans 4	-	+	+	+
C. albicans 4 + furosemide 0.1 mg/mL	+	+	+	+
C. albicans 4 + furosemide 0.5 mg/mL	+	+	++	++
C. albicans 6	-	-	-	-
C. albicans 6 + furosemide 0.1 mg/mL	-	-	-	+
C. albicans 6 + furosemide 0.5 mg/mL	-	+	+	+

Legend: (-) no biofilm; (+) weak biofilm; (++) moderate biofilm; (+++) strong biofilm.

Table 2. Biofilm forming capacity of *E. coli* strains

Biofilm formation	8h	16h	24h	48h
E. coli 8	-	+	+	+
E. coli 8 + furosemide 0.1 mg/mL	-	+	+	++
E. coli 8 + furosemide 0.5 mg/mL	-	+	++	++
E. coli 9	+	+	+	+
E. coli 9+ furosemide 0.1 mg/mL	-	+	+	+
E. coli 9 + furosemide 0.5 mg/mL	-	+	+	++
E. coli control	-	-	-	-
E. coli control + furosemide 0.1 mg/mL	-	-	-	++
E. coli control + furosemide 0.5 mg/mL	-	-	-	+

Legend: (-) no biofilm; (+) weak biofilm; (++) moderate biofilm; (+++) strong biofilm.

IV. DISCUSSION

E. coli is recognized as the most frequent cause of UTIs, while *C. albicans* is the most prevalent fungal urogenital pathogen [13]. Both primarily colonize gastrointestinal tract, but they may sporadically migrate to other systems as well. Most infections are endogenous, meaning that *E. coli* and *C. albicans*, constituents of normal microbial flora, take the new opportunity of dysbiosis as the advantage to cause infections. These situations are increased in hospitalized and immunocompromised patients [14–17]. Moreover, numerous studies associate increased pathogenicity of microbiota with certain hormonal influences and drug therapies, including antibiotics [18,19]. When found in a new environment, microbes may acquire features allowing them to survive and eventually to progress. A special adaptation is microbial organization into communities called biofilm. Biofilm provides microbes with numerous benefits. It allows them to colonize various surfaces, and protects them from the environment, including defense against host immune system and antimicrobial therapeutics [20,21]. In addition, microbes produce proteases, a huge group of enzymes playing important roles in microbial life, stress response and pathogenicity [22]. They also represent an important protein component of the biofilm [23]. Extracellular proteases are secreted principally to provide food for the cells, but microbes may use them in the processes of cell colonization, penetration and antimicrobial peptide cleavage. One of the most significant extracellular proteases is secreted aspartyl proteinase [24]. In *C. albicans*, aspartyl proteinase production is linked to several virulence features such as hyphal formation, adhesion, and phenotypic switching [25,26]. It has also been reported a higher aspartyl proteinase excretion in virulent *Candida* species in comparison to less virulent species [27]. Additional study has shown the importance of aspartyl proteinase for microbial cell-cell contact and biofilm formation [28].

Several types of loop diuretics exist, along with furosemide, bumetanide, torsemide, and ethacrynic acid. They are characterized with different pharmacokinetics and pharmacodynamics. In general, they have rapid absorption after oral administration, achieving maximum concentrations within half to two hours. Oral bioavailability of torsemide and bumetanide surpass 80%, compared to 50% of that for furosemide. However, gastrointestinal absorption of furosemide is slower than its elimination, making its duration of action longer [2]. There are several studies demonstrating a tight connection between diuretics use and their effect on microbiota, including spontaneous bacterial peritonitis episodes in cirrhotic patients and even anaphylactic reactions in some patients [3,4]. Nevertheless, there are no available studies about potential effects of diuretics on microbiota of urogenital tract. Since the administration of diuretics can cause gender-related differences by which women need lower doses of diuretics [5,6], we focused our study on microbial strains isolated from female patients suffering from UTI as more dominant type of infections in this population [7,8].We investigated the effect of furosemide on microbial growth,

metabolism and biofilm formation. Furosemide, as a member of loop diuretics, is commonly used drug in clinical settings, primarily in the treatment of high blood pressure, edema and several other cardiovascular, kidney and liver diseases [29].In developed countries, cardiovascular diseases are among the most common ones and their incidence is constantly increasing [30,31]. Consequently, a number of people using furosemide is on the rise. Therefore, a potential stimulatory effect of this drug on microbial flora would represent interesting translational information. Indeed, our results suggest that furosemide is able to increase the microbial virulence and even turn commensal microbes into opportunistic pathogens. Additionally, the results propose that virulent enzyme aspartyl proteinase might act as a signal molecule for the biofilm formation and thus help microbes to increase their chances of survival and progression. *E. coli* strains increased their aspartyl proteinase excretion under furosemide treatment. This effect was notably observed after 16 hours of incubation at 37ºC (Figure 3). Similarly, all tested *C. albicans* strains were metabolically upregulated under furosemide treatment (Figure 4). This drug has also increased the biofilm forming capacities of *E. coli* and *C. albicans* strains. Interestingly, non-biofilm former strains, *E. coli control* and *C. albicans* 6 gained the capacity of biofilm formation when treated with furosemide. *E. coli control* became a weak biofilm former after 48 hours of incubation (Table 1). *C. albicans* 6 became a weak biofilm former in dose-dependent fashion, after 48 hours incubation with furosemide in concentration of 0.1 mg/mL and after 16 hours of incubation with furosemide in concentration of 0.5 mg/mL (Table 2). To our knowledge, this represents the first association of the loop diuretic drug with increased microbial virulence. Similar findings were observed in another study on insulin and diabetes-related *E. coli* pathogenesis [10]. Moreover, several drugs have been associated with increased aspartyl proteinase excretion in *C. albicans*, including analgesic medications paracetamol, analgin, brufen and difen, as well as iron supplements. Interestingly, antibiotic ampicillin stimulates the growth of *C. albicans in vitro*, but it is not capable of inducing aspartyl proteinase excretion [32].

V. CONCLUSION

Since secreted aspartyl proteinase serves as one of the key virulence factors involved in the microbial pathogenesis, they represent a potential target for new antimicrobial drug development. Moreover, enzymatic activity of secreted aspartyl proteinase may be utilized as a component of biosensor chips used for microbial detection. This detection of microbial-specific enzymatic activity represents a rapid and inexpensive screening method using very small amounts of clinical samples [33,34]. In conclusion, our results show that drug furosemide, in addition to its primary effects for which it is prescribed, may act as the signal molecule for the biofilm formation, and thus, increase the microbial pathogenicity. These findings also indicate additional demand for research on microbial metabolic activity and their proteins as potential virulent factors and modulators of microbial pathogenicity.

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