



## ANTIBACTERIAL ACTIVITIES OF METABOLITES FROM CORYNEBACTERIUM SPP. STRAINS ISOLATED FROM THE REPRODUCTIVE TRACT OF A HEALTHY WOMAN AGAINST HUMAN PATHOGENIC BACTERIA

**IRINA V. GLADYSHEVA\*, SERGEY V. CHERKASOV,  
YURIY A. KHLOPKO**

*Institute for Cellular and Intracellular Symbiosis,  
Ural Branch of Russian Academy of Sciences,  
Orenburg, Russia.*

### ABSTRACT

The genus *Corynebacterium* spp., also known as "diphtheroids" or "coryneform" includes diverse group of microorganisms inhabiting different biotopes of the human body. In this work we have studied the influence of metabolites from *Corynebacterium* spp. strains isolated from the reproductive tract of a healthy woman on growth, biofilm formation and on developed biofilms (24h grown) *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. aureus*. We found that all human bacterial pathogens showed slower growth in the presence of metabolites of *Corynebacterium*. All the isolated *Corynebacterium* strains to inhibit biofilm formation and destroy developed biofilms (24h grown) *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. aureus*. The obtained data reveals the important role of *Corynebacteria* in protecting the vaginal biotope from infection in those women who do not have *Lactobacilli* and suggest a probiotic potential of these *Corynebacterium* strains as an anti-bacterial agent in the vagina.

**KEYWORDS:** *Corynebacterium*, antibacterial activity, antibiofilm activity, pathogenic bacteria



**IRINA V. GLADYSHEVA\***

**Institute for Cellular and Intracellular Symbiosis, Ural Branch of Russian  
Academy of Sciences, Orenburg, Russia.**

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## INTRODUCTION

The genus *Corynebacterium* belongs to the family *Corynebacteriaceae* of the phylum *Actinobacteria* and includes straight or slightly curved rod-shaped, Gram-positive, catalase-positive, non-spore-forming, non-motile micro(aerobic) microorganisms.<sup>1</sup> Among *Corynebacteria*, the most significant human pathogen is *Corynebacterium diphtheriae*, which causes diphtheria worldwide.<sup>2</sup> In addition, there are nondiphtheriae *Corynebacteria*, major components of the normal flora of human skin and mucous membranes, are commonly isolated from clinical specimens. It is widely known that nondiphtheriae *Corynebacteria* associated with cases of endocarditis, meningitis, arthritis, sinusitis, skin wounds and intrauterine infections in both immunocompromised and immunocompetent patients.<sup>3</sup> At the same time, several authors have described the important role the participation of particular strains of nondiphtheriae *Corynebacteria* in the protection of the different biotopes of the human body from infections. Uehara *et al.*, reported the elimination of nasal *Staphylococcus aureus* by application of *Corynebacterium* sp.<sup>4</sup> Bomar *et al.*, showed that *Corynebacterium accolens*, inhibits growth of *Streptococcus pneumoniae* by releasing antibacterial free fatty acids from host skin surface triacylglycerols.<sup>5</sup> Wysocki *et al.* demonstrated that lipophilic species of *Corynebacterium* inhabiting skin as residents produces substances that can regulate the composition of natural flora. In the course of research an inhibiting substance (BLIS) was isolated with its evident effect on *S. aureus*, *S. epidermidis*, *C. diphtheriae* and *Propionibacterium* spp.<sup>6</sup> Matthew M. Ramsey *et al.* found that *S. aureus* interactions with *Corynebacterium* spp. diminish *S. aureus* virulence.<sup>7</sup> In vaginal biotope nondiphtheriae *Corynebacteria* are found in women regardless of age and microecological status of the biotope.<sup>8,9,10</sup> In girls before puberty nondiphtheriae *Corynebacteria* along with *Staphylococcus epidermidis* constitute the main part – 80% of the vaginal microbiota.<sup>11</sup> Their number also is increased in pregnant and postpartum women.<sup>12</sup> Previously, we carried out a pilot study on intermicrobial interaction *Corynebacteria* and *Lactobacilli* isolated from the reproductive tract of women. We found that the metabolites of *Corynebacteria* greatly increased antagonistic activity of the peroxide producing *Lactobacilli* against pathogenic and opportunistic microorganisms.<sup>13</sup> These results show an important role nondiphtheriae *Corynebacteria* in the protection of the female reproductive tract against infection. However, research on their own antagonistic activity of *Corynebacteria* isolated from the reproductive tract of women to pathogenic and opportunistic microorganisms has not yet been conducted. Therefore, the aim of the current study is to determine the antibacterial activity of metabolites from *Corynebacterium* spp. against four important human bacterial pathogens.

## MATERIALS AND METHODS

### *Isolation and identification of Corynebacterium*

This study was conducted in the Laboratory of study mechanism formation microbiocenosis of human,

Institute for Cellular and Intracellular Symbiosis UrB RAS, Orenburg, Russia. The four *Corynebacterium* strains used in this study were previously isolated from vaginal smears of healthy women and were previously characterized by their properties increased the antagonistic activity of peroxide producing *Lactobacilli*. These strains are *C. amycolatum* ICIS 9, *C. amycolatum* ICIS 53, *C. minutissimum* ICIS 5 and *C. xerosis* ICIS 99.<sup>13</sup> *Corynebacteria* were identified on a set of morphological, cultural and biochemical properties (API Coryne 2.0 biochemical identification system (bioMérieux, France) according to manufacturer's instructions).

### *Bacterial strains*

Four human bacterial pathogens obtained from microbial culture collection, Institute for Cellular and Intracellular Symbiosis UrB RAS, Orenburg, Russia. These strains are *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. For the experiment, the strains were prepared according to Prabakaran and Kalimuthu (2013).<sup>14</sup>

### *Preparation of metabolites*

*Corynebacterium* strains was grown in tryptic soy broth (TSB) for 24 h at 37°C. Metabolite was obtained by centrifuging the culture (10000×g, 20 min, 4°C), purified by filtration (0.22 µm, Millipore Nihon, USA).

### *Inhibitory effect of metabolites from Corynebacterium on human bacterial pathogens*

The direct antagonism metabolites from *Corynebacterium* strains against human bacterial pathogens was monitored by turbidimetry. Aliquot of 100 µL of preinoculated pathogens (about 10<sup>6</sup> CFU/ml) in TSB broth was transferred to each well of 96 well polystyrene plates, the same volume of metabolites from *Corynebacterium* strains added to each well in replica and incubated at 37°C for 7h. Control wells contained culture medium and the tested strain without adding metabolites. Samples were taken at intervals of 1 hour, and their optical density (OD) was determined using a microplate photometer Thermo Scientific Multiskan FC at 600 nm.

### *Inhibitory effects of metabolites from Corynebacterium on biofilm formation of human bacterial pathogens*

Inhibitory effect of metabolites from *Corynebacterium* strains isolates against biofilm formation by human bacterial pathogens was determined using the commonly used 96 wells polystyrene plate method and was performed according to O'Toole and Kolter (1998)<sup>15</sup> with slight modifications. Aliquot of 100 µL of preinoculated pathogens (about 10<sup>6</sup> CFU/ml) in TSB broth was transferred to each well of 96 well plates, the same volume of metabolites from *Corynebacterium* strains added to each well. Control wells contained culture medium and the tested strain without adding metabolites. Further, the experiment was performed according to the procedure described by Wojnicz and Tichaczek-Goska (2013)<sup>16</sup>. After incubation at 37°C for 24 h the wells were rinsed thoroughly with phosphate buffered saline (PBS) in order to remove nonadherent bacteria. Bacterial cells bound to the walls of the wells were stained with 1% (w/v) crystal violet (Sigma) for 15

min, and then rinsed thoroughly with PBS. Afterwards the dye bound to the adherent bacterial cells was resolubilized with 95% (v/v) ethanol. The OD of each well was measured at 570 nm using a microplate photometer. The results were expressed as percentage of biofilm inhibition according to the following equation:

$$\text{Percentage inhibition} = (A1-A2)/A1 \times 100$$

where A1— the absorbance of the control group (without bacterial metabolites).

A2— the absorbance of the treatment group (with media containing bacterial metabolites).

#### **The influence of metabolites from *Corynebacterium* strains on pre-formed biofilms**

The influence of metabolites from *Corynebacterium* strains on pre-formed biofilms quantified according to the procedure described by Dalili *et al.*, (2015)<sup>17</sup> with slight modifications. Aliquot of 200  $\mu$ L of bacterial pathogens (about  $10^6$  CFU/ml) in TSB broth was transferred to wells of 96 well plates. After incubation for 24h, the media were poured out from the wells and washed with sterile PBS buffer. Then, 200  $\mu$ L of metabolites was added to each well following the incubation for 24 h. The culture without metabolites was served as control. The percentage of biofilms inhibition at metabolites for each microorganism was calculated according to the above equation.

#### **STATISTICAL ANALYSIS**

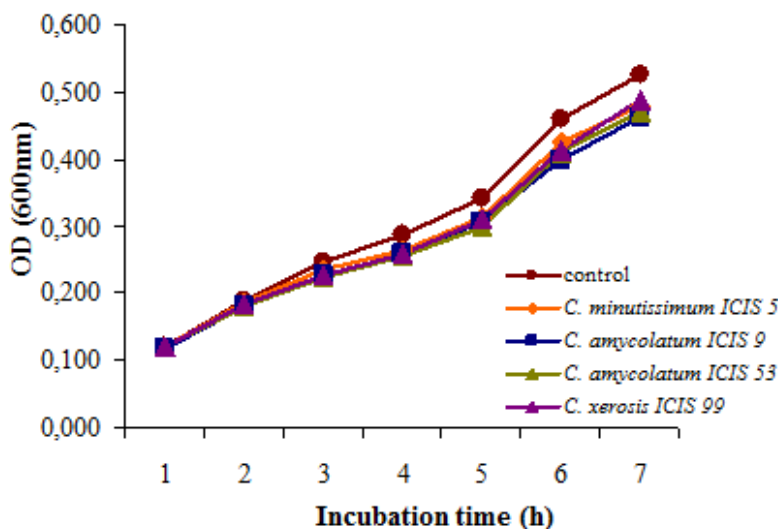
Statistical analysis was done using the statistical software "STATISTICA 10.0". Differences were considered significant when  $P < 0.05$ .

#### **RESULTS**

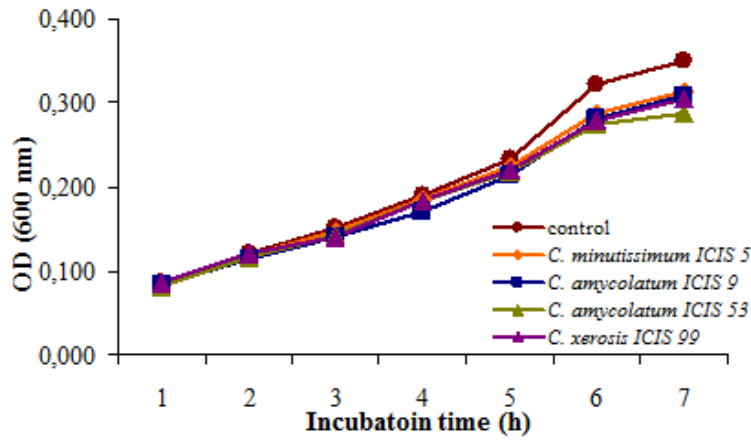
Four *Corynebacterium* strains previously were isolated from the reproductive tract of healthy women and were analyzed with regard to their morphology, cultural and biochemical characteristics. All strains were Gram-positive, catalase-positive, non-spore-forming, non-motile straight or slightly curved rod-shaped cells. The identification of *Corynebacterium* isolates was confirmed by determining the biochemical properties (API Coryne 2.0 biochemical system (bioMérieux, France) according to manufacturer's instructions. These strains are *C. amycolatum* ICIS 9, *C. amycolatum* ICIS 53, *C. minutissimum* ICIS 5 and *C. xerosis* ICIS 99.

#### **Inhibitory effect of metabolites from *Corynebacterium* strains on human bacterial pathogens**

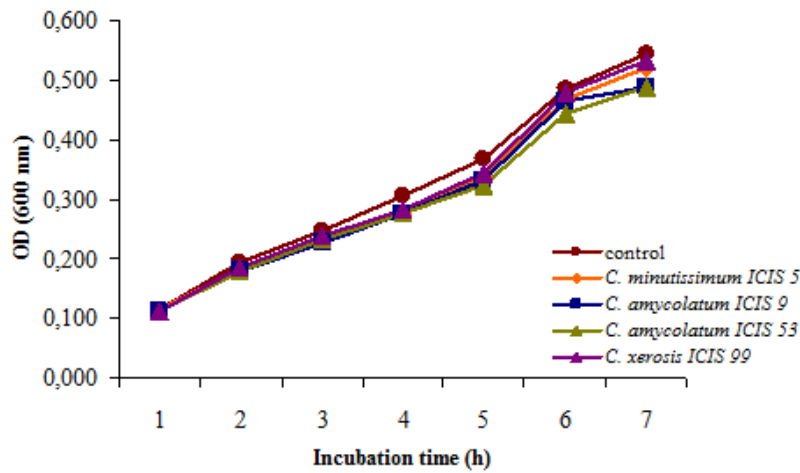
The direct antagonism of metabolites from *Corynebacterium* strains against four human bacterial pathogens were studied using 96 wells polystyrene plate method. Figure 1–4, shows the optical density of *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. aureus* grown in media containing the metabolites from different strains of *Corynebacterium*. The absorbance values demonstrate that all pathogenic strains were able to grow in media containing the metabolites of *Corynebacterium*. However, the pathogenic strains exhibited slower growth rate and reached lower final OD values of metabolites from *Corynebacterium* compared to the control.



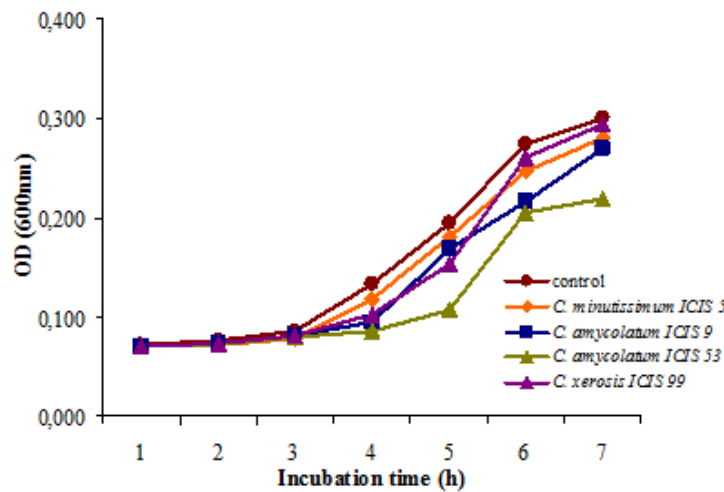
**Graph 1**  
**Growth of *K. pneumoniae* in cultural media containing the metabolites from *Corynebacterium* strains.**



**Graph 2**  
Growth of *E. coli* in cultural media containing the metabolites from *Corynebacterium* strains.



**Graph 3**  
Growth of *P. aeruginosa* in cultural media containing the metabolites from *Corynebacterium* strains.

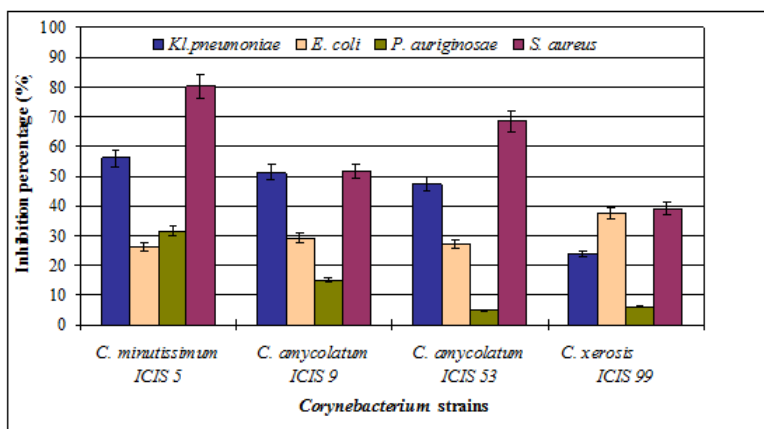


**Graph 4**  
Growth of *S. aureus* in cultural media containing the metabolites from *Corynebacterium* strains.

### **Inhibitory effects of metabolites from *Corynebacterium* on biofilm formation of human bacterial pathogens**

The results of inhibitory effects of metabolites from *Corynebacterium* strains on biofilm formation of human bacterial pathogens presented in Graph – 5. The metabolites from all four *Corynebacterium* strains inhibited the biofilm formation all test bacterial pathogens, but the degree of inhibition of the biofilm formation varied among the *Corynebacterium* strains. The results showed that all the isolated *Corynebacterium* strains, except *C. xerosis* ICIS 99, exhibited strong inhibition of the biofilm formation of *K. pneumoniae* and *S. aureus*. Weak inhibition of the biofilm formation all the isolated *Corynebacterium* strains showed against *P. aeruginosa*. The metabolites

from *C. minutissimum* ICIS 5 inhibited biofilm formation of *K. pneumoniae* (56.1%), *E. coli* (26.3%), *P. aeruginosa* (31.7%) and *S. aureus* (80.3%). The metabolites from *C. amycolatum* ICIS 9 caused inhibition of biofilm formation *Kl. pneumoniae* (51.3%), *E. coli* (29.2%), *P. aeruginosa* (15.2%) and *S. aureus* (51.7%). The metabolites from *C. amycolatum* ICIS 53 inhibited the biofilm formation of *K. pneumoniae* (47.2%), *E. coli* (27.2%), *P. aeruginosa* (4.9%), and *S. aureus* (68.4%). Among the all *Corynebacterium* strains, the metabolites from *C. xerosis* ICIS 99 in less degree inhibited biofilm formation *K. pneumoniae* (23.7%) and *S. aureus* (39.1%). This strain inhibited biofilm formation of *E. coli* up to 37.5% and *P. aeruginosa* up to 6.3%



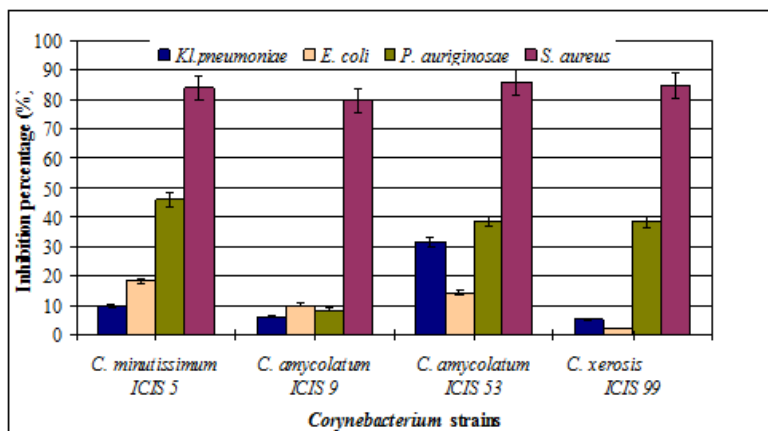
**Graph 5**

**The influence of metabolites from *Corynebacterium* strains on biofilm formation of human bacterial pathogens. Data represent the mean and SD of three independent experiments each in triplicate format.**

### **The influence of metabolites from *Corynebacterium* strains on pre-formed biofilms**

The results of influence of metabolites from *Corynebacterium* strains on developed biofilms (24h grown) of human bacterial pathogens presented in Graph – 6. Treatment of preformed biofilms of *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. aureus* in polystyrene microtiter plates with metabolites from all four *Corynebacterium* strains resulted in biofilm

disruption. To a greater extent this effect was observed under the influence metabolites from all four *Corynebacterium* strains on preformed biofilms *S. aureus* and *P. aeruginosa* (79.4 to 85.6% and 8.5 to 45.9%, respectively). To a lesser extent, metabolites from *Corynebacterium* strains influenced on preformed biofilms *K. Pneumoniae* and *E. coli* (5.1% to 31.6% and 2.4% to 18.5%, respectively).



**Graph 6**

**The influence of metabolites from *Corynebacterium* strains on developed biofilms (24h grown) of human bacterial pathogens. Data represent the mean and SD of three independent experiments each in triplicate format.**

## DISCUSSIONS

The vaginal ecosystem is dynamic and contains microbiota, that are protective against invading pathogens, including those causing urinary tract infections and sexually transmitted infections.<sup>18,19,20,21</sup>

*Lactobacillus* strains are thought to play a major role in protecting in the microenvironment of the vagina and in inhibiting the overgrowth of potentially pathogenic organisms.<sup>22,23,24</sup> Using culture-independent techniques several investigators have demonstrated that a significant proportion (7-33%) of healthy women lack appreciable numbers of *Lactobacillus* species in the vagina<sup>25,26</sup> which may be replaced by other lactic acid producing bacteria such as *Atopobium vaginae*, *Megasphaera*, *Leptotrichia* species and *Corynebacterium*.<sup>27</sup> Although the structure of the communities may differ between populations, health can be maintained provided the function of these communities i.e. the production of lactic acid, continues. Consequently, the absence of lactobacilli or the presence of certain organisms such as *G.vaginalis*, or species of *Peptostreptococcus*, *Prevotella*, *Pseudomonas*, *Streptococcus* and/or *Corynebacterium* does not constitute an abnormal state.<sup>18,26</sup> In this case, it is probably these microorganisms play the role in the protection of the vaginal biotope from pathogens. In present investigation, we study the antibacterial activity of metabolites from *Corynebacterium spp.* strains isolated from the reproductive tract of a healthy woman against four human pathogenic bacteria. The all *Corynebacterium* strains showed significant influence on growth, biofilm formation and on one-day developed biofilms *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. aureus*. Decrease in growth all pathogenic strains in media containing the metabolites of *Corynebacterium* is probably due to the ability of strains of *Corynebacterium* to produce organic acids. Funke *et al.*,<sup>28</sup> described, that the types of organic acids generated in the fermentation process depends on *Corynebacterium* species or strains, culture composition and growth conditions. The antimicrobial effect of organic acids lies in the reduction of pH, as well as the un-dissociated form of the molecules. It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the membrane. The un-dissociated acid acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability, which results in disruption of substrate transport systems.<sup>29</sup> Besides, the antibacterial activity of metabolites of *Corynebacterium* may often be due to the production of bacteriocins or bacteriocin-like compounds. Nakano *et al.*,<sup>30</sup> discovered in *C. hydrocarboclastus* the presence of Corynecin (an analog of chloramphenicol), characterized by bactericidal activity against Gram positive and Gram negative bacteria (cocci and rods). Kwaszewska *et al.*,<sup>31</sup> determined the presence of a bacteriocin-like substance in 90% of the investigated *Corynebacteria* isolated from the skin of patients. The spectrum of its activity was covered by Gram positive bacteria, in particular *S. aureus*. Probably, thanks to the presence

of bacteriocins, the corynebacteria are able not only to suppress the increase in the biomass of the pathogenic bacteria, but also to affect biofilm formation. It is known that the biological activity of bacteriocins depends on the pH level.<sup>32</sup> Probably, the corynebacteria isolated under normocenosis conditions influence the biofilm formation of pathogenic bacteria by synthesizing bacteriocins, while creating optimal conditions for their biological activity due to the acidification of the vaginal medium by the formation of organic acids. In addition, the ability of corynebacteria to inhibit biofilm formation and destroy one-day developed (24h grown) biofilms *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. aureus* can also be associated with the production of surface-active substances (surfactants) of different nature. Zajic *et al.*<sup>33</sup> found that *C. hydrocarboclastus* produces compounds having both surfactant and emulsifying properties. Thavasi *et al.*<sup>34</sup> demonstrated the emulsification capacities of the biosurfactant produced by *C. kutscheri* against hydrocarbons. Dalili *et al.*<sup>17</sup> described the lipopeptide coryxin isolated from *C. xerosis* NS5, isolated from the cubital fossa of a healthy person, which significantly suppressed the adhesive ability and biofilm formation *S. aureus*, *Streptococcus mutans*, *E. coli* and *P. aeruginosa*. The ability of *Corynebacteria* to influence growth, biofilm formation, and also to destroy biofilms of pathogenic microorganisms probably reveals one of the mechanisms of formation of normocenosis and determines the key role of *Corynebacteria* in protecting the vaginal biotope from infection in those women who do not have *lactobacilli*. In conclusion, the data suggest a probiotic potential of these *Corynebacterium* strains as an anti-bacterial agent in the vagina and *in vivo* studies, such as clinical trials designed to test their capacity to prevent and control urogenital tract infections in females.

## CONCLUSION

In present study, we have reported the antibacterial activity of metabolites from *Corynebacterium spp.* strains isolated from the reproductive tract of a healthy woman against four human pathogenic bacteria. The all *Corynebacterium* strains showed significant influence on growth, biofilm formation and on developed biofilms (24h grown) *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. aureus*. So, there is a need for further studies to isolate and characterize the bioactive compounds present in metabolites of *Corynebacterium* strains and these bioactive compounds can be used to develop effective drugs against these human pathogenic bacterial strains.

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## CONFLICT OF INTEREST

Conflict of interest declared none.

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