III. MICROORGANISMS AND ENVIRONMENT

INFLUENCE OF BACTERIAL DIVERSITY AND INTERSPECIES INTERACTIONS ON DRINKING WATER-ASSOCIATED BIOFILMS OF *YERSINIA ENTEROCOLITICA*

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Abstract: The capability of the enteropathogenic *Yersinia enterocolitica* 8081 bio/serotype 1B/O:8 to form binary and multi-species biofilms with defined bacterial strains was studied. The interspecies interactions in the binary biofilms of the enteropathogen with three bacterial isolates from drinking water and water-associated biofilms were assessed. The effect of each individual partner strain for the *Y. enterocolitica* involvement in the four-species bacterial biofilm was evaluated by excluding one by one the isolates from the sessile community.

It was found out that *Y. enterocolitica* and the tested bacterial strains interact each other in the binary biofilm formation. Moreover, the *Y. enterocolitica* involvement in the biofilms depends on the partner strain. In the multi-species biofilms, a synergetic effect of one of the bacterial partner strains on the *Y. enterocolitica* attachment was detected in contrast to the weak inhibitory effect of another one.

Key words: biofilm, drinking water, *Yersinia enterocolitica*, interspecies interactions, heterotrophs

INTRODUCTION

Drinking water purification processes have received their optimal technological solutions, but bacterial re-growth and biofilm formation occur in drinking water supply systems (DWSS) regardless of a residual disinfectant in water (most often active chlorine). Bacterial biofilms over the surfaces of pipelines and facilities of DWSS account for up to 95% of total active biomass and through biofilm detachment can enhance bacteria number in drinking water [4]. Specific microenvironments in the biofilm structure create conditions for recovery of bacteria injured through disinfection, ensure niches for survival or proliferation of water-borne pathogens and protection against predators and environmental stress, as well [4, 8, 9]. In this way, drinking water-associated biofilms can act as a reservoir of bacteria, including pathogenic ones, promoting bacterial contamination of drinking water, which in turn can create a health risk for water consumers and cause social and economic damages [4, 9].

Despite numerous scientific publications, an effective method for complete eliminating the drinking water-associated biofilms has not been developed yet. A possible reason is the lack of detailed knowledge of the mechanisms of interaction between various bacterial species coexisting in biofilm community. In addition, the data on planktonic growth and potential of the individual bacterial species to form single-species biofilms in drinking water cannot easily be extrapolated to multi-species biofilm community [13].

Over the last decade, significant research efforts are aimed at clarifying the social relationships and the driving forces in various natural bacterial biofilms and assessing the benefits and losses for the individual

species in these multi-species communities [3, 5, 10]. The examinations are mainly based on pure bacterial cultures isolated from their natural habitats like soil, surface water, waste water, etc. It has been found that interspecies interactions have a strong influence on composition, structure and physiology of mixed bacterial biofilms [1, 11, 13]. For example, non-biofilm forming *E. coli* O157:H7 strains could adhere owing to the partnership with biofilm forming strains [6, 15]. It has been demonstrated that a water-borne *E. coli* O157:H7 requires colonization partner, since it is not able to attach alone. However, it could co-adhere with *P. aeruginosa*, and best adhere to the surfaces pre-colonized by the partner strain [6].

Whereas the survival of water-borne pathogens *Vibrio*, *Shigella* and *Salmonella*, including the opportunistic *Mycobacterium avium*, *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila* in the drinking water-associated biofilms is well studied [8, 9], the fate of the newly recognized pathogens, that emerged increasingly in various drinking water sources [2, 12] is still insufficiently explored. Integration of zoonotic enteropathogens *Campylobacter jejuni*, *Yersinia enterocolitica* and *E. coli* O157:H7 in drinking water-associated biofilms can create health risk through water consumption, however, the studies on their survival at such conditions are still in their early stages [7]. Our previous study detected more successful attachment and longer survival of *Yersinia enterocolitica*, *Escherichia coli* O157 and *Salmonella dublin* in bacterial biofilms pre-existing in drinking water compared to non-colonized pipe surfaces [14]. However, the effects of the

enteropathogen interactions with the individual species of indigenous aquatic bacteria in the multi-species biofilms have remained unexplored [14]. For this reason, the present study aims at clarifying the integration of *Yersinia enterocolitica* 8081 bio/serotype 1B/O:8 into binary and multi-species biofilms with defined species composition, as well as to evaluate the pathogen interactions with the individual bacterial partners, taking part in building of the biofilm community.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The study was carried out with pathogenic strain *Yersinia enterocolitica* 8081 bio/serotype 1B/O: 8 and pre-isolated strains of indigenous aquatic bacteria. The pathogenic strain was kindly placed at disposal from the pure culture collection of the Stefan Angeloff Institute of Microbiology.

Isolation of heterotrophic bacteria from drinking water and drinking water-associated biofilm

Biofilm and water samples were collected from an operating model of domestic drinking water supply installation fed by tap water from the municipal water supply network of Veliko Tarnovo. The biofilm samples were homogenized in saline. Aliquots of the samples were spread on yeast extract agar (YEA) plates for isolation of single cells' bacterial colonies. Isolated bacterial strains (denoted as isolates 3, 6, 7 and 8) were checked for purity and stored as pure cultures at -20°C. The isolated heterotrophs were Gram (-) rods and oxidase (-), excepting the isolate 8. Only the isolate 6 formed yellowish colonies.

Preparation of calibrated bacterial suspensions (inoculums)

Overnight cultures of the tested bacteria, prepared by surface culturing of a single colony on the YEA plates were used. The bacterial biomass was removed and suspended in sterile drinking water (50 ml). Then, the suspensions were calibrated to 0.1 at optical density OD_{620nm} (approximately, 10⁸ CFU/ml).

Biofilm assay

Two types of batch experiments with pure or mixed cultures of the pathogenic *Y. enterocolitica* 8081 in drinking water were carried out: 1) Assessment of the pathogen potential to form binary biofilms with the isolated heterotrophs; 2) Assessment of individual influence of the bacterial partners for the pathogen involvement in four-species biofilm via excluding of one by one the isolates from the biofilm community.

1) Assessment of the biofilm formation potential of *Y. enterocolitica* 8081 and the interactions in its binary biofilms with distinct bacterial isolates

The capability of *Y. enterocolitica* to form binary biofilms with the tested heterotrophs (isolates 6, 7 and 8) was evaluated by analyzing the biofilm samples developed on sterile test surfaces immersed in inoculated drinking water over 12-days test period. Biofilm samples were taken regularly and analyzed for quantitative determination of the pathogen and isolate co-existing in the binary biofilms.

Test procedure: Six test surfaces (pieces cut from a polypropylene pipe, each one having a surface of 50 cm²) were immersed in a glass vessel containing 600 ml of sterile drinking water. Eight test vessels were prepared in total. Two of them were inoculated with the calibrated pathogen suspension (6 ml) for assessment the biofilm formation potential of *Y. enterocolitica* 8081. Another two vessels were inoculated with a pair of pathogen and an isolate (6 ml of each calibrated suspension) for assessment the pathogen inclusion in binary biofilm. This procedure was repeated for all tested isolates. The test vessels were incubated at 26°C for 12 days, and at the end of the 2nd day of the test, the inoculated water inside the vessels was replaced by sterile drinking water.

For biofilm analysis, one test piece was taken from each vessel on the 1st, 2nd, 3rd, 6th and 12th day of the test. The test surface was washed by 20 ml saline and then immersed in 40 ml saline. The biofilm was removed from the surface through sterile cotton swab and ultrasonic treatment (*Appronex*; 40 kHz) for 10 min. The biofilm suspension was ten-fold diluted and analyzed in triple repetition for the *Y. enterocolitica* number and heterotrophic plate count (YEA/36°C/3d).

2) Assessment of the interspecies interactions of *Y. enterocolitica* 8081 in multi-species biofilms

The individual effect of distinct bacterial strains on the *Y. enterocolitica* 8081 attachment in four-species biofilm was evaluated by excluding one by one each of the tested isolates from the biofilm communities. For the purpose, four glass vessels containing sterile drinking water with immersed test surfaces were prepared as described above. The first vessel was inoculated with equal volumes of the calibrated suspensions of the pathogen and each of the bacterial isolates (i.e. 3 ml of each of the 4 strains). Each subsequent vessel was inoculated in the same way, but one of the isolates was excluded (i.e. 3 ml of the 3 strains). Thus, the first vessel was inoculated with *Y. enterocolitica* 8081 and isolates 3, 6 and 7; the second vessel - with the pathogen and

isolates 6 and 7; the third one - with the pathogen and isolates 3 and 7, and the last one - with the pathogen and isolates 3 and 6. The test vessels were incubated for 9 days at 26°C. The biofilm sampling and analyses were performed according already described procedures.

Statistical data analysis

A significance *t*-test for comparison between a pair of biofilm samples was used, as the truth of the null hypothesis was tested. The difference between logarithms of a pair compared values was considered significant when the experimental value of $|t|$ was greater than the *t*-value from the *t*-distribution at *P* of 0.05 and degree of freedom *n*.

RESULTS AND DISCUSSION

Interspecies interactions in the binary biofilms of *Y. enterocolitica* bio/serotype 1B/O:8

The Fig. 1 presents the data on the *Y. enterocolitica* biofilms formed in pure culture or in pairs with the tested bacterial isolates 6, 7 or 8. The data show that the number of *Y. enterocolitica* in all binary biofilms (i.e. the pathogen density) was greater than its single-species biofilm. This tendency of higher pathogen density of the binary biofilms was detected for each sampling point over the entire test period and the difference was statistically significant for each compared pair ($|t| < t_{n,0.05}$), with only one exception.

Fig. 1. Dynamics of the culturable bacteria number of *Y. enterocolitica* 8081 (CFU/cm²) in its single-species biofilm and in the binary biofilms with bacterial isolates 6, 7 and 8 over the 12-days test period.

Comparing the *Y. enterocolitica* numbers in the binary biofilms allows to evaluate how the individual bacterial partners have influence on the pathogen attachment during the biofilm formation process. The data show that the partnership between *Y. enterocolitica* and isolate 6 stimulated most actively the pathogen attachment and involvement in the binary biofilm, while isolate 8 had the weakest stimulating effect. The pathogen density in the binary biofilms with isolate 6 increased 3÷4.5 times comparing to the single-species biofilm, while it increased up to 3 times in the binary biofilms with isolate 7, and up to 2.5 times in the ones with isolate 8. Despite the greater number of viable pathogenic cells in the binary biofilms with isolate 6 comparing with ones with isolate 7, the differences between them on the 3rd day and 12th day were statistically insignificant. However, the difference between the pathogen density of the binary biofilms with isolates 6 and 8 predominantly was significant.

The obtained data demonstrate the importance of the bacterial diversity on the *Y. enterocolitica* attachment and survival in drinking water-associated biofilms. They are in conformity with the diverse effects from the inter-species interactions reported for binary and multispecies biofilms of bacterial isolates from drinking water or waste water [1, 11], independently from the different experimental approach used for biofilm quantification (crystal violet biomass assay in microtiter plates for 24-72 hours).

Despite the varying synergistic effect that the particular heterotrophs had on the *Y. enterocolitica* attachment in their binary biofilms, the total culturable bacteria number of both partners in the biofilms most often differed negligible (Fig. 2). A bigger difference between all biofilms was observed only on the 1st day, when the cells surface properties of the tested isolates and the surface properties of the polypropylene test material probably could have a significant influence on

the initial attachment. Then, the difference between the binary biofilms with isolates 6 or 7 was statistically insignificant during the entire test period, likewise, the difference between the biofilms with isolates 7 or 8, excepting on the 12th day, and for the binary biofilms

with isolates 6 or 8 on the 3rd and 6th day. The maximum bacterial density of the binary biofilms is within the range already reported for the drinking water-associated biofilms at nutrient limitation [14].

Fig.2. Dynamics of the total culturable bacteria number of both partners (CFU/cm²) in the binary biofilms of *Y. enterocolitica* 8081 with isolates 6, 7 or 8 over the 12-days test period.

When considering the dynamics of either the tested isolate or *Y. enterocolitica* in the composition of their binary biofilms (Fig.3), the data show the equal initial contribution of isolates 6 or 8 and the pathogen, and a lower one - of isolate 7 (26%). On the 2nd day, the part of isolate 8 in the binary biofilm was 46%, whereas the part of isolates 6 and 7 - 12% and 15%, respectively. During the entire test period, the part of isolate 8 in the binary biofilm samples was the largest (on average 51%), while the participation of isolate 6 was on average 26% or one of isolate 7 - on average 34%. Consequently, competing with the isolates 6 or 7 during the biofilm formation, the *Y. enterocolitica* cells hindered the involvement of their cells, but had weak influence on the isolate 8.

On the basis of the discussed data it can be summarized that in drinking water:

- ✓ The single-species biofilm of *Y. enterocolitica* 8081 has a lower density in comparison with the binary biofilms formed with the tested bacterial isolates;
- ✓ The *Y. enterocolitica* participation in the binary biofilms depends on the partner bacterial strain;
- ✓ *Y. enterocolitica* and the tested bacterial isolates interacts with each other during the biofilm formation;
- ✓ The tested bacterial isolates have a beneficial effect on attachment and viability of the

Y. enterocolitica cells, and the isolate 6 had a greatest synergistic effect.

✓ In its turn, the co-existence with *Y. enterocolitica* hinders the isolates 6 and 7, and had neutral effect on the isolate 8.

Interspecies interactions in multi-species biofilms

The data on the multi-species biofilms formed by combining *Y. enterocolitica* and the examined bacterial isolates (Fig. 4) reveal the individual influence of each bacterial partner on the pathogen inclusion in the biofilm community. On the 1st day, in spite of the weakest initial attachment of *Y. enterocolitica* in the four-species biofilm, statistically insignificant differences were found when comparing with both biofilms formed by exclusion of isolate 3 or isolate 6. However, the exclusion of isolate 7 provided a larger number of attached pathogenic cells and kept the same effect on the 2nd day, as well. Thus, the co-existence with isolate 7 in the four-species biofilm could have a weak inhibitory effect on the initial attachment of *Y. enterocolitica*. On the 6th day and thereafter, in the absence of an external pathogen source, the *Y. enterocolitica* density has decreased most rapidly in the biofilms, formed without isolate 6. Therefore, the partnership with isolate 6 could affect synergetically the *Y. enterocolitica* inclusion in the multi-species biofilm. This observation is consistent

with the data on the *Y. enterocolitica* interaction with the isolate 6 in the binary biofilms (Fig.1). The exclusion of isolate 6, who best favor the pathogen inclusion in its binary biofilms, caused in turn the weakest pathogen attachment and survival in the multi-species biofilm community. In this way the

both type experimental data verify the synergistic effect of isolate 6. At the end of the test period, isolate 3 weak synergistically affected the pathogen involvement in the multi-species biofilm, while isolate 7 had a neutral effect.

Fig. 3. Dynamics of the culturable cells number of either *Y. enterocolitica* or bacterial isolate in the binary biofilm of the pathogen with isolate 6 (a), isolate 7 (b), and isolate 8 (c) over the 12-days test period

Fig.4. Comparison between the *Y. enterocolitica* 8081 density (CFU/cm²) in the multi-species biofilms, formed with the tested bacterial isolates: four-species biofilm (*Y. enterocolitica* plus isolates 3, 6 and 7) and three-species biofilms after individual exclusion of one of the bacterial partners, during the 9-days test period; d – day.

The data present at Table 1 illustrate the above discussed dynamics of the *Y. enterocolitica* cells number in the biofilms, formed in different partnership combinations with isolates 3, 6 or 7. They also demonstrate the putative contribution of

each individual partner, excluded from the biofilm community, for the *Y. enterocolitica* involvement: a great synergistic effect of isolate 6, a weak synergistic effect of isolate 3 and from a weak inhibitory to neutral effect of isolate 7.

Table 1. Changes in the *Y. enterocolitica* 8081 participation in the biofilms formed in the absence of one of the bacterial partners (isolates 3, 6 or 7) compared to the four-species biofilm

Biofilm composition	Participation of <i>Y. enterocolitica</i> (%)* in the biofilms over the test period			
	1 st day	2 nd day	6 th day	9 th day
Four-species biofilm (i.e. pathogen + isolates 3, 6, 7)	100	100	100	100
Without isolate 3 (i.e. pathogen + isolates 6, 7)	150	129	95	56
Without isolate 6 (i.e. pathogen + isolates 3, 7)	158	77	20	8
Without isolate 7 (i.e. pathogen + isolates 3, 6)	265	234	95	106

* Calculations are based on the data of culturable pathogen cells number

The data on the dynamics of the *Y. enterocolitica* density (Fig. 5) in the multi-species biofilms formed in different partnership combinations with isolates 3, 6 and 7 demonstrate the highest values on the 2nd day still as a result of pathogen attachment from the inoculated water. After removal of the pathogen source (by replacing the water), the *Y. enterocolitica* density in the biofilms started to decrease, probably due to the pathogen

transition in a viable, but non-culturable state or dying, or biofilm detachment. The data show that the pathogen reduction depends on species diversity in the biofilm community, and changed least in the four-species biofilm. The *Y. enterocolitica* density in the three-species biofilms changed least in the absence of isolate 7, whereas the absence of isolate 6 resulted in the greatest pathogen reduction.

Fig.5. Dynamics of the *Y. enterocolitica* 8081 density (CFU/cm²) in the multi-species biofilms, formed in different partnership combinations with the isolates 3, 6 and 7 during the 9-days test period; d – day.

The assessment of the individual contribution of the tested heterotrophic isolates for attachment and survival of *Y. enterocolitica* in multi-species biofilms demonstrates the importance of the interactions between the multiple species: co-existence with isolate 6 could provide a synergistic effect, whereas with isolate 7 - a weak inhibitory or predominantly neutral effect.

CONCLUSION

On the basis of the conducted studies, the following main conclusions can be drawn:

- In drinking water, *Y. enterocolitica* 8081 forms single-species biofilm with a lower bacterial density compared to its binary biofilms with the partner bacterial strains;
- The participation of culturable *Y. enterocolitica* 8081 cells in binary and multispecies bacterial biofilms depends on the partner strains and the time.
- The interactions of *Y. enterocolitica* 8081 with the partner bacterial strains are important for its attachment and survival in multi-species biofilms.

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ВЛИЯНИЕ НА БАКТЕРИАЛНОТО РАЗНООБРАЗИЕ И МЕЖДУВИДОВИ ВЗАИМОДЕЙСТВИЯ ВЪРХУ БИОФИЛМИ В ПИТЕЙНА ВОДА АСОЦИИРАНИ С *YERSINIA ENTEROCOLITICA*

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Резюме: Проучена е способността на ентеропатогенния щам *Yersinia enterocolitica* 8081 от био/серотип 1B/O:8 да образува бинарни и многовидови биофилми с определени бактериални щамове. Охарактеризирани са и вътревидовите взаимодействия в бинарни биофилми между посочения ентеропатоген и три бактериални изолати от питейна вода. Ефектът на всеки отделен бактериален вид с участието на *Y. enterocolitica* бе оценен чрез изключване на един по един на изолатите от микробната общност в биологични филми, състоящи се от четири вида. Установено е, че *Y. enterocolitica* и изследваните бактериални щамове си взаимодействат в условията на формиране на бинарните биофилми. Освен това, участието на *Y. enterocolitica* в биофилмите зависи от партньорския щам. При многовидовите биофилми се открива както синергичен ефект на един от бактериалните партньорски щамове върху прикрепването на *Y. enterocolitica*, така и слаб инхибиторен ефект на друг.

Ключови думи: биофилм, питейна вода, *Yersinia enterocolitica*, взаимодействия между отделните видове, хетеротрофи

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