IN VITRO MONOSPECIES, MULTISPECIES AND NECROTROPHIC BIOFILM PRODUCTION OF *Legionella pneumophila* IN WATER SUPPLY SYSTEMS

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ABSTRACT

Biofilms provide a shelter for microorganism to survive in unsuitable environments. *Legionella pneumophila* can produce monospecies biofilms in vitro, but usually exist in multispecies biofilms in nature. In this study, biofilm production capabilities of *L. pneumophila* strains isolated from water supply systems, the main resource of legionnaire's disease, was investigated. They were detect-ted to form biofilms in sterile tap water and BYE medium. In addition, it was shown that *L. pneumophila* had the ability of biofilm production using dead bacteria cells as food. Multispecies biofilm production and attachment to preformed biofilms were studied with six other bacterial species. *L. pneumophila* was found to have positive biofilm interactions for multispecies biofilm production with *Klebsiella pneumoniae, Enterococcus faecalis, Pseudomonas putida* and *Pseudomonas flourescens,* and for attachment to preformed biofilms with *K. pneumoniae, E. faecalis* and *P.aeruginosa.* The results suggest that *L. pneumophila* could produce biofilms and join to biofilms of other bacteria, even in the conditions that it is not able to grow.

KEYWORDS:
Legionella pneumophila; Biofilm; Biofilm production; Necrotrophic biofilm; *Pseudomonas aeruginosa*; *Klebsiella pneumoniae*

1. INTRODUCTION

Biofilm can be simply defined as a community of microorganism attached to a surface [1]. Biofilm-formed microorganisms are in an extracellular polymeric (EPS) matrix that produced by themselves. Therefore, they become more resistant to environmental factors [2], and to antibiotics [3]. Biofilms may be formed as a population of single species, and may be formed from different microbial species [4]. They are called monospecies and multispecies biofilms, respectively.

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Biofilms formed in the water supply pipes, ensure many pathogenic microorganisms to survive for a long time, and even interfere with detection of these microorganisms by culture methods [5]. *Legionella pneumophila*, one of these pathogens, can grow in the biofilms of water supply systems [6]. Colonization of potable water systems by *L. pneumophila* is the first step in the transmission of legionnaire's disease to humans. There are two important phenomena providing survival of *L. pneumophila* in potable waters: biofilms [7] and parasitism in protozoa [8, 9].

L. pneumophila is widespread in aquatic environments [10], even in extreme conditions [11]. Biofilms take an important place in their life cycle by providing an escape from unfavorable conditions [12]. It was shown that *L.pneumophila* could form monospecies biofilms [13, 14], and attach to preformed multispecies biofilms [15]. Biofilm production capacity of *L. pneumophila* was found higher than other *Legionella* species [16], and biofilm derived *L. pneumophila* was detected to be more virulent than planktonic forms [17, 18]. Additionally, Temmerman et al. [19] revealed that *L. pneumophila* is able to reproduce using dead bacteria cells as nutrient. It provides an opportunity for rapid multiplication after disinfection of water supply pipes.

Legionnaire's disease is travel associated, and most of the reported sources of the disease are tourist facilities. Thus, in this study, the biofilm production features of *L.pneumophila* strains isolated from various buildings in Muğla, a developed city in terms of tourism, was investigated. In vitro monospecies and multispecies biofilms of *L. pneumophila* were studied in BCYE, sterile tap water and necrotrophic medium.

2. MATERIALS AND METHODS

2.1 Bacterial strains and media

L. pneumophila strains employed in this study were isolated from water systems of various buildings in Muğla, Turkey. Isolation was performed by culture with membrane filtration and acid treatment [20], and then inocula-

tion to BCYE and MWY media [21]. Eleven isolated strains were assigned with numbers from L1 to L11. Four of strains (L2, L5, L6, L10) were identified as serogroup 1 and the rest (L1, L3, L4, L7, L8, L9, L11) as SG 2-14, with latex agglutination test (Oxoid) [22].

Pseudomonas aeruginosa (ATCC 10145), *P.fluorescens* (MU87), *P.putida* (MU171), *Klebsiella pneumoniae* (ATCC 13883), *Enterobacter aerogenes* (RSKK), *Enterococcus faecalis* (ATCC 19433) was employed in multispecies biofilm studies.

Buffered yeast extract broth (BYE), tryptic soy broth (TSB), sterile tap water (STW), and sterile tap water containing dead bacteria cells (Necrotrophic) were used as biofilm media. Tap water was sterilized by filtration with 0.2 nm filter.

2.2 Monospecies biofilm formation

Microtiter plate biofilm assay [23] and method of Mampel et al. [24] was modified and performed in glass test tubes (100x160mm). Microorganisms were diluted to give 0.2 OD at 600 nm. 2 ml of the bacteria suspension was dispersed into each test tube, and incubated at 37°C for biofilm production. Tubes containing no bacteria were used as negative control.

Necrotrophic biofilm production was studied in sterile tap water containing 10^9cfu/mL heat killed cells of *P. fluorescens, P. putida, K. pneumoniae, E. aerogenes and E. faecalis.*

2.3 Multispecies biofilm formation

Biofilm production of *L. pneumophila* jointly with other bacteria was studied in TSB as described in monospecies biofilm production. Differently, 1 mL *L. pneumophila*, and 1 mL other bacteria was dispersed to each tube.

Attachment of *L. pneumophila* to preformed 24h biofilms of other bacteria was studied in TSB. Planktonic cells and media were removed from 24h incubated tubes, and 2mL of *L. pneumophila* was dispersed. Own suspension of the bacteria forming the 24h biofilm or sterile TSB were dispersed to tubes as positive control. Tubes containing TSB but no microorganism were employed as negative control.

2.4 Measurement of biofilm

After incubation, planktonic cells and media were removed, and attached cells were fixed at 80°C, 10 minutes. All tubes were stained with 2ml of 0.3% crystal violet (CV) during 15 minutes, and rinsed with sterile distilled water three times. Tubes were left to dry upright at room temperature. Stained CV was dissolved in ethanol for 15 minutes and transferred to 12x92 mm PS tubes for measuring optical density (OD) in 570 nm.

Biofilm production capabilities of bacteria were evaluated as described by Stepanovic et al. [25]. Three standard deviations above of mean OD of the negative controls were defined as cut-off OD (OD_C). Biofilm production capabilities were scored as follows:

$OD_S < OD_C$	No biofilm production, (-) score
$OD_C \leq OD_S < 2xOD_C$	Weak biofilm, (+) score
$2xOD_C \leq OD_S < 4xOD_C$	Moderate biofilm, (++) score
$4xOD_C \leq OD_S$	Strong biofilm, (+++) score

Sample OD: OD_S, Cut-off OD: OD_C

3. RESULTS

3.1 Monospecies biofilms of *L. pneumophila*

Biofilm production capabilities of 11 *L.pneumophila* strains at 37°C 72h, in filter sterilized tap water and BYE broth were shown in Table 1. Best biofilm producers were detected as L7, L8 and L11 in STW, and L8, L10 and L7 in BYE. Biofilm production was observed in STW for all strains except L1. On the other hand, it was found that five strains could produce weak (+) biofilm, while the other six produce moderate biofilm (++) in BYE. Biofilm production rates in BYE were approximately two fold higher than in STW for most of the strains.

TABLE 4 - Biofilm production capabilities of *L.pneumophila* strains at 37°C, 72h.

Strains	Sterile Tap Water			BYE Broth		
	OD570nm	OD/Cut off	Score	OD570nm	OD/Cut off	Score
L1	0.40±0.04	0.96	-	0.84±0.10	1.72	+
L2	0.53±0.06	1.26	+	0.91±0.06	1.85	+
L3	0.53±0.03	1.26	+	1.07±0.04	2.19	++
L4	0.57±0.07	1.34	+	1.10±0.06	2.24	++
L5	0.56±0.09	1.32	+	1.05±0.05	2.15	++
L6	0.49±0.08	1.15	+	0.97±0.04	1.98	+
L7	0.61±0.06	1.45	+	1.11±0.04	2.26	++
L8	0.61±0.04	1.44	+	1.35±0.07	2.77	++
L9	0.54±0.09	1.27	+	0.95±0.06	1.94	+
L10	0.58±0.03	1.36	+	1.27±0.08	2.60	++
L11	0.60±0.03	1.41	+	0.92±0.04	1.88	+

3.2 Necrotrophic biofilms of *L. pneumophila*

Six strains determined to produce maximum biofilm was studied in five different necrotrophic media. Compared to negative controls, necrotrophic biofilm production was detected in almost all of the trials, except L3 in *P. putida* dead cells (Table 2). Only L8 was produced moderate (++) score biofilm in *E. aerogenes* dead cells, while the remaining was weak (+). "P" values were calculated according to biofilm values in STW and shown in Table 2.

Biofilm production capabilities of *L. pneumophila* strains are ranked as L7, L8, L10, L5, L4 and L3 (Fig. 1). Necrotrophic biofilm values are slightly higher than STW biofilms, but quite lower than BYE biofilms.

3.3 Multispecies biofilm production of *L. pneumophila*

L. pneumophila was found to produce multispecies biofilms jointly with *P. fluorescens, P. putida, K. pneumoniae, E. faecalis* significantly higher than monospecies biofilms (Fig. 2). However, multispecies biofilm production with *P. aeruginosa* was lower than monospecies biofilms.

Multispecies biofilms of *L. pneumophila* with *P. fluorescens* and *E. faecalis,* were found to have moderate (++) biofilm production score, and the rest were found weak (+).

3.4 Attachment of *L. pneumophila* to preformed biofilms

L. pneumophila suspensions were added to tubes containing preformed biofilms of other bacteria and the results were compared with control studies that sterile TSB or suspension of biofilm production bacteria were used instead of *L. pneumophila*.

L. pneumophila was found to attach and produce significantly biofilms on *P. aeruginosa, E. faecalis and K. pneumophila* biofilms, compared to the control results (Fig. 3). Maximum biofilm production was shown on *K. pneumoniae* biofilms, approximately 70% higher than control values. Increase was found not remarkable for *E. aerogenes* and *P. fluorescens*. Addition of *L. pneumophila* on *P. putida* biofilms caused a decrease of biofilm values.

Two control studies showed similar results, except for *P. fluorescens*. Addition of TSB was found higher than

TABLE 2 - Biofilm production capabilities of *L.pneumophila* strains in necrotrophic media (OD/Cutoff)

Strains	Necrotrophic Media (STW + Dead Bacteria Cells 10^9 cfu mL^{-1})				
	E.faecalis	*E.aerogenes*	*K.pneumoniae*	*P.putida*	*P.fluorescens*
L3	1.23 (+)	1.37 (+)	1.30 (+)	0.99 (-)	1.10 (+)
L4	1.11 (+)	1.32 (+)	1.30 (+)	1.12 (+)	1.20 (+)
L5	1.42 (+)	1.29 (+)	1.42 (+)	1.53 (+)*	1.42 (+)*
L7	1.48 (+)*	1.60 (+)	1.47 (+)	1.63 (+)*	1.25 (+)
L8	1.17 (+)	2.03 (++)**	1.60 (+)	1.19 (+)	1.45 (+)**
L10	1.45 (+)**	1.35 (+)	1.59 (+)*	1.49 (+)*	1.40 (+)*

**P<0.05; **P<0.01*

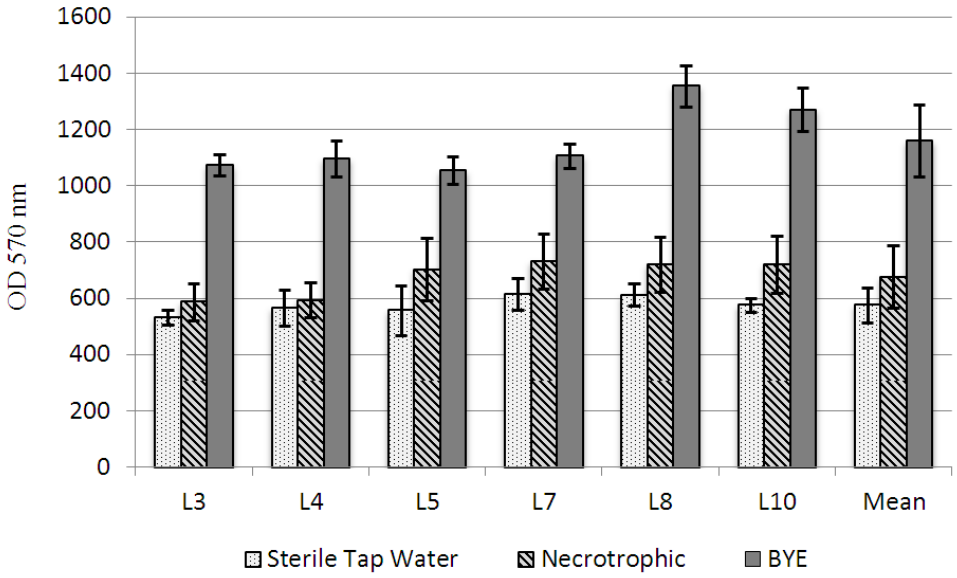

FIGURE 1 – Comparison of necrotrophic biofilms of *L. pneumophila* with biofilms in STW and BYE.

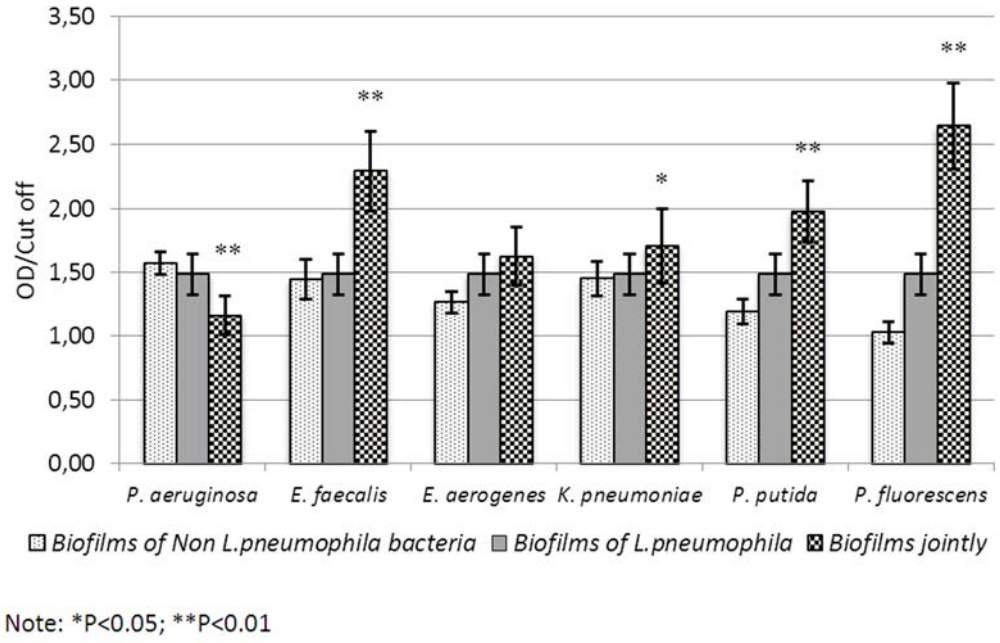

FIGURE 2 – Comparison of monospecies and multispecies biofilms produced by *L. pneumophila* with other bacteria.

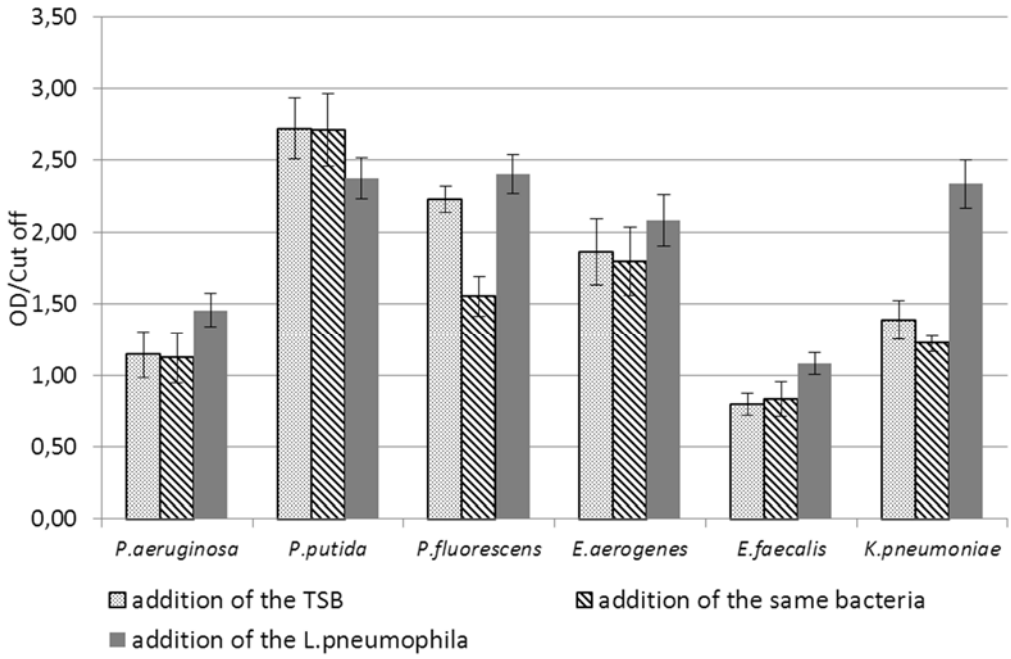

FIGURE 3 – Biofilm production of *L. pneumophila* on biofilms of other bacteria.

addition of *P. fluorescens* on 24h biofilms of *P. fluorescens*.

4. DISCUSSION

L. pneumophila has fastidious growth characteristics, and cannot multiply in usual media that other bacteria can grow easily. According to biofilm results of our study, *L.pneumophila* can produce weak biofilms in STW. OD results was found at 570nm between 0.404 - 0.614, and biofilm values between (OD/cutoff) 0.96 – 1.45.

Six of our *L. pneumophila* strains showed moderate (++) biofilm production in BYE, while the other five was weak (+). The maximum OD/cutoff value was 1.45 in STW

TABLE 3 - Comparison of multispecies biofilm interactions of *L.pneumophila* with other bacteria

	Jointly biofilm production with *L.pneumophila*	Attachment of *L.pneumophila* to 24h biofilms
P.aeruginosa	—	+
P.putida	+	—
P.fluorescens	+	N
E.aerogenes	N	N
E.faecalis	+	+
K.pneumoniae	+	+

("+": Positive interaction, "—": Negative interaction, "N": No interaction)

and the minimum in BYE was 1. 72. These results enhance the importance of biofilm production in STW.

Mampel et al. [24] had found 3.0 OD at 600 nm, and Tai et al. [14] between 0.2 - 0.8 OD at 550 nm, with similar methods in BYE. However, former had used clinical and environmental isolates, while latter had used potable water systems isolates, as in our study. The three-fold difference between biofilm results was probably depends on the isolates rather than methods.

Because the majority of the microorganisms die after disinfection in potable water systems, an environment occurs for microorganisms which get rid of disinfection, where there is no competition, and plenty of organic waste. It was reported that *L pneumophila* could reach high concentrations in water supply systems after high temperature disinfection [26, 27]. Temmerman et al. [19] detected that in a necrotrophic media contains 10^6 cfu/ml *L. pneumophila* and 10^9 ml^{-1} *P. putida* dead cells, concentration of *L. pneumophila* increased to 1.1 x 10^7 cfu/ml after 96h incubation. The result means that *L. pneumophila* could use of dead cells to multiply.

In our study, biofilm production capability of *L. pneumophila* was investigated in necrotrophic media similar with Temmerman et al. [19] had used. Seven of total 30 results were found higher than negative results as $P<0.05$, and three as $P<0.01$. It means, *L. pneumophila* could produce necrotrophic biofilms significant as statistically in 1/3 of experiments. To our knowledge, this is the first study reporting the necrotrophic biofilm production of *L. pneumophila.*

Microorganisms have complex relationships with each other either in nature or in water supply systems biofilms. Likewise, *L. pneumophila* is related to other bacteria as well as protozoa in biofilms. Murga et al. [28] showed that *L. pneumophila* could attach and survive fifteen days in biofilms produced by *P. aeruginosa, K. pneumoniae* and *Flavobacterium sp*. Whereas, Stewart et al. [15] reported that *L. pneumophila* could not attach to *P. aeruginosa* biofilms, while could survive in *P. putida, K. pneumoniae* and *Flavobacterium* biofilms. It was also reported that Quorum sensing molecules of *P. aeruginosa* prevented the growth and biofilm production of *L. pneumophila* [29].

Our results have showed that *L. pneumophila* had positive biofilm interaction with *E. faecalis*, *K. pneumoniae, P. fluorescens* and *P. putida*. The biofilm produced together with mentioned species were found higher than negative controls as significant statistically. All multispecies biofilm interactions were shown in Table 3.

L. pneumophila and *P. aeruginosa* have negative (-) biofilm interaction between them in consistent with literature. However, when *L. pneumophila* added to preformed *P. aeruginosa* biofilms, a remarkable increase was observed in comparison with negative controls. The results suggest that *L. pneumophila* could attach *P. aeruginosa* biofilms in contrast to the literature.

According to our results, *L. pneumophila* could attach and grow at most in *K. pneumoniae* biofilms among the bacteria in this study. *K. pneumoniae* and *E. faecalis* have positive interactions with *L. pneumophila* in terms of either biofilm production or attachment to preformed biofilm. On the other hand, *E. aerogenes* was observed to have no biofilm interaction with *L. pneumophila*.

It was found that *L. pneumophila* could produce biofilms jointly with *P.putida* and *P.fluorescens*, but could not colonize to their preformed biofilms. Our results suggest that interaction between two different species during biofilm production, could be opposite during attachment to preformed biofilms (Table 3).

5. CONCLUSION

In brief, *L.pneumophila* was shown to be able to produce biofilm in vitro in STW, BYE and necrotrophic media and jointly with other bacterial species *K.penumoniae, E.faecalis, P.putida* and *P.fluorescens,* and to be able to attach and grow in preformed biofilms of *K.pneumoniae, P.aeruginosa* and *E.faecalis.* Results indicate that *L.pneumophila* has the capacity of biofilm production and attachment to biofilms, even in the environments that could not grow. So that the control measures to prevent colonization of *L.pneumophila* in water supply systems, must include the treatments to eradicate biofilms, and must be repeated to inhibit necrotrophic growth and biofilms.

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