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An *in vitro* evaluation of the interactions of *Legionella pneumophila* serogroups 2 to 14 strains with other bacteria in the same habitat

Ayten KIMIRAN ERDEM*, Aysegül YAZICI

Istanbul University, Faculty of Science, Department of Biology, Section of Basic and Industrial Microbiology, 34134, Vezneciler, Istanbul, Turkey

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Abstract - The aim of the present study is to determine *in vitro* inhibitory and/or stimulatory effects of different bacteria on concomitant *Legionella pneumophila*. The interactions between *Legionella* and other bacteria were investigated by using bacteria culture and cell-free supernatants (CFSs) on Buffered Charcoal Yeast Extract agar (BCYEA) and/or in BCYEA lacking L-cysteine. Additionally, CFSs of non-*Legionella* bacteria that possess inhibitory effect on *L. pneumophila* were characterised using enzyme and heat treatments. The inhibition ratio of the CFSs and the cultures of Gram-negative rod bacteria (GNRB) and Gram-positive rod bacteria (GPRB) on the growth of *L. pneumophila* strains were 26-47% and 33-67%, respectively, on BCYEA. It was detected that the cultures of *Aeromonas hydrophila*, *Aeromonas* spp. strain and *Bacillus pumilus* stimulated the growth of one of the investigated *L. pneumophila* strains, but their CFSs did not show any stimulatory effect. The results indicated that growth and multiplication of legionellae could be affected by different bacteria sharing the same habitat and the level of this effect varies among the species. To our knowledge, this is the first study which determined the inhibitory effects of *B. pumilus* and *Brevibacillus brevis* against *Legionella*. The biologically active substances produced by the bacteria could play an important role in the control of *L. pneumophila*. This phenomenon may be used an alternative approach for controlling legionellae in man-made environments.

Key words: *Legionella*, biofilm, non-*Legionella* spp., cultivation *in vitro*, influence of shed non-legionellae components.

INTRODUCTION

Legionella pneumophila frequently inhabits man-made aquatic environments such as cooling towers, shower heads and nebulizators where it lives a complex social life (Den Boer *et al*., 2007). Although, water systems generally have low nutrient content, legionellae are widespread within these environments (Toze *et al*., 1990; Sanli-Yurudu *et al*., 2007). Legionellae are fastidious in their growth requirements, and their diet must contain L-cysteine and iron salts (Tesh and Miller, 1981). They obtain these nutrients from the environment either directly from other living organisms which produce them in excess or indirectly from the decomposition of organic matter, or both (Wadowsky and Yee, 1983, 1985, 1988). Several investigations (Stout *et al*., 1985; Yamamoto *et al*., 1992) have suggested that certain abiotic factors such as pH, temperature, organic matter, and certain metals play important roles as growth enhancers or inhibitors of legionellae in the environment. On the other hand, existence of other organisms such as cyanobacteria, some heterotrophic bacteria and protozoa in the microbial community are believed to be effective for survival or multiplication of legionellae in natural and man-made aquatic environments (Fliermans *et al*., 1981; Bohach and Snyder, 1983; Stout *et al*., 1985; Koide *et al*., 1989; Toze *et al*., 1990; Yamamoto *et al*., 1992; Gomez-Lus *et al*., 1993; Cotuk *et al*., 2005). Thus, it is suggested that metabolites produced by other microorganisms are utilised by *L. pneumophila*. On the other hand, some other metabolites might be inhibitory on the growth of *L. pneumophila.* It is believed that bactericidal proteins, collectively known as bacteriocins are continuously produced by both Gram-positive and Gram-negative bacteria. The bacteriocin family includes a diversity of proteins with different size, microbial targets, modes of action, and immunity mechanisms (Riley and Chavan, 2007). Since man-made water systems are known as the major sources of Legionnaires' disease outbreaks, alternative approaches must be considered to control/prevent and treat legionellae (Delgado-Viscogliosi *et al*., 2005; Sanli-Yurudu *et al*., 2007). Bactericidal proteins, which inhibit the growth of other strains of the same organism or related and different species from the same ecological niche (Barja *et al*., 1989; Todorov *et al*., 2005), play important roles in the lives of various bacterial species and may be useful as an alternative approach for preventing legionellosis outbreaks, in man-made environments.

The purpose of the current study is to investigate inhibitory and/or stimulatory effects of different bacteria on *L. pneumophila in vitro*. Furthermore, our aim is to characterise anti-*Legionella* compound for their chemical properties and to determine their antibacterial activities against the GNRB and GPRB isolated.

MATERIALS AND METHODS

Isolation of *Legionella pneumophila*. Ten water and biofilm samples have been collected from seven different hotels in the vicinity of Istanbul and were analysed within two days.

* Corresponding author. Phone: +90 212 455 57 00;
Fax: +90 212 528 05 27; E-mail: aytenkimiran@yahoo.com, kimiran@istanbul.edu.tr

The *Legionella* analysis was conducted according to the British Standard (1998). Two litres of a water sample were concentrated by membrane filtration (0.2 µm pore sized polyamide filter; Sartorius). Acid (1:1 HCl-KCl buffer at pH 2.2 for 10-15 min) and heat treatments (at 50 °C for 30 min) for selective inhibition of non-*Legionella* bacteria were performed, then aliquots of the treated samples were inoculated onto Buffered Charcoal Yeast Extract agar (BCYEA, Oxoid Ltd., Basingstoke, Hampshire, UK, DR800M) supplemented with glycine, vancomycin, polymyxin, and cycloheximide (GVPC). All plates were incubated at 37 °C in an atmosphere of 2.5% CO_2 for 10-14 days and examined daily for evidence of growth. Colonies consistent with *Legionella* morphology were subcultured to Tryptone Soy agar (Oxoid) and BCYEA plates. All suspected *Legionella* isolates were characterised using *Legionella* Latex Agglutination Test Kit (Oxoid) allowing separate identification of *Legionella pneumophila* serogroup 1 and serogroups 2-14 and detection of seven other *Legionella* species.

Biofilms forming on the surfaces of cooling tower tanks were removed by swabbing, suspended in sterile phosphate buffer, and vortexed (60 s) for detachment of biofilm bacteria. Biofilm homogenate was treated as described above for the isolation of *L. pneumophila* (Türetgen and Cotuk, 2007).

Isolation of Gram-negative and Gram-positive rod bacteria strains. Water samples collected from different locations (cooling tower water, tap water) in the hotels, were spread on plates containing five different isolation media from Oxoid (Mac Conkey agar, Nutrient agar, R2A agar, *Pseudomonas* selective media with CFC supplement, and *Aeromonas* selective media with ampicillin selective supplement) to recover as many different bacteria as possible. Plates were then incubated aerobically at a suitable temperature and duration. Representatives of all bacterial colonies were recorded. Strains determined as Gram-negative rod bacteria (GNRB) and Gram-positive rod bacteria (GPRB) were selected and subcultured on Plate Count agar at 20 °C for 24-48 days to determine oxidase activity and identification. Gram-negative bacteria were identified using API 20E strips (BioMerieux, Marcy-I'Etoile, France) and API 20NE strips for oxidase negative and positive strains, respectively. Species identification of the Gram-positive bacteria was done via testing for reduction of potassium tellurite and for acid production from 50 different carbohydrates in API 50 CH and API 20E strips (BioMerieux).

Homogenated biofilm samples were diluted in 1/200 ratio, from diluted biofilm homogenates, 100 µl liquid was drawn, transferred to the different media and incubated as described above.

Cell-free supernatants (CFSs) preparation. To obtain cell-free supernatants of all the GNRB and GPRB cultures, bacteria were suspended in sterile tap water to obtain a concentration of 1.5 x 10^8 colony forming unit ml^{-1} (CFU ml^{-1}). This suspension (100 µl) was transferred to Buffered Yeast Extract Broth (BYEB) and incubated 37 °C for 24 h. Cells were then removed by centrifugation in Eppendorf tubes at 10000 *x g* at 4 °C for 20 min. Supernatants were filtered using membrane filters (0.22 µm pore size, Sartorius) (Cotuk *et al*., 2005; Hechard *et al*., 2005; Yokoyama *et al*., 1998). All filtrates were immediately used or stored at -70 °C until use.

Inhibition of *Legionella* strains by GNRB and GPRB strains. Twenty four strains of GNRB and GPRB were tested for their capability of inhibition of *L. pneumophila* growth by measuring inhibition zone diameters.

Both *L. pneumophila* strains (S1 and S2) were suspended in sterile tap water to a concentration of 1.5 x 10^8 CFU ml^{-1} and then 100 µl of inocula were spread onto BCYEA plates. *Legionella* inoculated BCYEA plates were used for the determinations of the inhibitory effects of both culture and cell-free supernatants of GNRB and GPRB strains.

i - Experiments with the GNRB and GPRB cultures. GNRB and GPRB strains were cultivated at 37 °C for 18 h on Nutrient agar. Inocula were turbidimetrically adjusted in sterile tap water to 3 x 10^8 CFU ml^{-1} concentrations, and then 100 µl of inocula were spread on the surface of Nutrient agar plates using a Drigalski rod, individually. Agar cubes (6 x 6 mm) containing GNRB and GPRB colonies from Nutrient Agar was patched onto the surface of the *Legionella* inoculated BCYEA plates with a nichrome wire loop. A sterile Nutrient agar cube (6 x 6 mm) was patched as control in another plate. Inoculated plates were aerobically incubated at 37 °C for 3 days.

ii - Experiments with the CFSs of GNRB and GPRB cultures. Wells (6 x 6 mm) were aseptically cut in the *Legionella* inoculated BCYEA plates and each was filled with the filtered CFSs obtained from of GNRB and GPRB cultures. Sterile tap water was used as control. Plates were aerobically incubated at 37 °C for 3 days. After the incubation period, diameters of the inhibition zones of *Legionella* growth formed around each of the GNRB and GPRB strains tested were measured with a plate stereomicroscope (Toze *et al.*, 1990). Results were recorded as positive when there was a clear inhibition zone and negative when there was no discernible inhibition. The inhibition zone diameters (ø, cm) were compared with the control experiment.

Determination of sensitivity to heat and proteolytic enzymes of inhibitory cell-free supernatants. Cell-free supernatants of GNRB and GPRB cultures which exhibited bactericidal effect against tested *L. pneumophila* strains were characterised by their sensitivity to heat and proteolytic enzymes.

Sensitivity to heat. In order to evaluate the heat sensitivity, CFSs were kept at 70, 90 or 121 °C (autoclaving) for 20 min, separately. Then, a disc diffusion test was performed as described above, to detect residual activity (Hechard *et al*., 2005).

Sensitivity to proteolytic (hydrolytic) enzymes. CFSs were treated with proteinase K and trypsin, each at a final concentration of 0.5 mg ml^{-1} (Laukova, 2001). The samples with and without proteases were incubated at 37 °C for 1 h, followed by the determination of the residual activity. Discs impregnated with CFSs (treated with proteases) were placed onto the surface of the inoculated BCYEA plates using a nichrome wire loop. The activity of each sample was compared to the non-treated CFSs by the disc diffusion assay. Plates were incubated in an air atmosphere, at 37 °C for 3 days.

After the incubation period, diameters of the inhibition zones of *Legionella* growth around each disc were measured using a plate stereomicroscope (Toze *et al.*, 1990). Results were accepted as positive when there was a clear inhibition zone, and negative when there was no discernible inhibition. The inhibition zone diameters (cm) were compared with the control.

Stimulation of *Legionella* strains by GNRB and GPRB strains. The same GNRB and GPRB strains tested for inhibitory actions were also assayed to test their stimulatory effect on *L. pneumophila* growth.

Both strains of *L. pneumophila* were suspended in sterile tap water to obtain a concentration of 1.5 x 10^8 CFU ml^{-1}, then, 100 µl of inocula were spread on the surface of BCYEA lacking L-cysteine plates. Stimulatory effects of the complete culture or the CFSs of GNRB and GPRB strains on *L. pneumophila* were investigated. These assays were carried out as described above for *Legionella* growth inhibition assays. Cultures or CFSs (20 µl) were spotted on BCYEA lacking L-cysteine plates already inoculated with *L. pneumophila.* Each experiment was done in triplicate. After inoculation, the plates were sealed in polyethylene bags to prevent dehydration (Surman *et al*., 1994) prior to incubation at 37 °C for 14 days. The plates were then examined for the existence of *Legionella*-like colonies proximal to the GNRB and GPRB colonies. *Legionella*-like colonies were tested by *Legionella* Latex Aglutination Test Kit (Toze *et al*., 1990; Paszko-Kolva *et al*., 1991). Results were considered positive when a satellite growth of *Legionella* colonies was observed around colonies of the GNRB and GPRB strains (Wadowsky and Yee, 1983; Toze *et al*., 1990).

RESULTS AND DISCUSSION

In the current study, ten samples were collected and examined from different locations (cooling tower water, biofilm from cooling tower tank, tap water) in seven different hotels in the vicinity of Istanbul city. Among all isolates, two strains were found as *L. pneumophila* serogroup 2-14 (S1 and S2), fifteen as GNRB and nine as GPRB.

The fifteen GNRB strains were identified as three *Aeromonas hydrophila* strains (A1, A2 and A3), two *Aeromonas* spp. (A4 and A5), *Acinetobacter* spp. (Ac1), *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) (Bur1), *Comamonas testosteroni* (formerly *Pseudomonas testosteroni*) (C1), *Chryseobacterium indologenes* (Ch1) (formerly *Flavobacterium indologenes*), *Escherichia coli* (E1), *Moraxella lacunata* (M1), *Pasteurella* spp. (P1), *Pasteurella pneumophila* (P2), *Pantoea* spp. (Pa1) (formerly *Enterobacter* spp.) and *Pseudomonas* spp. (Ps1). According to the results of API CH and API 20E strips, five of the nine GPRB strains were identified as *Bacillus pumilus* strains (B1, B2, B3, B4 and B5) and one *Bacillus circulans* strain (B6); and the remaining three strains were identified as *Brevibacillus brevis* (Br1, Br2, Br3) (formerly *Bacillus brevis*).

Legionella pneumophila is the most common pathogenic species of *Legionella*, with 15 serogroups described (Brenner *et al*., 1988). Results from various epidemiological and ecological studies relevant to *Legionella* bacteria indicate that the most frequently isolated environmental *Legionella* type is *L. pneumophila* serogroup 2-14 (Baskin *et al*., 1998; Zeybek *et al*., 2003). In the current study, because among all the bacteria found, two strains were isolated as *L. pneumophila* serogroup 2-14, to evaluate GNRB and GPRB strains antagonistic activities were used *L. pneumophila* serogroup 2-14.

Among a total of 24 strains screened for inhibitory activity, *Aeromonas* spp. (A5), *P. pneumophila* (P2), *Pantoea* spp. (Pa1) and *B. pumilus* (B2, B3, B5) culture strains inhibited the growth of both tested *Legionella* strains (S1 and S2) (Table 1). CFSs of all the 24 strains did not inhibit the growth of S1 strain (Table 1). On the other hand, it has been shown that both cultures and the CFSs of *A. hydrophila* (A1) and *Aeromonas* spp. (A4) only inhibited

TABLE 1 - The inhibitory effect of the cultures and CFSs of isolated GNRB and GPRB against the growth of the *Legionella pneumophila* SG 2-14 strains S1 and S2

	Non-*Legionella* bacteria	Cultures		Cell-free supernatant	
		S1	S2	S1	S2
GNRB	*Aeromonas hydrophila* (A1)	-	+(2.0)	-	+(1.9)
	Aeromonas hydrophila (A2)	-	-	-	-
	Aeromonas hydrophila (A3)	-	-	-	-
	Aeromonas spp. (A4)	-	+(1.5)	-	+(1.5)
	Aeromonas spp. (A5)	+(3.2)	+(1.9)	-	+(1.4)
	Acinetobacter spp. (Ac1)	-	-	-	-
	Comamonas testosteroni (C1)	-	+(2.0)	-	+(1.4)
	Chryseobacterium indologenes (Ch1)	-	-	-	+(1.3)
	Escherichia coli (E1)	-	+(2.8)	-	-
	Moraxella lacunata (M1)	-	-	-	-
	Pasteurella spp. (P1)	-	-	-	-
	Pasteurella pneumophila (P2)	+(1.0)	+(1.1)	-	-
	Pantoea spp. (Pa1)	+(3.4)	>4	-	+(1.3)
	Pseudomonas spp. (Ps1)	-	-	-	-
	Burkholderia cepacia (Bur1)	+(3.0)	-	-	-
GPRB	*Bacillus pumilus* (B1)	-	>4	-	+(1.3)
	Bacillus pumilus (B2)	>4	>4	-	+(1.0)
	Bacillus pumilus (B3)	+(3.7)	+(4.0)	-	-
	Bacillus pumilus (B4)	-	-	-	-
	Bacillus pumilus (B5)	+(1.8)	+(2.1)	-	-
	Bacillus circulans (B6)	-	-	-	-
	Brevibacillus brevis (Br1)	-	+(1.6)	-	+(1.6)
	Brevibacillus brevis (Br2)	-	+(1.6)	-	+(1.7)
	Brreibacillus brevis (Br3)	-	-	-	+(1.5)

+: inhibitory effect, -: no inhibitory effect, ( ): inhibition zone (ø, cm).

TABLE 2 - Inactivation, after treatment with proteolytic enzymes and high temperature exposition, of the CFSs of GNRB and GPRB cultures which exhibited inhibitory effect against tested *Legionella pneumophila* strain S2

	Non-*Legionella* bacteria	Enzymes		Temperatures		
		Proteinase K	Trypsin	70 ºC	90 ºC	121 ºC
GNRB	*Aeromonas hydrophila* (A1)	-	-	-	-	-
	Aeromonas spp. (A4)	-	-	+	+	-
	Aeromonas spp. (A5)	-	+	-	-	-
	Chryseobacterium indologenes (Ch1)	-	-	+	+	+
	Comamonas testosteroni (C1)	-	-	-	-	-
	Pantoea spp. (Pa1)	-	-	-	-	-
GPRB	*Bacillus pumilus* (B1)	-	-	-	-	-
	Bacillus pumilus (B2)	-	-	-	-	-
	Brevibacillus brevis (Br1)	-	-	-	-	-
	Brevibacillus brevis (Br2)	-	-	-	-	-
	Brevibacillus brevis (Br3)	-	-	-	-	-

+: inhibitory effect, -: no inhibitory effect.

the growth of S2 strain. Similarly, both cultures and the CFSs of *B. pumilus* (B1) and *B. brevis* (Br1 and Br2) and *Comamonas testosteroni* (C1) only inhibited the growth of S2 strain (Table 1). Our results agree with those of Gomez-Lus *et al*. (1993), Toze *et al*. (1993), and Cotuk *et al*. (2005) where the inhibitory effects of a broad spectrum of substances from *Aeromonas* were demonstrated to be effective against *L. pneumophila*. The inhibition ratio of the CFSs and the cultures of Gram-negative rod bacteria (GNRB) and Gram-positive rod bacteria (GPRB) on the growth of *L. pneumophila* strains were 26-47% and 33-67%, respectively, on BCYEA. Toze *et al*. (1990) found that between 16 and 32% of the heterotrophic bacterial strains were able to inhibit the growth of *Legionella* species grown on BCYEA, similarly our results. In contrast to the results of other studies (Toze *et al*., 1990; Gomez-Lus *et al*., 1993), where *Pseudomonas* strains were able to inhibit the growth of *Legionella* species on BCYEA, our *Pseudomonas* strain was unable to affect the growth of *L. pneumophila*.

The inhibitory activity exhibited by the complete bacterial cultures was higher than that exhibited by the supernatants (Table 1), and this may be due to the production of bacteriocins (or bacteriocin-like substances), organic acids, and surfactants-like compounds (Bevilacqua *et al*., 2003). According to the results, this activity appeared to be associated with the cell wall, from where it was probably transferred into culture supernatant (Rokka *et al*., 2006).

It was found that strains that belong to the same genus behaved differently during the inhibition tests; confirming the results of previous studies (Bevilacqua *et al*., 2003; Cotuk *et al*., 2005), supporting the idea that even within the same species the inhibitory activity is strain-dependent. On the other hand, it is also possible that one isolate may produce more than one antagonistic substance with different physico-chemical and biological properties (Gaetti-Jardim and Avila-Campos, 1999).

It has been reported in several studies that different *Bacillus* species produced antibacterial proteins against various bacteria (Lee *et al*., 2001; Cetinkaya *et al*., 2003; Cladera-Olivera *et al*., 2004; Zeybek *et al*., 2005; Yilmaz *et al*., 2006). Nevertheless, to our knowledge, this is the first study in view of the determinations of the inhibitory effects of *B. pumilus* and *B. brevis* against *Legionella*.

Inactivation after treatment with proteolytic enzymes is a key criterion in antibacterial compounds (Rokka *et al*., 2006). It was shown that the antibacterial compounds by bacteria were inactivated by both proteolytic enzymes (trypsin and proteinase K) except trypsin for *Aeromonas* spp. (A5) strain (Table 2). The inhibitory compounds are proteinaceous constitution, which is a general property of bacteriocin family (Savadogo *et al*., 2004). The inhibitory activity of CFSs of *Chryseobacterium indologenes* (Ch1) was completely stable after being heated at 70, 90 °C and autoclaving 121 °C. Activity of substances produced by *Aeromonas* spp. (A4) remained stable when held at 70 and 90 °C but lost its activity against *L. pneumophila* (S2) after autoclaving at 121 °C. This is in agreement with the results of Hechard *et al*. (2005) who defined that an antibacterial protein produced by bacteria might be a bacteriocin. These results demonstrated that the inhibitory activity of *Chryseobacterium indologenes* (Ch1) and *Aeromonas* spp. (A4) was due to a proteinaceous substance sensitive to proteinases but exhibiting heat stability. The results from the heat treatments suggest that inhibitory substances produced by our isolated strains could be a peptide rather than a protein since peptides are less sensitive to high temperature treatments.

There are a few studies related to stimulatory effect of bacteria on the growth of *L. pneumophila* (Wadowsky and Yee, 1983; Stout *et al*., 1986; Cotuk *et al*., 2005). In these studies, it was reported that formation of the satellite colonies of *L. pneumophila* around the peripheral edge of a bacterial growth zone indicated nutritional symbiosis. Since cysteine is an essential amino acid for the growth of *L. pneumophila*, the stimulation test (satellite growth) confirms the ability of bacteria to provide this metabolic substitute and demonstrates the potential for a symbiotic relationship between microorganisms (Wadowsky and Yee, 1983; Stout *et al*., 1986; Toze *et al*., 1990).

In the current study, it was detected that one *A. hydrophila* (A3), one *Aeromonas* (A5) and *B. pumilus* (B4) (Fig. 1) exhibited stimulatory effect on only the S2 strain and developed satellite growth. This is in agreement with the results of Cotuk *et al*. (2005). In these experiments, it was found that 13% of the strains were capable of stimulating the growth of *L. pneumophila*. Although it was previously shown that the growth of several *Legionella* species had been inhibited by *Bacillus* species, there is no result to demonstrate their stimulatory effect on the growth of *Legionella* species. Therefore, to our knowledge, this is the first report about the growth stimulation of *Legionella* by bacilli. It was

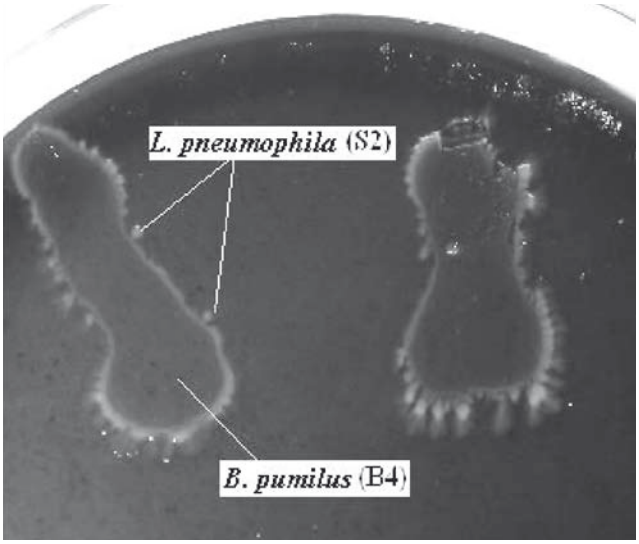

FIG. 1 - The stimulatory effect of isolated *Bacillus pumilus* (B4) (Culture) against the growth of *Legionella pneumophila* (S2) on BCYEA lacking L-cysteine.

detected that none of the CFSs showed any stimulatory effect on the growth of *L. pneumophila* on BCYEA lacking L-cysteine (Table 3). While *Pseudomonas* strains were previously reported as stimulants for the growth of *L. pneumophila* (Koide *et al*., 1989; Cotuk *et al*., 2005), results of the current study demonstrated no evidence for this kind of an effect.

In the present study, it has been demonstrated that *Aeromonas* spp. (A5) which inhibited the growth of several *Legionella* species could also stimulate the growth of the same species when both the test strain and the *Legionella* species were grown on BCYEA lacking the essential amino acid L-cysteine (Table 1). The results of Toze *et al*. (1990) also supported these findings and indicated that production of the inhibitory substances by heterotrophic bacteria depend on the presence of amino acid L-cysteine, in some cases.

The results indicated that growth and multiplication of legionellae could be affected by different bacteria sharing the same habitat and the level of this effect varies among the species. These data are not surprising, because two investigated *Legionella* strains were isolated from different locations (cooling tower water, tap water) from the hotels and may be different serogroups of *L. pneumophila* (serogroup 2, serogroup 3,...etc). This is in agreement with the opinions of Maillard (2002) who defined that antimicrobial activity varies significantly among different types of microorganisms; moreover, it might also differ between different strains of the same species.

Overall, it could be postulated that some of the characteristics of these compounds are consistent with ecological functions (Barja *et al*., 1989; Toze *et al*., 1990; Bevilacqua *et al*., 2003; Cotuk *et al*., 2005). They might play an important role in the control of *L. pneumophila* outbreaks, and their influence could be either inhibitory or stimulatory. The inhibitory substances produced by bacteria might contribute into the natural inactivation of microorganisms in water systems. In addition to their ecological functions, the biologically active substances produced by the bacteria might provide new compounds with yet to be explored industrial applications. Moreover, bacterial substances supportive for *L. pneumophila* that could be isolated from cultures might facilitate further studies comparing the activity of disinfectants and other control strategies on naturally occurring against *L. pneumophila*.

TABLE 3 - The stimulatory effect of the cultures and CFSs of isolated GNRB and GPRB against the growth of the *Legionella pneumophila* SG 2-14 strains S1 and S2

	Non-*Legionella* bacteria	Cultures		Cell-free supernatant	
		S1	S2	S1	S2
GNRB	*Aeromonas hydrophila* (A1)	-	-	-	-
	Aeromonas hydrophila (A2)	-	-	-	-
	Aeromonas hydrophila (A3)	-	+	-	-
	Aeromonas spp. (A4)	-	-	-	-
	Aeromonas spp. (A5)	-	+	-	-
	Acinetobacter spp. (Ac1)	-	-	-	-
	Comamonas testosteroni (C1)	-	-	-	-
	Chryseobacterium indologenes (Ch1)	-	-	-	-
	Escherichia coli (E1)	-	-	-	-
	Moraxella lacunata (M1)	-	-	-	-
	Pasteurella spp. (P1)	-	-	-	-
	Pasteurella pneumophila (P2)	-	-	-	-
	Pantoea spp. (Pa1)	-	-	-	-
	Pseudomonas spp. (Ps1)	-	-	-	-
	Burkholderia cepacia (Bur1)	-	-	-	-
GPRB	*Bacillus pumilus* (B1)	-	-	-	-
	Bacillus pumilus (B2)	-	-	-	-
	Bacillus pumilus (B3)	-	-	-	-
	Bacillus pumilus (B4)	-	+	-	-
	Bacillus pumilus (B5)	-	-	-	-
	Bacillus circulans (B6)	-	-	-	-
	Brevibacillus brevis (Br1)	-	-	-	-
	Brevibacillus brevis (Br2)	-	-	-	-
	Brevibacillus brevis (Br3)	-	-	-	-

+: stimulatory effect, -: no stimulatory effect.

The benefits possibly offered by our isolates and the chemical characterisation of the antibacterial compounds produced by the non-*Legionella* bacteria are subject to further studies, according to results of these examinations, a new source can be obtained for industrial applications.

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