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Research Article

Inhibition of Legionella pneumophila by *Bacillus* sp.

This study presents the potential of a biological control of *Legionella pneumophila*. It was verified to what extent the enrichment of various *Bacillus* species in water may decrease or prevent *L. pneumophila* from growing in water. During in vitro tests, *B. subtilis* BS104 was able to induce an average decrease in *L. pneumophila* numbers of $1.9 + 0.2$ log units after 120 h. Furthermore, the spore and cell free filtrate of *B. subtilis* BS104 also decreased *L. pneumophila* by $2.6 + 0.4$ log units after 120 h. An addition of *Bacillus* BS104 to a cooling tower test system with a water volume of 8 m^3 resulted in a *L. pneumophila* level below 1000 CFU/L after 3 weeks, whereas the starting point was 5.3×104 CFU/L. Further experiments also indicated that *B. subtilis* BS104 might inhibit the internal replication of *L. pneumophila* in *Acanthamoeba castellanii*. This paper demonstrates that certain strains of *Bacillus* have the potential of reducing the number of viable *L. pneumophila* in water, or at least prevent its increase. These results are the first indication that a biological abatement of *L. pneumophila* could be possible.

Keywords: Biological control, Health care, Pathogenic microorganisms

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1 Introduction

Improved detection and reporting of *Legionella* infections demonstrate that *Legionella pneumophila* poses a rising threat to human health, despite large investments in its abatement [16, 29]. Because person-to-person transmission of *Legionella* has not yet been observed, measures to prevent the bacterium from spreading have concentrated on the elimination of this (opportunistic) pathogen from water sources [6]. *Legionella* eradication seems to be very difficult and multiple alternative water treatment techniques have been designed in addition to the conventional heat treatment. These include chemical and physical techniques such as UV radiation, ozonization, sonication, Copper/Silver ionization, chlorination and filtration, all showing variable efficiency, safety and costs [14]. Heat treatment remains the most applicable and recommended technique, although recent studies demonstrate contradictory effects due to the re-growth of *Legionella* in water systems [30]. Newly discovered survival strategies of the pathogen entail an additional shortcoming of many water treatment techniques towards *Legionella* eradication [6]. Hence, innovative approaches to interfere with the normal microbial ecology of *L. pneumophila* are warranted.

A new approach in the abatement of certain pathogenic microorganisms is the use of antagonistic microorganisms. This approach is based on the application of probiotic bacteria in human and animal intestines to create a pathogen unfriendly environment, stimulating the host's overall health. Expansion of the probiotic concept towards the environment has designated a biocontrol, when the application is antagonistic towards a certain pathogen [11]. One of the most successful applications today is the use of *Bacillus* sp. in aquaculture to reduce the detrimental effects by the pathogenic *Vibrio* bacteria [13, 31, 33]. Another example of an environmental application was recently described by Zhao and co-workers [36], using probiotic *Enterococcus* and *Lactococcus* strains in drains of meat processing facilities to reduce the incidence of *Listeria* sp. infections. Despite the negative image due to *B. anthracis*, the genus *Bacillus* comprises multiple probiotic species such as *B. subtilis, B. claussii, B. coagulans, B. licheniformis* and *B. pumilus*, being commercially available for human consumption for years [8]. During the complex processes of sporulation and desporulation, *Bacillus* species produce multiple components demonstrating an antagonistic effect towards other microorganisms in its environment [1, 5, 10, 21, 24–25, 35].

The goal of this study was to verify whether a range of probiotic *Bacillus* species could exert an antagonistic effect on the

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number of *L. pneumophila* in water and, as such, be used as a biological abatement strategy.

2 Materials and Methods

2.1 Strains and Cultivation

The strains used in the course of this study were *L. pneumophila* sg1 (ATCC 33152), *Bacillus subtilis* (BS10 and BS104), *Bacillus pumilus* (BP16), *Bacillus cereus* (B104, ATCC 14893) and *Acanthamoeba castellanii* (ATCC 30234). Strains BS10, BS104, BP16 and B104 were supplied as freeze-dried cultures by Artechno (Liège, Belgium). *L. pneumophila* was grown aerobically in BYE broth or on BCYE agar plates for 72 h at 37 °C (Edelstein, 1981). *Bacillus* sp. was grown on Nutrient Agar (Oxoid, Basingstoke, UK) for 24 h at 30 °C. *A. castellanii* was grown aerobically in ATCC medium 711 for 5 days at 28 °C. The cell suspensions for the subsequent experiments were prepared by centrifugation of a fully grown culture for 4 minutes at 5000 × g for bacteria and 600 × g for *Acanthamoeba* (Minispin, Eppendorf, Hamburg, Germany) and a suspension of the pellet in 0.22 μm filter sterilized tap water. In order to remove all nutrients originating from the growth medium, each suspension was washed twice by centrifugation and again suspended in sterile tap water. Optical density measurements at 610 nm (ISIS9000, Dr. Lange, Berlin, Germany) were performed to obtain the desired starting concentration of each cell suspension, as determined initially by means of plate counting.

2.2 *Legionella* Inhibition Tests

Several experiments were performed to verify the ability of four *Bacillus* species to reduce the number of *L. pneumophila* in water. Using sterile glass test tubes, 5 mL of sterile tap water was inoculated with a final concentration of approx. 10^5 CFU/ml *L. pneumophila* and approx. 10^7 CFU/mL *Bacillus* sp. One tube contained only *L. pneumophila*, which was used as a negative control. Incubation was performed aerobically at 37 °C. Samples of 1 mL were taken at 24 h and 120 h in order to determine the number of *L. pneumophila* by means of real-time PCR.

Using the best performing strain, *Bacillus subtilis* BS104, a new experiment was conducted in order to verify the optimal concentration of *Bacillus* required to maximally reduce *L. pneumophila*. Again, 5 mL of sterile water was supplemented with 10^5 CFU/mL *L. pneumophila* and *B. subtilis* BS104 concentrations ranging from 10^4 to 10^9 CFU/mL.

Finally, from a 24 h inoculated *B. subtilis* BS104 suspension (approx. 10^{10} CFU/ml) at 37 °C, a spore and cell free filtrate was prepared and 100 μL was added to 10^5 CFU/mL *L. pneumophila* containing tap water. Again, a *L. pneumophila* supplemented test tube of sterile water served as a negative control. Samples of 1 mL were taken at 24 h and 120 h in order to determine the number of *L. pneumophila* by means of real-time PCR and plate counting on BCYE agar plates. Supplementally to plate counting and real-time PCR, flow cytometry was applied to demonstrate the inhibition of *L. pneumophila* by *B. subtilis* BS104. To detect and quantify the number of live and dead *L. pneumophila* cells, 1 mL samples were stained for 10 minutes with the LIVE/DEAD® BacLight™ Bacterial Viability kit (Invitrogen, Merelbeke, Belgium), according to the manufacturer's instructions. Stained samples were analyzed with a Cyan™ LX flow cytometer (Dakocytomation, Heverlee, Belgium) [2].

2.3 Cooling Tower

In order to verify the effect of *Bacillus* on the number of *L. pneumophila* in larger volumes of water, a test system was used which simulated an industrial cooling tower. The system consisted of 6 square water reservoirs of 1 m^3 and 1 reservoir of 2 m^3 on which a small cooling tower was mounted. The reservoirs were open to the air and filled with water originating from an electrical power plant cooling tower. Each reservoir was equipped with a heating device to prevent the water temperature from dropping below 21 °C. The refreshment rate of the water was 50 L per day. The number of *L. pneumophila* in the water was monitored weekly until the addition of *Bacillus* BS104, after which the sampling frequency was increased to every 4 days during 3 weeks and again weekly for another 3 weeks. *Bacillus* BS104 was added twice, with a 10 day interval, to a final concentration of 10^6 CFU/L in the water.

2.4 Amoeba

To verify the effect of the amoeba on the *Legionella* inhibitory effect of *Bacillus subtilis* BS104, test tubes containing 5 mL of sterile tap water were inoculated with *(i) L. pneumophila*, *(ii) L. pneumophila* + *A. castellanii* and *(iii) L. pneumophila* + *A. castellanii* + *B. subtilis* BS104. The starting concentrations as determined using OD measurements for *L. pneumophila*, *B. subtilis* and *A. castellanii* were approx. 10^5 CFU/mL, 10^7 CFU/mL and 10^5 CFU/mL, respectively. Incubation was carried out at 30 °C. Samples for real-time PCR quantification of *L. pneumophila* were taken after 0, 24 and 120 h.

2.5 Proteases

To verify whether the *Legionella* inhibitory effect of *Bacillus* might be due to the production of proteases, an experiment using the protease inhibitor was performed. A protease inhibitor mix consisting of Pefabloc SC (Serva, Heidelberg, Germany) and Na_2-EDTA (Merck, Darmstadt, Germany) was prepared to block serine proteases and metalloproteases, respectively. Test tubes containing 5 mL of sterile tap water were inoculated with *(i) L. pneumophila*, *(ii) L. pneumophila* + *B. subtilis* BS104, *(iii) L. pneumophila* + *B. subtilis* BS104 + protease inhibitors and *(iv) L. pneumophila* + protease inhibitors. Starting concentrations as determined by OD measurements for *L. pneumophila* and *B. subtilis* were approx. 10^5 CFU/mL and 10^7 CFU/mL, respectively. Incubation was carried out at 30 °C. Samples for real-time PCR quantification of *L. pneumophila* were taken after 0, 24 and 120 h. Furthermore, the effect

of the protease inhibitors on the viability of *B. subtilis* BS104 was determined by adding 100 µL of the inhibitor mix to a 5 mL 10^7 CFU/mL *Bacillus* suspension in sterile tap water. Plate counting of a 10-fold dilution series after 24 h was performed.

2.6 Identification of *L. pneumophila*

In order to verify the purity of the *L. pneumophila* culture used throughout the experiments, a latex agglutination test kit (Oxoid, Basingstoke, UK) was employed following the manufacturer's instructions. Furthermore, real-time PCR, flow cytometry and DGGE analyses [4] gave some additional information on the identity of the strain used.

2.7 DNA Extraction

The majority of experiments provided 1 mL samples which were directly suitable for DNA extraction [2]. In the case of the cooling tower experiment, 1 liter of water was filtered using sterile filtration units (250 mL, Nalgene, Rochester, USA) equipped with a 0.22 µm filter (47 mm, Millipore, Bedford, USA). Subsequent DNA extraction was performed directly on the filter paper based on the protocol described in [2].

2.8 Real-Time PCR

Real-time PCR was based on the protocol described by Wellinghausen and co-workers [34] for the specific quantification of *L. pneumophila*, amplifying the *mip* gene. PCR was performed in 25 µL reaction mixtures using the qPCR™ Core Kit for Sybr™ Green I as described by the manufacturer (Eurogentec, Liège, Belgium) in MicroAmp optical 96-well reaction plates with optical caps (PE Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). The thermal profile was as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 sec, 57 °C for 1 min and 60 °C for 1 min. Amplicon dissociation curves were determined by constant fluorescent measurement during a final 60 °C to 95 °C heating at a 0.1 °C/sec ramping speed. The template DNA in the reaction mixtures was amplified in triplicate and monitored with an ABI Prism SDS 7000 instrument (PE Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). The construction of the *mip* gene standard curve was performed by means of a dilution series of an *L. pneumophila* culture in sterile tap water.

3 Results

Bacillus subtilis (BS10 and BS104), *Bacillus pumilus* (BP16) and *Bacillus cereus* (B104, ATCC 14893) were screened for their potential to decrease the numbers of *L. pneumophila* in sterile tap water. From a set of multiple *L. pneumophila* strains, the most actively growing strain (determined by growth curve analysis) was chosen for all experiments. Measurements after 24 h did not show a significant decrease in *L. pneumophila* and are not presented. Compared to the *L. pneumophila* control, after 120 h both *B. subtilis* BS104 and *B. pumilus* BP16 were able to induce a maximum decrease in the *Legionella* numbers of 2.1 and 1.2 log units, respectively, whereas *B. cereus* B104 and *B. subtilis* BS10 did not show any significant result (see Fig. 1). The experiments were performed in triplicate and the average drop in *L. pneumophila* numbers for the best performing *Bacillus* strain (*B. subtilis* BS104) was 1.9 ± 0.2 log units. Besides real-time PCR, also flow cytometry using Live/Dead staining was used to determine the effect of *B. subtilis* BS104 on *L. pneumophila* in sterile tap water (see Fig. 2). A clear separation between *L. pneumophila* cells (lower left region) and *B. subtilis* cells (upper right region) in the scatter plot was possible and facilitated the quantification of intact cells from both bacterial species. When comparing Fig. 2A with Fig. 2B it becomes clear that the fraction of *L. pneumophila* in the suspension drops drastically, whereas *B. subtilis* remains stable. Because of the disappearance of dots in the *Legionella* region of the scatter plot, it appears that *Bacillus* induces a cell lysis within the *L. pneumophila* population. Using flow cytometry, a 1.8 log unit drop in *L. pneumophila* after 120 h was measured, confirming earlier results by means of real-time PCR.

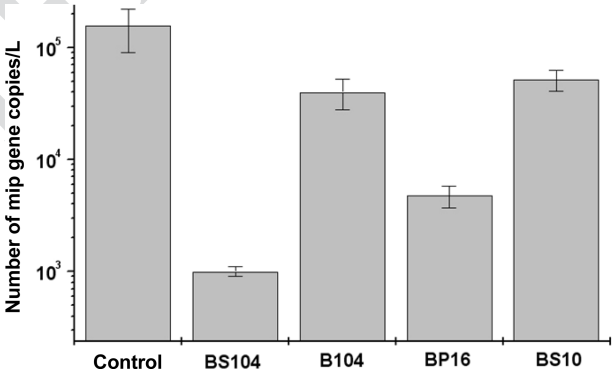

Figure 1. Inhibitory action of *Bacillus* sp. towards *L pneumophila*. The values are means of triplicate experiments and result from real-time PCR quantification of the *mip* gene after 120 h. Control: *L. pneumophila* in water; BS104: *B. subtilis* BS104; B104: *B. cereus* B104; BP16: *B. pumilus* BP16; BS10: *B. subtilis* BS10.

Owing to the clearly better results obtained with *B. subtilis* BS104, compared to other species, it was decided to perform further experiments with this strain. In order to determine the optimal concentration of *Bacillus* required to induce a significant *Legionella* reduction, a 10-fold dilution series of *Bacillus* was mixed with a fixed concentration of *L. pneumophila*. At least 10 times more *Bacillus* (10:1) is required to obtain a significant inhibitory effect against *Legionella*, with a maximum effect at a 100:1 ratio or higher (data not shown).

In order to verify whether the inhibitory effect is due to competitive exclusion or the result of an extracellular component produced by *Bacillus*, a spore and cell free filtrate was prepared from a dense *B. subtilis* BS104 suspension (10^{10} CFU/mL). This filtrate was screened for its *L. pneumophila* inhibitory effect, compared to the effect of a *B. subtilis* BS104 suspen-

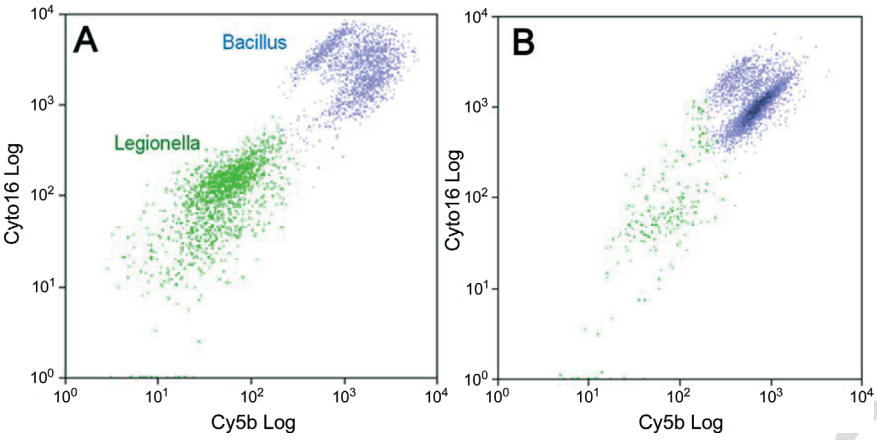

Figure 2. Inhibitory action of *B. subtilis* BS104 towards *L. pneumophila* as determined by means of Live/Dead™ staining using flow cytometry.
A. Measurement at the starting point, the blue dots represent *B. subtilis*, the green dots represent *L. pneumophila*; B. measurement at 120 h.

sion itself. From Fig. 3 it can be concluded that the spore and cell free filtrate has a significant *Legionella* inhibitory effect. The remaining numbers of *L. pneumophila* were near the detection limit of real-time PCR and were therefore confirmed by means of plate counting. This showed a decrease in the *L. pneumophila* numbers from an initial concentration of $1.5 \times 10^5 \pm 3.3 \times 10^4$ CFU/mL to 650 ± 280 CFU/mL in the case of the addition of the filtrate, whereas the negative control remained nearly stable at $7.2 \times 10^4 \pm 1.6 \times 10^4$ CFU/mL after 120 h. Filtrate originating from a more feasible 10^6 CFU/mL *Bacillus* suspension had no significant effect (data not shown).

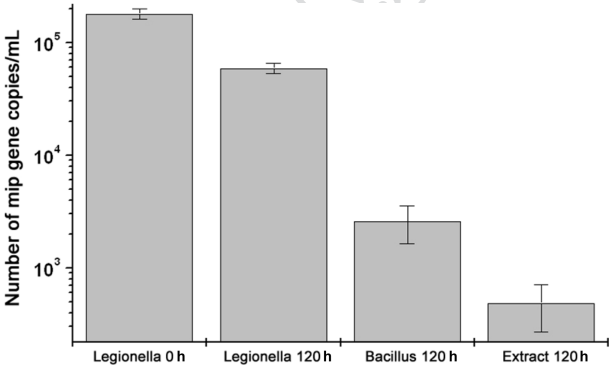

Figure 3. Inhibitory action of *B. subtilis* BS104 filtrate towards *L. pneumophila* as determined by means of real-time PCR.
Bacillus represents *B. subtilis* BS104.

The strong *L. pneumophila* reducing capacity of the *Bacillus* filtrate suggests that at least one active component needs to be produced extracellularly. An experiment introducing a mix of the protease inhibitors Pefabloc SC and Na_2-EDTA was performed to block serine proteases and metallo-proteases. The results are presented in Fig. 4 and indicate indeed that proteases are involved in the *L. pneumophila* inhibitory effect of *B. subtilis* BS104. The control again demonstrates a stable *L. pneumophila* population in the tap water, whereas the positive control shows the inhibitory effect of *B. subtilis* BS104. The addition of the protease inhibitor mix neutralizes the effect of *Bacillus*, with no significantly larger decrease in *L. pneumophila* after 120 h compared to the control. Moreover, when the protease inhibitors were added to an axenic *L. pneumophila* suspension in tap water, they did not affect the pathogen at all, demonstrating that the effect of the inhibitors indeed works through *Bacillus*. Also, the protease inhibitors did not influence the viability of a *Bacillus* suspension in water as determined by means of plate counting (data not shown). *L. pneumophila* quantification was performed using real-time PCR only.

The major route for replication of *L. pneumophila* in the environment is by means of internalization in protozoan hosts such as amoeba [19]. To verify whether *B. subtilis* BS104 also has an inhibitory effect towards *Legionella* in the presence of *Acanthamoeba castellanii*, a new experiment in sterile tap water was conducted. In Fig. 5 it can be observed from the control that *L. pneumophila* remains stable in water, whereas the pathogen shows a significant growth of 1 log unit in the presence of *A. castellanii* after 120 h. The addition of *B. subtilis* BS104 did not induce a decrease in the numbers of *L. pneumophila*, although the observed growth in the positive control was inhibited and the level of *Legionella* remained equal to the starting concentration. These results indicate that *B. subtilis* BS104 also counteracts the replication of *L. pneumophila* in protozoa.

A first up-scaling and application of the concept in a representative water system was performed by means of a small cooling tower water circuit of 8000 liters. After 3 weeks, a stable *L. pneumophila* population of approx. 4.5×10^4 CFU/L (see Fig. 6) was obtained. A first addition of *Bacillus* BS104 was performed at day 21, with a final concentration in the water system of 10^6 CFU/L. After 10 days, a 1 log reduction in *L. pneumophila* numbers was witnessed and at day 32, a second similar amount of *Bacillus* BS104 was added to the system. This resulted in a level of *L. pneumophila* below 1000 CFU/L after a further 10 days, indicating that within 3 weeks, the number of *Legionella* can be reduced below the legislative threshold values. Since no third addition of *Bacillus* was carried out, the number of *Legionella* increased again after 1 week. Furthermore, no experiments were performed using the *Bacillus* filtrate because previous experiments showed that the required concentration for these kinds of water volumes was unfeasible.

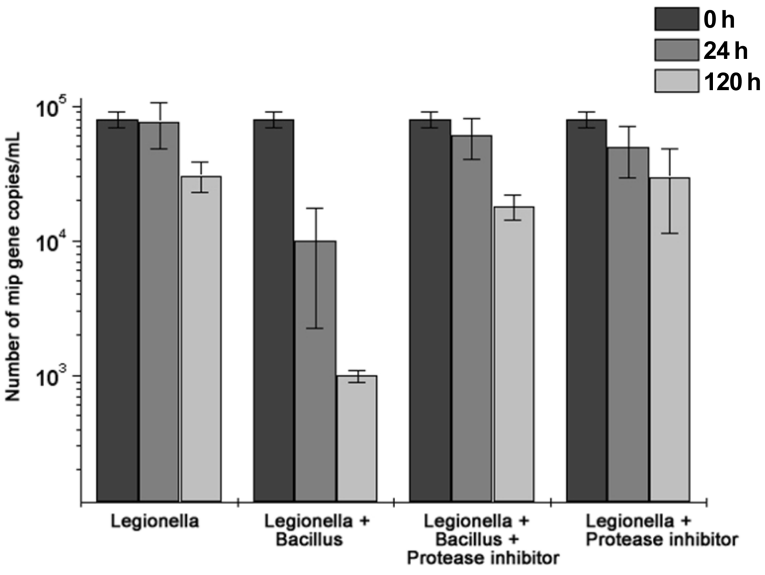

Figure 4. Effect of protease inhibitors on the inhibitory action of *B. subtilis* BS104 towards *L. pneumophila*.
Bacillus represents *B. subtilis* BS104. Measurements by means of *mip* gene specific real-time PCR at 0, 24 and 120 h.

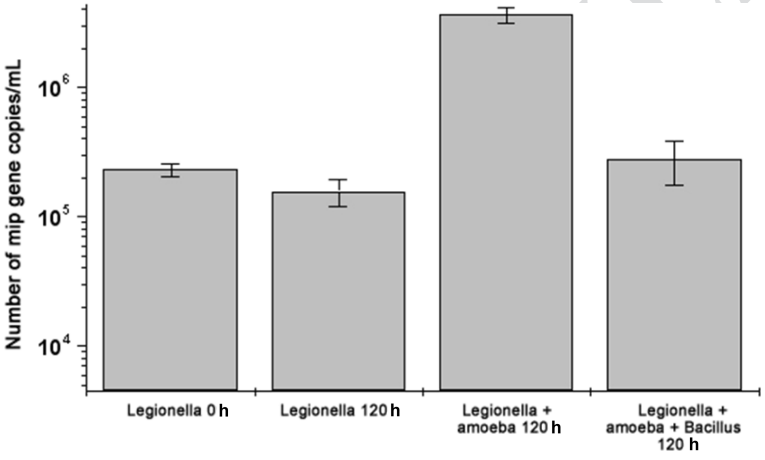

Figure 5. Effect of *A. castellanii* on the inhibitory action of *B. subtilis* BS104 towards *L. pneumophila*.
Amoeba represents *A castellanii*; *Bacillus* represents *B. subtilis* BS104.
Measurements by means of *mip* gene specific real-time PCR.

4 Discussion

Legionella eradication is difficult and multiple alternative water treatment techniques have been designed besides heat treatment. However, the newly discovered survival strategies of the pathogen entail a shortcoming of many water treatment techniques towards *Legionella* eradication [28]. This paper demonstrates the potential of a biological control strategy for *L. pneumophila* in addition to the existing physical and chemical water treatment techniques.

Based on the successful application of certain *Bacillus* species as probiotics in aquaculture [13, 31, 33] four *Bacillus* strains were screened for their potential to counteract *L. pneumophila* in water. Compared to the negative control, two strains induced a significant decrease in the number of *L. pneumophila* after 120 h. Experiments were repeated threefold over time and showed highly reproducible results. Remarkably, the best performing strain (*B. subtilis* BS104) belongs to the same species as the strain not having any effect on *Legionella* (*B. subtilis* BS10). This indicates that the inhibitory action against *Legionella* is strain-specific, a feature often described for probiotic strains [26]. Because of the proof-of-principle nature of this study, additional *Bacillus* strains were not screened in the course of this study. Instead, it was decided to focus on *B. subtilis* BS104 and gain information on the conditions and nature of its activity.

Using flow cytometry, it was shown that *B. subtilis* BS104 induces cell lysis in the *L. pneumophila* population, indicating that certain active compounds might be released by *Bacillus*, or a specific stress response is induced in *Legionella*. Within the *Bacillus* population, some shifts were visible in the scatter plot as well, although microscopic analyses could not reveal whether this reflects changes in the balance between the spores or vegetative cells of *Bacillus*. A mixture of both morphologies remained present throughout the experiments. Additional experiments using multiple ratios of *B. subtilis* BS104/*L. pneumophila* indicated that at least ten times more *Bacillus* is required to induce an effect on *Legionella*. The maximum effect was already obtained as soon as 100 times more *Bacillus* cells are present. Given the fact that *L. pneumophila* rarely exceeds 10 000 CFU/L in water systems, this might indicate that future applications in certain water systems are feasible. Based on our results, the addition of 100,000 CFU/L of an active *Bacillus* strain could already induce a reduction in *L. pneumophila*. This was verified by means of an experiment using an 8 m^3 water system simulating an industrial cooling tower. It was shown that a repetitive addition of *Bacillus* in a feasible final concentration of 10^6 CFU/L decreased the number of *Legionella* to values below most legislative threshold values within 3 weeks. Recolonization of the system by *L. pneumophila* after finishing the addition of *Bacillus* indicates that a repetitive addition of the bacterium is required to keep *Legionella* levels under control, the frequency of which being dependent on the test system. Parameters such as the type of water, volume, temperature, biofilm formation, and total microbial load will influence the efficiency of the biological control and a case-specific preliminary study prior to an actual application to a certain water system is required.

Further insight into the mechanism of this biological control strategy for *L. pneumophila* was obtained using the filtrate of a

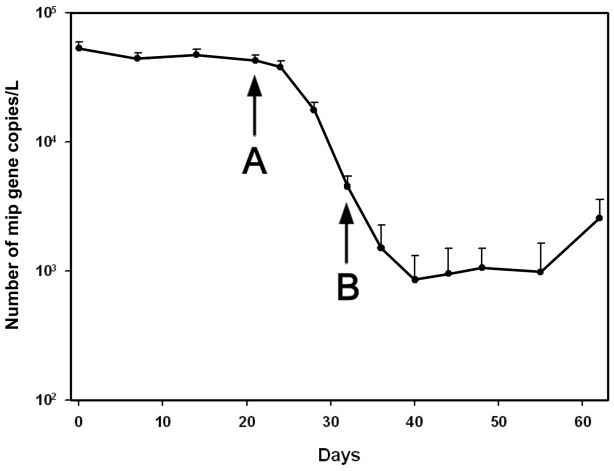

Figure 6. Biological control of *L. pneumophila* in a cooling tower water system by means of *Bacillus* BS104.
A and B: First and second addition of *Bacillus* BS104 in a final concentration of 10^6 CFU/L. The presented values indicate the number of *L. pneumophila* as determined by means of real-time PCR, with error bars representing the standard deviations of the triplicate PCR analyses.

dense *B. subtilis* BS104 suspension. Using this filtrate, an even higher decrease in *L. pneumophila* numbers could be obtained after 120 h compared to *Bacillus* itself. This indicates that an active component is excreted by *Bacillus* into the matrix. Several authors have described that *Bacillus* species produce multiple components demonstrating an antagonistic effect towards other microorganisms in its environment. Some of these components are produced during the complex processes of sporulation and desporulation, whereas others are part of the regular growth and metabolism [1, 5, 10, 21, 25, 35]. Whether the effect towards *Legionella* in our study is the result of one or a mixture of such components, has to be determined in future research. However, the active filtrate originated from a dense 10^{10} CFU/mL *Bacillus* suspension and additional experiments using filtrate from a more realistic 10^6 CFU/mL *Bacillus* concentration did not show any significant effect towards *Legionella*. This indicates that in order to explain the recorded reduction of *Legionella* using a 10^7 CFU/mL *Bacillus* suspension, a combination of competitive exclusion with active components might be involved. Also, the use of the filtrate in water systems, which should be recommendable because of safety regulations, is economically not feasible because of the very high concentration needed.

Bacillus sp. are specific producers of extra-cellular proteases [23]. In order to verify whether these proteases may be partly responsible for the inhibitory effect towards *L. pneumophila*, a mixture of protease inhibitors was added to the experiments. From these results it became clear that proteases may indeed be partly responsible for the *Bacillus* effect on *L. pneumophila*. Viability of both *Bacillus* and *Legionella* in tap water was not influenced by the protease inhibitors themselves, indicating that the *Legionella* reduction is not due to an effect of the inhibitors on *Bacillus* itself. A possible mode of action against *Legionella* is the production of siderophores by *Bacillus subtilis* [18] that may capture the iron required by *Legionella* for growth. EDTA applied in this test might interfere with this siderophore action and counteract the effect on *Legionella*. However, given the large amount of components and enzymes produced by *Bacillus* species throughout their life cycle, further extensive research is needed to identify all components that might contribute to the recorded *L. pneumophila* inhibitory effect.

Various authors assume that in aquatic environments, *L. pneumophila* grows within protozoan hosts and that this interaction is central to the pathogenesis and ecology of *L. pneumophila* [12, 15, 19–20]. Therefore, this study tried to find out whether the addition of *Bacillus* could also negatively influence the replication of *L. pneumophila* in protozoa. Although no actual decrease in *Legionella* numbers was measured, *Bacillus* seemed to be able to inhibit the growth of *L. pneumophila* in *A. castellanii*. Whether this effect is due to an inhibition of the internal replication process or the killing of newly grown *L. pneumophila* exiting *A. castellanii* is not known. Most likely, the latter option will be more realistic, because an inhibition of the internal replication can only be achieved when *Bacillus* itself is internalized. In this way, *Bacillus* could serve as a feed to protozoa, increasing their numbers and in the end also those of *Legionella.*

In conclusion, the results obtained in this study clearly show that certain strains of *Bacillus* are capable of decreasing the number of viable *L. pneumophila* in water. Compared to the recent finding that *Legionella* is able to grow on dead organic matter, this study further demonstrates that in the environment both *Legionella* promoting (e.g. protozoa) and inhibiting (e.g. *Bacillus*) microorganisms are present. Therefore, a well-considered microbial management of the environment is crucial to maintain a healthy (microbial) community.

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