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Research Article

Adverse Effects of Immobilised *Pseudoalteromonas* on the Fish Pathogenic *Vibrio anguillarum*: An *In Vitro* Study

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As a prerequisite for use in marine aquaculture, two immobilisation systems were developed by employing the probiotic bacterium *Pseudoalteromonas* sp. strain MLms_gA3. Their impact on the survivability of the fish pathogen *Vibrio anguillarum* was explored. Probiotic bacteria either grown as a biofilm on ceramic tiles or embedded in alginate beads were added to sterile artificial seawater that contained the fish pathogen. While immobilisation on ceramics followed a recently developed protocol, a medium allowing for alginate microencapsulation was newly developed. Anti-*Vibrio* activities were obtained with both immobilisation systems. The viable cell counts of *V. anguillarum* constantly decreased within the first two weeks of the treatments evidencing the potential of the immobilisation systems for providing probiotic-based protection against this pathogen.

1. Introduction

It is not surprising that probiotics gain more and more importance for human and animal nutrition and welfare as well as for aquaculture, even for marine ornamental fish purposes [1]. Antibiotics, the alternative, frequently provoke resistant phenotypes in harmful germs and, thus, their administration must in general be considered as a critical issue (reviewed in [2]). Also, antibiotics should, and customarily are, being administered solely upon emergence of clear signs of microbial diseases, whereas bacteria with "antagonism toward pathogens" [3] that is probiotics can prophylactically exert their protective capacities already from the time of stocking and subsequently during all phases of life. There are promising previous studies from temperate areas, revealing probiotic protection for a number of different fish species, such as Atlantic cod, Atlantic salmon, and turbot [4–9].

The hitherto commercially available probiotic products for use in aquaculture are usually powders or fluids (such as Sanolife® MIC-F (http://shrimpcare.com/newwp/inve-mic-f-probiotic Access: 12th August 2016) or DrTim's Aquatics® Eco-Balance Probiotic Bacteria for Reef, Nano and Seahorse Aquaria (http://store.drtimsaquatics.com/Eco-Balance-Probiotic-Bacteria-for-Reef-Nano-and-Seahorse-Aquaria_p_389 html Access: 9th July 2015.), resp.), which are readily water soluble and, thus, are immediately available for the fish. It remains, however, at least uncertain whether probiotic planktonic bacteria can withstand intestinal environments, where they encounter low pH values as well as oxygen

limitation. Also, there is a risk of being (completely) absorbed within the aquarium's filter system. Hence, immobilised probiotics, such as *Shewanella putrefaciens* in alginate beads, were previously tested for oral administration in sole [10]. Moreover, it was proven *in vitro* that microencapsulation protects bacteria against the action of digestive enzymes and low pH values [11]. Immobilisation is in general considered to give the cells shelter from harmful conditions while the immobilised bacteria are in the stationary growth phase and efficiently produce secondary metabolites [12]. Although such secondary metabolites are known to be generated by free-living bacteria as well, immobilised microorganisms are probably more potent producers [13, 14]. Secretion of bioactive molecules is indeed rather common for bacteria embedded in particles/beads or fixed on solid surfaces [8, 15–18]. A number of bacteria immobilised in extracellular polymer substances (EPS) were seen to detach from the biofilm and upon dispersal into the environmental fluid they became directly fish-available similar to the nonimmobilised (fluid and powder) formulations [19]. There is also the risk that bacterial bioactive substances in fish tanks have short-term effects only, as the water, including its free-living bacterial charge, is continuously circulated by the filtration system, resulting in a high dilution rate. As a consequence probiotic bacteria may deposit in the filtration system and, thus, soon become hardly available in the water.

In this study we deal with the microencapsulation and immobilisation of a putative probiotic bacterium belonging to the genus *Pseudoalteromonas* for use in marine ornamental aquaculture to analyse its capability to defeat the fish pathogenic *Vibrio anguillarum*. Various species of *Pseudoalteromonas* are known to exert antagonistic actions on a number of bacteria harmful for fish [22–24] and, more specifically, anti-*Vibrio* substances were isolated from a *Pseudoalteromonas* representative [22, 25]. By applying *Pseudoalteromonas* culture supernatants, Vynne [26] demonstrated the inhibition of *V. anguillarum*, a pathogen that affects "various marine and fresh/brackish water fish" [27] and which ubiquitously occurs in marine environments. In this study we use a *Pseudoalteromonas* strain that was previously identified by means of agar diffusion assays to display antagonistic properties against *V. anguillarum* [20].

Pseudoalteromonas sp. strain MLms_gA3 was immobilised on porous ceramic tiles [28] for the potential long-term supply with the probiotic strain and alternatively they were embedded in alginate beads for ensuring also oral administration. From previous studies [20] it is anticipated that the isolated *Pseudoalteromonas* cells will negatively impact *V. anguillarum* and that this effect will be enhanced when immobilised cells are administered.

2. Material and Methods

The following experimental steps were carried out to test the antagonistic effect of the putative probiotic *Pseudoalteromonas* systems against *V. anguillarum*: (i) production of alginate beads and biofilm-coating of ceramic tiles with the isolated *Pseudoalteromonas* strain, (ii) inoculation of sterilised artificial seawater (supplied by Leibniz Center for Tropical Marine Ecology, ZMT, Bremen) with a predefined viable cell count of *V. anguillarum*/*Pseudoalteromonas* sp. strain MLms_gA3 and the insertion of alginate beads/ceramic tiles, respectively, and (iii) monitoring of viable *V. anguillarum* and *Pseudoalteromonas* cell counts over a period of 28 days, on days 0, 1, 3, 7, 14, 21, and 28 in the different setups (Table 1). There were two biological replicates with two parallel test series in each setup. Error bars represent standard deviations of the arithmetic average.

2.1. Strain Selection and Cultivation. *P.* sp. strain MLms_gA3 was originally isolated from aquarium water samples provided by the Leibniz Center for Tropical Marine Ecology GmbH, Bremen, Germany (ZMT). Therefore, the water samples were serial diluted and incubated on marine agar (MA, Carl Roth GmbH and Co. KG, Karlsruhe, Germany) at 30°C (incubator WTC, Binder GmbH, Tuttlingen, Germany). Morphologic different colonies with appropriate growth rate were tested by agar diffusion assay towards fish pathogens relevant for aquaculture [20]. A bacterium, forming yellow colonies, with activity towards the tested fish pathogens was identified as *Pseudoalteromonas* by 16S rRNA analyses [20]. *Pseudoalteromonas citrea* (~95% genome agreement) shaped up as the nearest known neighbour, but the apparent phylogenetic distance did not allow integration of the strain into the same species. MLms_gA3 was routinely cultivated on marine agar at 30°C. The strain was stored on MA at 21°C and inoculated on fresh MA every month. *Vibrio anguillarum* was obtained from the German Collection of Microorganisms and Cell Culture (Leibniz Institute DSMZ, DSM 21597, [29]) and routinely cultivated at 30°C on Nutrient Agar (Carl Roth GmbH and Co. KG, Karlsruhe, Germany) supplemented with 3% w/v sodium chloride (NaCl) and stored at −20°C in Roti®-Store cryo vials (Carl Roth GmbH and Co. KG, Karlsruhe, Germany), respectively. Precultures were incubated for 24 h at 30°C in a water bath shaker at 150 rpm (GFL® 1092, Gesellschaft fuer Labortechnik mbH, Burgwedel, Germany).

2.2. Encapsulation of Pseudoalteromonas sp. Strain MLms_gA3 in Alginate Beads. The extrusion technique [30] was used for microencapsulation of MLms_gA3 cells. For harvesting, cells from 10 mL precultures were collected by centrifugation (10 min, 4500 rpm; Universal 320R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) and the obtained cell pellet was r-suspended in 2 mL marine solution containing NaCl, Tris-HCl, peptone, and yeast extract. Sodium alginate 2% w/v (Carl Roth GmbH and Co. KG, Karlsruhe, Germany) was dissolved at ~70°C in the marine solution to obtain a hydrocolloidal solution. After cooling the solution to ~35°C, 2 mL of the resuspended cell pellet was added to the marine solution with sodium alginate. The mixture was added dropwise into the maturing solution containing 1.5 M calcium chloride [30] by making use of a syringe with an exit diameter of 2 mm (20 mL/Luer Solo, B. Braun Melsungen AG, Melsungen, Germany), which resulted in the formation of alginate beads (supplemental data, Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3683809). The resulted beads (diameter of ~3 mm) were washed in sterilised artificial seawater prior to use. As reference samples,

Table 1: Setups in the different test tubes, each containing 30 mL sterilised artificial seawater ($n = 2$). Blank = alginate beads; neither ceramic tiles were inoculated with *P.* sp. strain MLms_gA3 cells.

	Control						Experiment		
	Pseudoalteromonas sp. strain MLms_gA3			*Vibrio anguillarum*			Sea water inoculated with *V. anguillarum* ($\sim 10^5$ cfu mL^{-1} planktonic cells)		
Test tube number	1	2	3	1	2	3	1	2	3
Sea water inoculated with	Planktonic cells ($\sim 10^4$ cfu/mL)	15 alginate beads containing *P.* sp. strain MLms_gA3	Ceramic tiles with MLms_gA3 biofilm	Planktonic cells ($\sim 10^5$ cfu/mL)	15 blank alginate beads	Blank ceramic tiles	Planktonic MLms_gA3 cells ($\sim 10^4$ cfu/mL)	15 alginate beads containing *P.* sp. strain MLms_gA3	Ceramic tiles with MLms_gA3 biofilm

the effect of "blank" alginate beads (without bacterial addition) on the viable cell count of *V. anguillarum* was tested.

2.3. Biofilm Formation on Ceramic Tiles. As described previously [28] porous ceramic tiles made of alumina (diameter of 17 mm, height of 5 mm, open porosity of ~80%, average pore size of ~5 μm) served as model structure for bacterial immobilisation and the procedure for bacteria immobilisation was performed in accordance with our established protocol [20]. Only MLms_gA3 cells were immobilised for a possible comparison of the effect with alginate beads. The biofilm formation was analysed by scanning electron microscopy (SEM; LEO Type 1530 VP, Carl Zeiss Microscopy GmbH, Jena, Germany) performed in cooperation with nanoAnalytics GmbH (Münster, Germany). As a control the influence of "blank" ceramics on the viable cell count of *V. anguillarum* was tested by incubating a tile in MB containing 1% w/v starch for a period of 14 d at 30°C with a renewal of the medium after 7 d.

2.4. Determination of Viable Cell Counts. Planktonic bacteria (*P.* sp. strain MLms_gA3 and *V. anguillarum*) and released *P.* sp. strain MLms_gA3 cells from alginate beads or from ceramic tiles were determined as colony forming units per millilitre (cfu mL^{-1}). This method would give a gross underestimate of the number of viable cells. For such purpose samples were serially diluted with sterilised artificial seawater in a 96-well plate (Sarstedt AG & Co., Nümbrecht, Germany) and dropped on marine agar (MA) in the case of *P.* sp. MLms_gA3 and on Nutrient Agar containing 3% w/v sodium chloride in the case of *V. anguillarum* followed by incubation for 24 h at 30°C. The colonies were distinguished by their colony morphology: *P.* sp. strain MLms_gA3 forms yellow colonies and *V. anguillarum* forms white colonies. Two aliquots of each sample were serially diluted and 10 μL of the relevant dilutions was plated in triplicate. The antagonistic effects of immobilised and planktonic cells of *P.* sp. strain MLms_gA3 against *V. anguillarum* were tested *in vitro* in 30 mL sterilised artificial seawater in 50 mL tubes (Sarstedt AG & Co., Nümbrecht, Germany), inoculated with ~ 10^5 cfu mL^{-1} of *V. anguillarum* (according to [31]). Planktonic cells of *P.* sp. MLms_gA3 (~10^4 cfu mL^{-1}) or the probiotic *Pseudoalteromonas* immobilisation systems (15 alginate beads or one coated ceramic tile) were added subsequently.

Untreated alginate beads and ceramic tiles as "blank" samples were applied to sterilised artificial seawater inoculated with ~10^5 cfu mL^{-1} of *V. anguillarum*, respectively, to analyse a possible effect of the material on growth or survival of *V. anguillarum*.

The viable cell counts of *V. anguillarum* (~10^5 cfu mL^{-1}) and *P.* sp. strain MLms_gA3 (~10^4 cfu mL^{-1}) were adjusted in 30 mL sterilised artificial seawater. The optical density at 585 nm (OD_{585}) referring to the associated viable cell counts of the precultures was determined after 24 h. When an adequate OD_{585} was reached (~0.190 rel. AU in 1 : 10 dilution for MLms_gA3 and ~0.090 rel. AU in 1 : 10 dilution for *V. anguillarum*; supplemental data, Table S1), the cfu of the bacterial cultures were adjusted to ~10^4 cfu/mL in the case of the *Pseudoalteromonas* strain and ~10^5 cfu/mL in the case of *V. anguillarum* by adding sterilised artificial seawater.

The survival of the bacteria was calculated as cfu mL^{-1}.

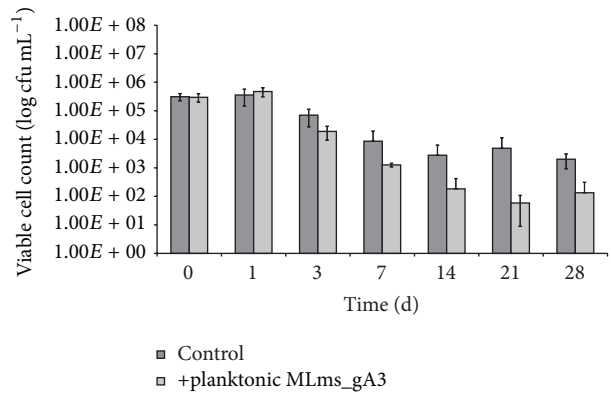

Figure 1: Logarithmic presentation of *V. anguillarum* viable cell counts [cfu mL^{-1}] over a period of 28 days in the presence of planktonic *Pseudoalteromonas* sp. strain MLms_gA3 cells (light grey bars). Viable *V. anguillarum* obtained without the *Pseudoalteromonas* strain served as the control (dark grey bars). The starting inoculum of *V. anguillarum* was for all experiments ~10^5 cfu mL^{-1}; see Table 2 for definite numbers.

3. Results

3.1. Effect of Planktonic P. sp. Strain MLms_gA3 on V. anguillarum. Prior to immobilisation, the survival of *V. anguillarum* was monitored as viable counts over a period of 28 days with and without the addition of planktonic *Pseudoalteromonas* sp. MLms_gA3 cells in order to check the putative probiotic bacterium's capability to exert an antagonistic effect on its pathogenic counterpartin our *in vitro* experimental setup (Figure 1).

For the control setup of *V. anguillarum*, which lacked *Pseudoalteromonas* cells, viable cell counts decreased within the monitored period of 28 days to 2.0×10^3 cfu mL^{-1} ($\pm 1.07 \times 10^3$ cfu mL^{-1}), whereas in the presence of planktonic MLms_gA3, the cfu of the pathogenic bacterium decreased to 1.34×10^2 cfu mL^{-1} ($\pm 1.76 \times 10^2$ cfu mL^{-1}). An antagonistic effect was already evident between day three and day seven as the survival of *V. anguillarum* decreased to 1.26×10^3 cfu mL^{-1} ($\pm 1.83 \times 10^2$ cfu mL^{-1}), while the control still displayed a high level that is 8.63×10^3 cfu mL^{-1} ($\pm 1.08 \times 10^4$ cfu mL^{-1}). Although *V. anguillarum* viable counts constantly decreased over time, irrespective of the addition of *P.* sp. MLms_gA3, the presence of the latter reduced colony formation of the *V. anguillarum* further and more rapidly. Between days 7 and 21 their number clearly decreased in the presence of the probiotic strain and was at any respective reading point between days 7 and 21 lower than for the control.

3.2. Influence of Empty Alginate Beads and Uncoated Ceramic Tiles. To check a possible detrimental or beneficial effect of the material itself on the tested bacterial strain, the viable

Table 2: Viable cell counts (as colony forming units, cfu mL^{-1} on agar plates) of *Vibrio anguillarum* and *Pseudoalteromonas* sp. strain MLms_gA3 in the control, as well as in the treatments with added *P.* sp. strain MLms_gA3 and *V. anguillarum*.

		V. anguillarum		*P.* sp. strain MLms_gA3	
		Control [cfu mL^{-1}]	Treatment (+ *P.* sp. strain MLms_gA3) [cfu mL^{-1}]	Control [cfu mL^{-1}]	Treatment (+ *V. anguillarum*) [cfu mL^{-1}]
Planktonic	0 d	$3.1 \times 10^5 \pm 8.8 \times 10^4$	$3.0 \times 10^5 \pm 9.7 \times 10^4$	$2.7 \times 10^4 \pm 8.8 \times 10^3$	$2.8 \times 10^4 \pm 1.7 \times 10^4$
	28 d	$2.0 \times 10^3 \pm 1.1 \times 10^3$	$1.3 \times 10^2 \pm 1.8 \times 10^2$	$1.2 \times 10^5 \pm 6.5 \times 10^3$	$4.2 \times 10^5 \pm 3.2 \times 10^5$
Alginate	0 d	$3.1 \times 10^5 \pm 1.5 \times 10^5$	$2.1 \times 10^5 \pm 6.4 \times 10^4$	7.5 ± 3.5	2.5 ± 1.2
	28 d	$1.2 \times 10^5 \pm 1.1 \times 10^5$	$3.0 \times 10^3 \pm 3.8 \times 10^2$	$1.1 \times 10^6 \pm 5.1 \times 10^5$	$6.9 \times 10^5 \pm 3.0 \times 10^5$
Ceramic	0 d	$3.4 \times 10^5 \pm 2.0 \times 10^5$	$3.9 \times 10^5 \pm 2.7 \times 10^5$	$4.5 \times 10^4 \pm 2.9 \times 10^4$	$2.9 \times 10^4 \pm 1.1 \times 10^4$
	28 d	$6.8 \times 10^3 \pm 5.0 \times 10^3$	$6.7 \times 10^4 \pm 9.4 \times 10^4$	$1.2 \times 10^6 \pm 8.6 \times 10^5$	$1.5 \times 10^6 \pm 3.0 \times 10^5$

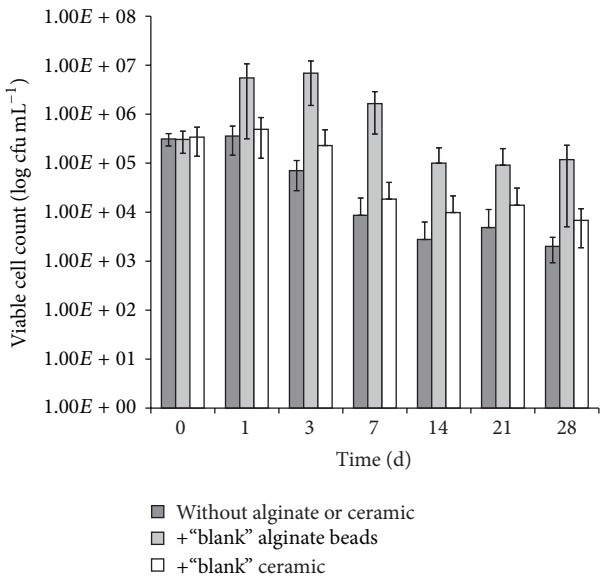

Figure 2: Logarithmic presentation of *V. anguillarum* viable cell counts [cfu mL^{-1}] in the presence of empty alginate beads and ceramic tiles, respectively, and without any material (control). The initial concentration of *V. anguillarum* is the same in both experimental setups (~10^5 cfu mL^{-1}; see Table 2).

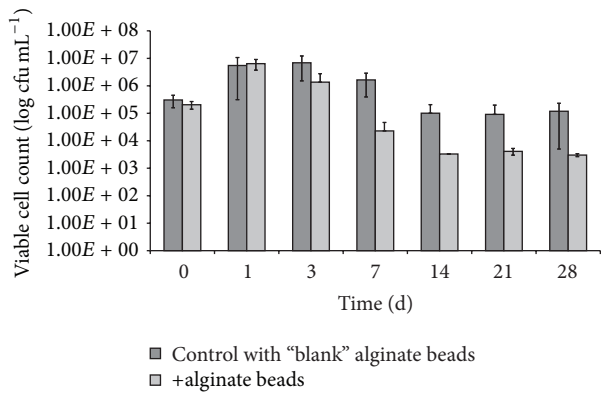

Figure 3: Logarithmic presentation of *V. anguillarum* viable cell counts [cfu mL^{-1}] in the presence of *P.* sp. strain MLms_gA3 loaded alginate beads over a period of 28 days (light grey bars). The survival is presented in comparison to a setup with empty alginate beads as the control (dark grey bars). The initial concentration of *V. anguillarum* was the same in both treatments (~10^5 cfu mL^{-1}; see also Table 2, 0 d alginate).

cell counts of *V. anguillarum* were monitored with "blank" alginate beads or ceramic tiles.

Starting with an almost identical initial viable cell count of *V. anguillarum* in all treatments (~10^5 cfu mL^{-1}; 0 d, Table 2), the cfu of *V. anguillarum* varied within the different samples during the time of incubation (Figure 2). Within the first 24 hours there was a strong increase in the number of *V. anguillarum* cfu in the presence of the "blank" alginate beads (from $3.05 \times 10^5 \pm 1.46 \times 10^5$ cfu mL^{-1} to $5.5 \times 10^6 \pm 5.19 \times 10^6$ cfu mL^{-1}) compared to the setups with the "blank" ceramic (from $3.4 \times 10^5 \pm 2.02 \times 10^5$ cfu mL^{-1} to $4.92 \times 10^5 \pm 3.65 \times 10^5$ cfu mL^{-1}) or to that with seawater only (from $3.13 \times 10^5 \pm 8.78 \times 10^4$ cfu mL^{-1} to $3.58 \times 10^5 \pm 2.12 \times 10^5$ cfu mL^{-1}). Cfu counts dropped only slightly till the end of the experiment in setups with the alginate beads (final $1.19 \times 10^5 \pm 1.14 \times 10^5$ cfu mL^{-1}), whereas in the other two setups (control: $2 \times 10^3 \pm 1.07 \times 10^3$ cfu mL^{-1}; ceramic tile: $6.83 \times 10^3 \pm 4.95 \times 10^3$ cfu mL^{-1}) a rather similar and more pronounced decrease became evident.

3.3. Impact of Immobilised Pseudoalteromonas Cells. As the survival of *V. anguillarum* was reduced in the presence of planktonic MLms_gA3 cells (Figure 1) and, concomitantly, alginate beads as well as the ceramic tiles did not negatively influence but rather increased the survival of the tester strain (Figure 2), the above, potential probiotic *Pseudoalteromonas* strain was encapsulated in alginate (Figure 3) and immobilised on ceramic tiles (Figures 4 and 5), respectively. Subsequently, the influence of the two immobilisation systems on the survival of *V. anguillarum* was tested. For this purpose, sterilised seawater was inoculated with *V. anguillarum* (~10^5 cfu mL^{-1}; see Table 2) and treated with loaded alginate beads or a biofilm coated ceramic tile.

Supporting the findings of the previous experiments using "blank" alginate beads, the number of cfu increased in the *V. anguillarum* control setup (again empty beads) within the first 72 hours (from $3.05 \times 10^5 \pm 1.46 \times 10^5$ cfu mL^{-1} to

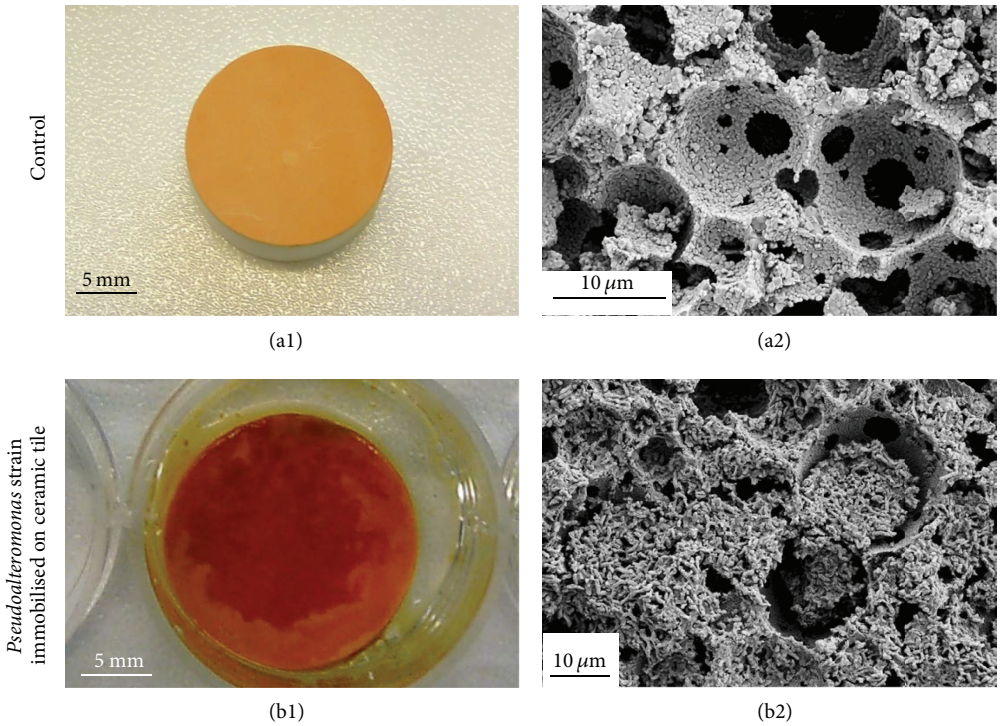

FIGURE 4: Biofilm formation of the *Pseudoalteromonas* sp. strain on a ceramic tile. Ceramics were photographed immediately after the incubation period needed for biofilm formation [20], prior to starting the competition experiment with *V. anguillarum*. The orange-coloured biofilm (b1) is typical for strain MLms_gA3. In parallel SEM analysis revealed the presence of rod-shaped bacteria. The caves of the tiles are filled cells displaying a length of ~2 μm and a width of ~1 μm (corresponding to the known size of *Pseudoalteromonas* with 1 μm × 2-3 μm, [21]). A blank ceramic tile incubated in MB-starch served as a control ((a1) and (a2)).

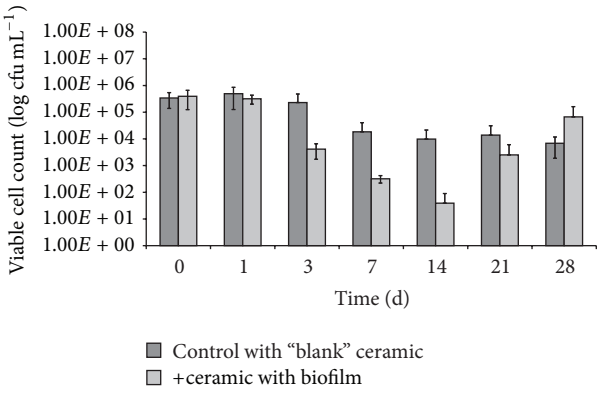

FIGURE 5: Logarithmic presentation of viable cell count measurements [cfu mL^{-1}] of *V. anguillarum* in the presence of a *P.* sp. strain MLms_gA3 biofilm coated ceramic tile over a period of 28 days (light grey bars). A blank ceramic tile served as the control (dark grey bars). The initial concentration of *V. anguillarum* was the same in both treatments (~10^5 cfu mL^{-1}; see also Table 2, 0 d ceramic).

$6.88 \times 10^6 \pm 5.36 \times 10^6$ cfu mL^{-1}) and only slowly decreased, starting rather lately from day 7 ($1.64 \times 10^6 \pm 1.25 \times 10^6$ cfu mL^{-1}) until the end of the experiment on day 28 ($1.19 \times 10^5 \pm 1.14 \times 10^5$ cfu mL^{-1}; Figure 3). In the treatment with the probiotic loaded alginate beads the survival of *V. anguillarum* initially again increased, but such increase was limited to the first 24 hours (reaching a level of $6.42 \times 10^6 \pm 2.71 \times 10^6$ cfu mL^{-1}). This became already evident on day 3 ($1.36 \times 10^6 \pm 1.4 \times 10^6$ cfu mL^{-1}) and until the end of the record (on day 28) the level reached $3.02 \times 10^3 \pm 3.77 \times 10^2$ cfu mL^{-1}. Striking differences to the control were seen already on day 7, when the survival of *V. anguillarum* in the presence of the loaded alginate beads decreased to a considerable lower level that is to 2.28×10^4 cfu mL^{-1} ($\pm 2.36 \times 10^4$ cfu mL^{-1}) versus $1.64 \times 10^6 \pm 1.25 \times 10^6$ cfu mL^{-1} of the control. Thus, the encapsulated *Pseudoalteromonas* strain clearly and effectively compromised the survival of the fish pathogenic *V. anguillarum*.

Immobilisation of MLms_gA3 cells on the ceramic tiles was performed [20]. Scanning electron microscopy (SEM, Figure 4) revealed the successful colonisation of the tile, as a plenty of bacteria were readily detectable on the ceramics that were incubated in Marine Bouillon-starch to which MLms_gA3 cells were added for immobilisation (Figure 4(b2)). The control sample was a "blank" tile that was treated likewise and incubated in MB-starch, but the lack of bacteria was obvious (Figures 4(a1) and 4(a2)).

Subsequently, survival of *V. anguillarum* was monitored in sterilised seawater to which a biofilm coated ceramic tile was added (Figure 5). On the first day there was no strong difference of the cfu between the biofilm coated ceramic ($3.18 \times 10^5 \pm 1.17 \times 10^5$ cfu mL^{-1}) and the "blank" sample ($4.92 \times 10^5 \pm 3.65 \times 10^5$ cfu mL^{-1}). Between day 3 and day 14

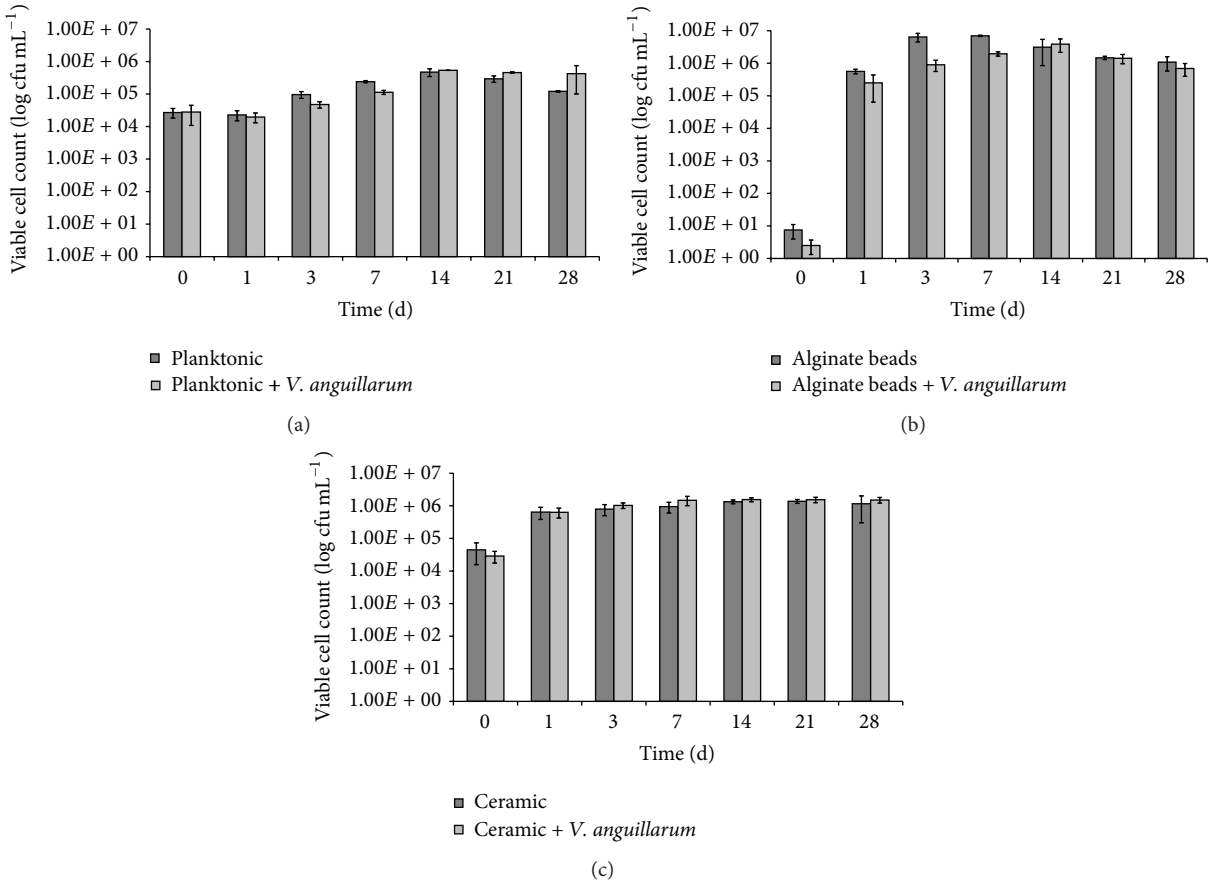

Figure 6: Logarithmic presentation of *P.* sp. strain MLms_gA3 viable cell counts [cfu mL^{-1}] ((a) planktonic, (b) immobilised in alginate beads, and (c) immobilised as an artificial biofilm on a ceramic tile) in the presence of *V. anguillarum* (light grey bars) compared to the viable cell counts without *V. anguillarum* (control, dark grey bars) over a period of 28 days. (a) Initial concentration of planktonic cells was ~10^4 cfu mL^{-1} on day 0. (b) 15 alginate beads were added to each treatment on day 0. (c) One ceramic tile was added to each treatment on day 0.

survival of *V. anguillarum* was clearly negatively affected by the immobilised *Pseudoalteromonas* (day 3: $4.14 \times 10^3 \pm 2.41 \times 10^3$ cfu mL^{-1}; day 7: $3.21 \times 10^2 \pm 1.0 \times 10^2$ cfu mL^{-1}; day 14: $3.92 \times 10^1 \pm 5.07 \times 10^1$ cfu mL^{-1}) in comparison to the control (day 3: $2.3 \times 10^5 \pm 2.53 \times 10^5$ cfu mL^{-1}; day 7: $1.85 \times 10^4 \pm 2.22 \times 10^4$ cfu mL^{-1}; day 14: $9.8 \times 10^3 \pm 1.17 \times 10^4$ cfu mL^{-1}). However, between days 21 and 28 the number of *V. anguillarum* cfu increased again (day 21: $2.52 \times 10^3 \pm 3.54 \times 10^3$ cfu mL^{-1}; day 28: $6.68 \times 10^4 \pm 9.42 \times 10^4$ cfu mL^{-1}).

3.4. Development of the P. sp. Strain MLms_gA3 Viable Cell Counts in the Treatments with V. anguillarum. The viable cell counts of MLms_gA3, either added as planktonic cells or immobilised in alginate beads or on a ceramic tile, were also plotted over a period of 28 days in the presence (Figure 6, light grey bars) and absence of *V. anguillarum* (control, dark grey bars, Figure 6) to determine if there is an effect of *V. anguillarum* on the survival of MLms_gA3. The viable cell counts of MLms_gA3 added as planktonic cells increased within 28 days and were slightly higher than the control starting from day 14. Other than in the control treatment, no decrease in viable cell counts was found between day 14 and day 28 (Figure 6(a)).

As anticipated, when MLms_gA3 was added in the form of alginate beads, almost no released MLms_gA3 bacteria were found in the control (7.5 cfu mL^{-1} ± 3.5 cfu mL^{-1}), as well as in the setup with *V. anguillarum* (2.5 cfu mL^{-1} ± 1.2 cfu mL^{-1}) on day 0 (Table 1 and Figure 6(b)). However, the bacterial counts increased within 24 hours to ~10^5 cfu mL^{-1} in both treatments with a further increase to ~10^6 cfu mL^{-1} until day 28 (Figure 6(b), Table 2). Thus, the survival of MLms_gA3 was almost the same in the setups between day 1 and day 28 but was slightly reduced in the presence of *V. anguillarum*.

Although the ceramics were rinsed three times with sterilised seawater, on day 0 there were ~10^4 cfu mL^{-1} resulting from released MLms_gA3 bacteria at the beginning. The cfu-number of the *Pseudoalteromonas* strain in the treatment with *V. anguillarum* increased as for the control (~10^6 cfu mL^{-1}, Figure 6(c)). In fact, it was even (slightly) higher when the seawater was inoculated with *V. anguillarum*. The MLms_gA3 immobilisation on the ceramic tile (after incubation for 28 days in sterilised seawater) was analysed by SEM to check the condition of the biofilm (Figure 7). Only very few cells were seen to reside in the small cavities of the ceramic. Thus, at the end (after 28 days) the *Pseudoalteromonas* biofilm did not cover the entire tile-surface as at the set out.

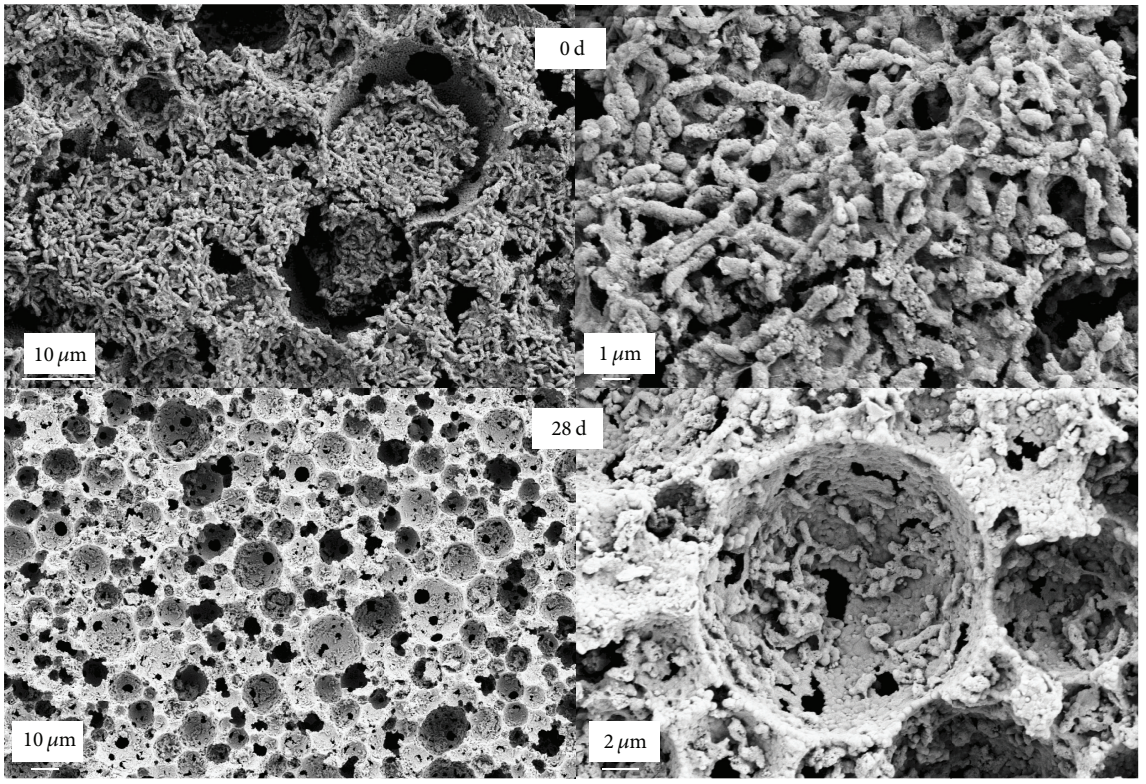

Figure 7: *P.* sp. strain MLms_gA3 biofilm on the ceramic tile after 0 and 28 days of incubation in sterilised artificial seawater. Compared to day 0, in which a clear biofilm was detected on the ceramic tile, there were only single cell aggregates found on the ceramic tile after 28 days of incubation.

4. Discussion

In marine aquaculture, particularly for the breeding of ornamental fish, probiotics are most frequently used as fluid or powder formulations. Presumably, such supplements exert only short-term effects as they may be culled by filtration and/or the free-living bacteria do not survive gastrointestinal passages. When potential probiotic microorganisms are caught by filter systems such beneficial cells are no longer directly available, neither for the parental fish, nor for their eggs or hatching larvae. Hence, aiming at a more continuous supply and availability, especially for substrate spawners, like clownfish and their egg clutches, we developed two immobilisation systems containing a putative probiotic *Pseudoalteromonas* strain that was previously proven to display anti-*Vibrio* activities [20].

Clear reduction of the *V. anguillarum* viable cell counts in the control setup (without any addition of *P.* sp. Strain MLms_gA3) was eventually due to the lack of nutrients in the artificial seawater (starvation). At starvation conditions bacteria are able to survive in smaller cell size [32], which could be checked by electron micrographs or the cell count is reduced, which agrees with findings of previous studies with *V. anguillarum* [33]. On the other hand, MLms_gA3 cell numbers increased in the treatments without *V. anguillarum*. Though the cells, as for *V. anguillarum,* meet starving conditions, viable cell counts increased until day 14. These results correspond with the observation that *Pseudoalteromonas* cells are able to survive for several years "in sterile, unsupplemented seawater" [34]. Cell division and growth were possibly supported by feeding on lysed *V. anguillarum* cells, as the viable cell counts of MLms_gA3 became slightly higher than for the control starting from day 14.

The initial increase of the *V. anguillarum* survival upon addition of "blank" alginate beads may be due to a feeding effect provided by the alginate itself. Extracellular lyases potentially produced by *V. anguillarum* can degrade polymannuronate and polyguluronate, the alginate compounds (reviewed by [35]). The resulting oligosaccharides may serve *V. anguillarum* as carbon and energy sources [36]. It is also conceivable that *V. anguillarum* colonised the "blank" beads, thereby gaining protection [10].

As the survival of *V. anguillarum* was, though slightly, positively influenced in the presence of a "blank" tile, SEM-analysis was performed (supplemental data, Figure S2). Although biofilm formation of *V. anguillarum* was not detectable, cell aggregate formation supported by the ceramic tiles and thereby giving shelter from starvation cannot be excluded. There is no argument that the used material, neither alginate nor ceramics, has a negative influence on *V. anguillarum*.

An alginate feeding effect may also account for the initial increase of the viable *V. anguillarum* counts in the setup with the entrapped *Pseudoalteromonas* cells, a finding that is possibly supported or facilitated by the low number of released probiotic bacteria at the outset of the experiment. In line with such assumption is the subsequently monitored

anti-*Vibrio* effect which is manifested as a reduction of the colony forming units of the latter with progressively decreasing numbers until day 14 and which was accompanied by the simultaneous increase of *Pseudoalteromonas* viable counts.

Pseudoalteromonas citrea, sharing an approx. 95% homology of its 16S rRNA with the strain used in this study [20], produces the rather hydrophobic antimicrobial compound 2-n-pentyl-4-quinolinol (PQ). Originally isolated from a marine pseudomonad [37], PQ was indeed shown to limit the growth not only of *Staphylococcus aureus* but also of *Vibrio* species [38]. As the hydrophobicity precludes its dispersal in the environmental water [14], direct cell-cell contacts are necessary to exert its antimicrobial action. Though a compound with properties similar to PQ lends itself a candidate for the observed antagonistic action, it remains to be elucidated whether the strain used here produces such molecules.

The almost complete lack of the biofilm on the ceramic tile seen after 28 days was probably due to the limited availability of nutrients in the sterilised seawater. Supposingly, upon continuous starvation motile bacterial cells leave the biofilm to seek for further nutrition. This is just the opposite of an adequately supplied biofilm in which cells coalesce and stick together [39]. It is to be anticipated that in *in vivo* experiments, when the *Pseudoalteromonas* biofilm is continuously supplied with nutrients from the seawater (organic and inorganic compounds [40], as well as microalgae [41]) a more stable and robust biofilm is formed. Hence, biofilms grown on ceramic tiles represent an option for a long-term supply with probiotic bacteria. Irrespective of a possibly increased stability of the biofilm in future *in vivo* experiments, the antagonistic action within the first two weeks monitored in the presented *in vitro* setups should already suffice to protect clown fish egg clutches, which mature within eight to nine days [42]. Moreover, it becomes possible to check the applicability and the effect of biofilms on ceramic tiles under aquaculture conditions. Since bacteria, immobilised as stationary phase cells in biofilms, are known to efficiently produce secondary metabolites [12], production of the latter possibly slowed down along with the *Pseudoalteromonas* biofilm breakup, which agrees with increasing *V. anguillarum* viable cell counts from 21 days. Furthermore, the dissolving biofilm may release alimentary substances, such as polysaccharides, DNA, and proteins, which may additionally accelerate the growth of *V. anguillarum*. As the *Pseudoalteromonas* viable cell counts kept nearly constant over the entire period, whereas the number of *V. anguillarum* increased, there seems to be a balance between growth of *Pseudoalteromonas* and concomitant cell death, which have to be checked by, for example, DAPI-staining that records the whole cell number. Since the viable cell count of *Pseudoalteromonas* is not suited to monitor the survival of the biofilm immobilised cells, a live/dead staining [43] should be performed in future experiments.

5. Conclusions

Pseudoalteromonas sp. MLms_gA3 successfully immobilised in alginate beads and on ceramic tiles exhibited promising *in vitro* anti-*Vibrio* activities and, thus, provides the basis for further *in vivo* experiments. Both systems are potentially suited to improve the water quality by the long-term supply with probiotics in marine aquacultures, as they impaired survival of *V. anguillarum* more than the planktonic *Pseudoalteromonas* cells. Alginate beads can furthermore serve as a food supplement, because embedded bacteria are protected from the fish gastrointestinal environment, ensuring their long-term beneficial action. In further experiments amino acids or vitamins could be tested as additives for supporting fish health and the living cell count in the beads could be detected by dissolving them in a sodium bicarbonate solution [10]. Biofilms on ceramic tiles are promising tools for ornamental aquaculture as they may directly protect egg clutches from infestation with pathogenic microorganisms.

Abbreviations

EPS:	Extracellular polymer substances
cfu:	Colony forming unit
d:	Day
Leibniz Institute DSMZ:	German Collection of Microorganisms and Cell Culture
MA:	Marine agar
MB:	Marine Bouillon
NaCl:	Sodium chloride
OD:	Optical density
P. citrea:	*Pseudoalteromonas citrea*
P. sp. strain MLms_gA3:	*Pseudoalteromonas* sp. strain MLms_gA3
rel. AU:	Relative arbitrary units
SEM:	Scanning electron microscopy
V. anguillarum:	*Vibrio anguillarum*
ZMT:	Leibniz Center for Tropical Marine Ecology GmbH, Bremen, Germany.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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