In vitro growth characteristics of five candidate aquaculture probiotics and two fish pathogens grown in fish intestinal mucus

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Abstract

The selection of probiotics for aquaculture is usually based on their antagonism towards pathogens. However, other criteria such as growth, attachment to intestinal mucus and production of beneficial compounds should also be considered. We suggest a protocol for the isolation and selection of potential probiotic bacteria based on their in vitro growth characteristics and propose a ranking index (RI) to screen potential aquaculture probionts. We suggest that the lag period and doubling time are the most important criteria for the comparison of growth curves, hence the RI is based on the doubling time (t_d) and lag period (λ) obtained from the growth profile of each bacterium. Bacteria were isolated from the gut of the common clownfish, Amphiprion percula, and screened for antagonistic activity towards seven aquatic pathogens. All five candidate probiotics showed antagonism to various aquatic pathogens. When grown in intestinal fish mucus no probiotic had a RI higher than the two tested pathogens (Aeromonas hydrophila and Vibrio alginolyticus). However, candidate probiont AP1 had a faster specific growth rate (μ) (0.05) than the pathogens (0.049 and 0.047 respectively), while AP5 grown in marine broth had a shorter lag period than the pathogens. Strategies to increase probiotic concentration include the inoculation of high concentrations and the preconditioning of these bacteria to reduce the lag period. It should be tested whether or not such strategies will allow the probiotic bacteria to dominate initially and thereby gain a competitive advantage. This could become an important aspect under in vivo conditions where both attachment and nutrient supply differ from that found in in vitro studies.

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Keywords: Aquaculture; Probiotic; Growth profile; Fish intestinal mucus; Doubling time; Lag period

1. Introduction

The use of probiotics in aquaculture is becoming increasingly popular, having recently been defined as ‘microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health’ [1]. To date, the screening of probiotics has been pragmatic, lacking meaningful selection criteria, and they are often assessed only on their ability to produce antimicrobial metabolites [2–7].

Isolating bacteria that produce antimicrobial metabolites is common practice, while experiments to determine at what stage of growth the bacteria produce the metabolites and whether the bacteria are able to compete for attachment sites [8] are seldom performed. Such experiments are necessary as bacteria may only produce metabolites during the stationary growth phase [9], and this phase may not occur in the gut due to constant flushing. Thus, in vitro studies may create a false impression of the ability of probiotics to inhibit pathogens in vivo. Any inability to compete for attachment sites on the mucus of the gut wall suggests that these bacteria may not multiply sufficiently fast to compensate for being flushed from the mucus during gut evacuation.

Therefore, screening of candidate probiotics preferably requires that the various selective criteria such as antagonism, production of beneficial compounds, attachment and growth are all considered. This paper suggests a protocol for the isolation and selection of potential probiotic bacteria based on their in vitro growth characteristics. Bacterial growth characteristics such as lag period and doubling time are influenced by more factors than were tested in this study. Potential variables are preincubation,
composition and concentration of ingredients in culture media and temperature. This experiment used the same parameters for all organisms tested thereby allowing for direct comparisons between organisms. Future studies could test and modify the proposed ranking concept depending on different experimental conditions or additional variables. Here, the concept of a ranking index (RI) is proposed to screen potential aquaculture probionts before being tested, thereby reducing the time and expense of in vivo studies.

2. Materials and methods

2.1. Isolation and basic screening

Five adult common clownfish, *Amphiprion percula*, were killed by severing the spinal column. Each fish was surface washed for 1 min with 0.1% benzalkonium chloride to remove external bacteria. Under sterile conditions, the gut region was dissected and separated into stomach and intestine, and were then sliced open. Samples from the two regions were individually masticated in 1 ml of sterile seawater. Serial dilutions were made of each sample to dilutions of $10^{-8}$ from which aliquots of 100 μl were placed on Petri dishes containing media chosen for their general and selective abilities: marine agar is a general non-selective, heterotrophic bacterial media, MacConkey agar is selective for Enterobacteriaceae, thiosulfate-citrate-bile salt agar (TCBS) selects for the genus *Vibrio*, and *Pseudomonas*-isolating agar selects for the genus *Pseudomonas*. The media were prepared according to the manufacturer’s directions (Difco). The Petri dishes were incubated for 48 h at 25°C after which colony-forming units (CFU) were counted. Counts between 30 and 300 CFU were used for analysis.

2.2. Antagonism to pathogens

A bacterial growth inhibition study to test for the production of antimicrobial metabolites by the isolates was performed using seven fish pathogens: *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Vibrio harveyi*, *Vibrio anguillarum*, *Vibrio damsela*, *Vibrio alginolyticus* and *Carnobacterium piscicola*. Pathogens were incubated in marine broth for 24 h at 25°C and incorporated into pour plates. Wells were cut into the agar and filled with 100 μl of the marine broth isolates. The presence of antimicrobial metabolites produced by the isolates inhibited the growth of the pathogen producing a zone of inhibition around the well. To determine at what stage of growth the antimicrobial metabolites were produced, a 50-ml volume of marine broth was inoculated with 4 ml from a 36-h culture and incubated on a shaker at 25°C. 5-ml samples were taken every 6 h for 48 h, centrifuged (5000 rpm for 10 min) and the supernatant was filtered through a 0.22-μm filter. Pathogen pour plates, made as described above, were then inoculated with 100 μl of the supernatant. After 48 h, zones of inhibition were recorded as being present or absent.

2.3. Growth

To further reduce the number of candidate probionts for in vivo studies, those that inhibited only one pathogen were excluded leaving 12 candidate probionts (from an original total of 106 isolates). Based on antagonism to the same pathogens, similar colony morphology characteristics (Gram stain, shape, color, motility, oxidase production, catalase production and motility) and similar growth profiles in marine broth, duplicate candidate probionts were eliminated. The growth profiles of the bacteria were determined by inoculating the wells of a sterile 96-well microtiter plate with 250 μl of marine broth to which a 20-μl volume of 24-h-old marine broth bacterial culture was added. The plate was incubated at 25°C and the optical density (OD) recorded at 640 nm (BioTek Powerwave ELX808 Microplate Reader/KC Junior software) every 15 min for 36 h. Each culture was inoculated in triplicate and the readings of the profiles were averaged.

Growth profiles of five candidate probionts and two pathogens (*A. hydrophila* and *V. alginolyticus*) were used to compare characteristics such as length of lag phase ($\lambda$) and maximum specific growth rate ($\mu$). These two probionts were selected as they are both known to grow on intestinal mucus [10,11].

To assess their potential ability to grow in the gut, all bacteria were grown in marine broth or in intestinal fish mucus that was filter-sterilized (0.22 μm) and diluted to 1 mg ml$^{-1}$ using Bradford’s protein assay [12]. The bacteria were inoculated in triplicate into wells of a sterile microtiter plate with 250 μl of sterile mucus or marine broth to which a 20-μl volume of a 36-h-old marine broth bacterial culture was added. The plate was incubated for 36 h at 25°C during which time the OD was read every 15 min at 640 nm.

The OD$_{640}$ reference values were fitted to the logistic (Eq. 1) and Gompertz (Eq. 2) functions [13]. These functions are non-linear models in which the exponential increase or decrease is corrected by an exponential term.

$$y(t) = \frac{a}{1 + be^{-ct}} \quad \text{(logistic growth model)}$$

$$y(t) = ae^{(-e^{-bt})} \quad \text{(Gompertz growth model)}$$

where $e$ = natural log (2.718), $y$ = population size, $t$ = the incubation time and $a$, $b$ and $c$ are estimated coefficients.

The estimates within most sigmoidal growth curves usually have no biological meaning and therefore both models were rewritten according to the method proposed by [14] who substituted the mathematical parameters with $A$ (maximum biomass yield), $\mu$ (maximum specific growth rate) and $\lambda$ (lag period) (Fig. 1). This is achieved by deriv-
ing the biological parameters as a function of the parameters of the basic function and then substituting them into Eqs. 1 and 2, respectively. An example using the logistic growth Eq. 1 follows:

To determine the inflection point of the curve, the second derivative of the function with respect to $t$ was calculated (Eq. 3).

$$\frac{d^2y}{dt^2} = abc e^{-ct} \frac{b e^{-ct} - 1}{(1 + be^{-ct})^2}$$

(3)

At the inflection point, where $t = t_i$, the second derivative is equal to zero (Eq. 4).

$$\frac{d^2y}{dt^2} = 0 \rightarrow t_i = \frac{\ln b}{c}$$

(4)

The maximum specific growth rate ($\mu$) was derived by calculating the first derivative at the inflection point ($t_i$).

$$\mu = \left(\frac{dy}{dt}\right)_{t_i} = \frac{ac}{4}$$

(5)

Here, $c$ can be substituted by $c = 4\mu a$.

The lag period ($\lambda$) is defined as the $t$-axis intercept of the tangent line through the inflection point and can be described as:

$$y = \frac{a}{2} \left( \frac{1}{2} \lambda t + 1 + \ln b \right)$$

(6)

Using Eqs. 4, 5 and 6 yields:

$$\lambda = \frac{2}{c} \left( \frac{\ln b}{2} - 1 \right)$$

(7)

The parameter $b$ in the logistic Eq. 1 was substituted by:

$$b = e \left( \frac{4\mu a \lambda}{a^2} \right)$$

(8)

The asymptotic value is reached for $t$ approaching infinity:

$$t \rightarrow \infty \rightarrow y \rightarrow a \Rightarrow \lambda = a$$

(9)

The parameter $a$ in the logistic Eq. 1 can be substituted by $\lambda$, yielding the modified logistic equation:

$$y = \frac{A}{1 + e^{-\frac{4\mu_b(\lambda - t_i) + 2}{A}}}$$

(10)

Similarly, the Gompertz Eq. 2 can be solved using the parameters $\mu$, $\lambda$ and $A$ to produce:

$$y = Ae^{-\frac{\mu}{A} e^{-\frac{t}{\lambda}} + 1}$$

(11)

The growth models were fitted to the data for the period from the start of incubation ($t=0$) to the start of the stationary phase since the osmotic swelling of bacteria in a hypotonic medium can considerably decrease the opacity, probably through lowering the refractive index of the cells [15]. Curve fitting and estimation of the kinetic parameters was performed using the Lavenberg–Marquardt method [16], which minimizes the sum of square differences between measured and predicted values. Starting values are estimated by searching for the steepest ascent of the curve between four data points (estimation of $\mu$) and where this intersects the $x$-axis (estimation of $\lambda$), the final data point is taken as an estimation for the asymptote ($A$).

The doubling time ($td$), which is the time taken to double the biomass during the exponential growth period is described as:

$$td = \frac{\ln 2}{\mu}$$

(12)

In two cases, one of the three growth profiles was distinctly different from the other two (probably due to contamination) and was discarded in the analysis. To provide an equal starting point for growth of each bacterium, the data were blanked using the average absorbance at $t=0$.

The model for each bacterium was chosen based on the results of the following four steps: (i) the calculation of $r^2$, the coefficient of determination, (ii) a residual pattern analysis, (iii) the biological interpretation of parameters $\mu$, $\lambda$ and $A$, and (iv) an estimation of the models’ $F$ value.

Since this experiment was designed to aid the screening of large numbers of potential fish probiotics, a RI was developed using the parameters’ doubling time ($td$) and lag period ($\lambda$) converted into integer hours and minutes (i.e., $3h40 = 3.67$).

$$RI = \frac{1}{\lambda \times td} \times 100$$

(13)

The RI hypothesizes that a bacterium with a short lag period and short doubling time has a better chance of outcompeting other bacteria based on their growth characteristics.
3. Results

3.1. Isolation and screening

Bacterial growth on the various media is summarized in Table 1.

3.2. Antagonism to pathogens

Pathogenic bacterial growth was greatest on marine agar. The number of vibrios in the intestine was high and contributed 59% as compared to pseudomonads, which contributed less than 10%. The stomach microbiota was composed of 4% pseudomonads and less than 0.001% vibrios. Antimicrobial metabolites were produced by all candidate probiotics during the stationary phase and no zones of inhibition were observed during the lag or exponential phase.

The five candidate probiotics collectively showed antagonistic activity against the seven pathogens. Candidate probiotic AP3 showed the greatest antagonistic activity, inhibiting five of the pathogens. The pathogens *V. damsela* and *C. piscicola* had the highest number of antagonists with four each (Table 2).

3.3. Growth

All models gave a visually good fit to the data \((P < 0.01)\). Only models with \(r^2\) values greater than 0.90 were considered for analysis. The residuals were plotted for each model and checked by eye to ensure they conformed to a normal distribution. \(F\) values were determined for each model with the best model being chosen based on the highest \(r^2\) value and highest \(F\) value. The Gompertz model was the best-fitting model for eight of the 15 growth profiles.

All bacteria grew in both the marine broth and gut mucus. The growth profiles in marine broth were typical, single-phase profiles. Growth in mucus, however, produced a diauxic profile for *V. alginolyticus* (Fig. 2). The lag period was greater for all bacteria in mucus when compared to growth in marine broth (Table 3): however, \(\mu\) was greater for AP1, AP4 and *V. alginolyticus* when grown in mucus. The growth parameters of candidate probiotic and pathogenic bacteria grown in marine broth and intestinal fish mucus are shown in Table 3. Fig. 2 depicts the growth of candidate probions AP2 and AP5, and pathogens *V. alginolyticus* and *A. hydrophila* grown in fish gut mucus. No model for *V. alginolyticus* was plotted because the profile is diauxic and rather two separate models were used, one for each \(\lambda\), \(\mu\), and \(A\) of the two stages. The growth curves in Fig. 2 are representative of others obtained in this study.

The RIs of AP1, AP2 and AP5 were highest for that among all candidate probiotics when grown in fish intestinal mucus (Table 3). The average RI for the pathogens was 2.65 for marine broth and 2.89 for mucus (only the first model of *V. alginolyticus* growth is included). The candidate probiont AP4 showed slow growth in marine broth and failed to reach an asymptote after 36 h. As a result, the line depicting growth was almost linear and the \(y = 0\) portion of the curve intercepts the \(t\)-axis on the negative quadrant to yield a negative \(\lambda\).

4. Discussion

The aim of this study was to provide a method for the

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### Table 1

Bacterial cell concentrations based on CFU isolated from the gut and stomach of the common clownfish *A. percula* on various standard microbiological media

| Medium                  | Gut (CFU ml\(^{-1}\)) | N  | Stomach (CFU ml\(^{-1}\)) | N  |
|-------------------------|------------------------|----|---------------------------|----|
| Marine agar 2216e       | \(6.78 \times 10^6\)   | 11 | \(2.72 \times 10^7\)     | 12 |
| MacConkey agar          | \(8.75 \times 10^4\)   | 6  | \(1.75 \times 10^5\)     | 1  |
| TCBS agar               | \(4.01 \times 10^8\)   | 4  | \(3.08 \times 10^9\)     | 2  |
| Pseudomonas-isolating agar | \(6.75 \times 10^5\)   | 2  | \(1.10 \times 10^6\)     | 1  |

\(N\) = number of plates on which CFU ranged between 30 and 300.

### Table 2

Antagonism (\(^*\)) between candidate probiotics (AP1–AP5) and fish pathogens grown on marine agar

| Probiotics | A. salmonicida | A. hydrophila | V. alginolyticus | V. harveyi | V. anguillarum | V. damsela | C. piscicola |
|------------|----------------|---------------|------------------|------------|----------------|------------|--------------|
| AP1        | \(\times\)     | \(\times\)    | \(\times\)       | \(\times\) | \(\times\)     | \(\times\) | \(\times\)    |
| AP2        | \(\times\)     | \(\times\)    | \(\times\)       | \(\times\) | \(\times\)     | \(\times\) | \(\times\)    |
| AP3        | \(\times\)     | \(\times\)    | \(\times\)       | \(\times\) | \(\times\)     | \(\times\) | \(\times\)    |
| AP4        | \(\times\)     | \(\times\)    | \(\times\)       | \(\times\) | \(\times\)     | \(\times\) | \(\times\)    |
| AP5        | \(\times\)     | \(\times\)    | \(\times\)       | \(\times\) | \(\times\)     | \(\times\) | \(\times\)    |

The plates were pathogen pour plates within which wells had been punched and the candidate probiotic was added in marine broth.
screening of potential probiotics that could be applied to combat fish pathogens under aquaculture conditions. To accomplish this, the growth of five probiotics in vitro was compared to that of two pathogens. Using estimates of coefficients from two best-fit non-linear growth models, a RI was produced by combining estimates for doubling time $t_d$ and lag period $\lambda$. The assumption was that a probiotic will be most competitive in vivo if it has a short lag phase and a fast growth rate. However, as growth is not the only criterion useful in screening potential probiotics, other modes of competition such as fast growth and ability to attach to the intestinal tract can be considered together with the probiotic’s ability to produce substances antagonistic to pathogens.

In marine broth, candidate probiotics with a shorter lag period and a greater doubling time than the pathogens were AP5 and AP1, respectively. In mucus, the $w$ value of AP1 (0.050) was slightly higher than that of the pathogen $A. hydrophila$.

Table 3

| Treatment       | Model               | Model parameters | $t_d$ | $r^2$ | $F$ value | RI |
|-----------------|---------------------|------------------|-------|-------|-----------|----|
| Marine broth    |                     |                  |       |       |           |    |
| AP1             | Gompertz            | 0.376 0.044      | 2.23  | 15.43 | 0.943     | 17138 2.67 |
| AP2             | logistic            | 0.397 0.062      | 3.31  | 11.14 | 0.996     | 38315 2.54 |
| AP3             | logistic            | 0.447 0.047      | 13.18 | 14.45 | 0.998     | 103220 0.51 |
| AP4             | Gompertz            | 0.208 0.006      | *     | 114.11| 0.983     | 40534 *  |
| AP5             | Gompertz            | 0.253 0.021      | 0.44  | 32.33 | 0.972     | 37260 4.14 |
| $A. hydrophila$ | Gompertz            | 0.342 0.035      | 2.08  | 19.58 | 0.991     | 112181 2.35 |
| $V. alginolyticus$ | Gompertz    | 0.333 0.036      | 1.45  | 19.28 | 0.994     | 189448 2.94 |

| Treatment       | Model               | Model parameters | $t_d$ | $r^2$ | $F$ value | RI |
|-----------------|---------------------|------------------|-------|-------|-----------|----|
| Mucus           |                     |                  |       |       |           |    |
| AP1             | logistic            | 0.376 0.050      | 3.49  | 13.54 | 0.981     | 47830 1.89 |
| AP2             | logistic            | 0.342 0.043      | 5.05  | 16.13 | 0.994     | 81669 1.21 |
| AP3             | Gompertz            | 0.822 0.017      | 14.47 | 39.41 | 0.975     | 7903 0.17 |
| AP4             | Gompertz            | 0.189 0.007      | 22.52 | 95.58 | 0.986     | 10055 0.05 |
| AP5             | logistic            | 0.327 0.019      | 8.19  | 36.33 | 0.993     | 118839 0.33 |
| $A. hydrophila$ | logistic            | 0.393 0.049      | 3.20  | 14.06 | 0.985     | 63874 2.13 |
| $V. alginolyticus$ first model | logistic | 0.250 0.047      | 1.51  | 14.50 | 0.986     | 8179 3.65 |
| $V. alginolyticus$ second model | Gompertz | 0.203 0.025      | 0.37  | 27.13 | 0.94      | 15910 6.04 |

Parameters $A$ (asymptote), $\mu$ (specific growth rate) and $\lambda$ (lag period) were estimated using either the logistic or Gompertz equations, which were selected based on criteria discussed in the text. $RI=1/(doubling \, time \, (t_d) \times \lambda) \times 100$.

*: Negative values not given.
growth should not be viewed in isolation since all probiotics and pathogens caused by the changing size and shape of the microorganisms since these features alter the degree and direction of light scatter [15]. Such species-specific absorbance values limit direct comparison of growth profiles.

These results confirm suggestions by other authors that growth should not be viewed in isolation since all probiotic bacteria produced antagonistic metabolites [17,18]. For example, the probiotic with the longest lag period (AP3) inhibited five of the seven pathogens although its RI was low. The two highest-ranking probiotics showed combined antagonism to three pathogens. Thus, the ability to inhibit the growth of competitors through the production of antimicrobial metabolites is one way a bacterial species is able to establish itself over competitors. In marine fish, the gut’s bacterial microflora varies from species to species with intraspecific interactions [19]. The major taxonomic groups contributing to the healthy intestinal flora of marine fish species include *Vibrio, Pseudomonas* and *Achromobacter*, followed by *Micrococcus, Bacillus* and *Enterobacteriaceae* making up the majority of the remainder [20–23]. In aquaculture, non-pathogenic strains of known pathogens have been successfully used as probiotics [24–26], however, testing whether strains are possibly pathogenic before large-scale use, is strongly advised. Due to the presence of pathogenic and opportunistic bacteria in the environment [27], these organisms need to be suppressed to reduce their proliferation and consequently the incidence of disease. This study provided for the first time an estimation of the growth pattern of two selected pathogens, *A. hydrophila* and *V. alginolyticus*, in fish intestinal mucus. The pathogen *A. hydrophila* exhibited a diauxic growth profile when grown in mucus and a single growth profile when grown in marine broth. This type of growth has been noted in *Escherichia coli* as it sequentially utilizes glucose and then lactose [15]. Fast-growing pathogenic bacteria like *V. alginolyticus* and *A. hydrophila* are considered r-selected species [28]. The rapid growth of these pathogens in intestinal mucus confirms that the intestinal tract serves as a site of growth [29]. If these bacteria are present in the culture environment in low numbers, and given that their lag period appears to be shorter and their maximum specific growth rate higher than that of other bacteria, these species could reach potentially harmful concentrations under in vivo conditions.

The results have practical applications. For example, based on the RIs of the candidate probiotics grown in mucus, AP1 has one growth parameter (μ) greater than that of the pathogens tested. However, the manner in which the potential probiont grows in a bacterial community may be different. Strategies to increase probiotic concentration include the inoculation of probiotics and preconditioning of these bacteria to reduce the lag period. It should be tested whether or not such strategies will allow the probiotic bacteria to dominate initially and thereby gain a competitive advantage. This could become an important aspect under in vivo conditions where both attachment and nutrient supply differ from that found in vitro studies. Here, the growth of the bacteria may be less than the rate of flushing from the intestine, and the bacteria may become unable to attach to the intestinal mucus or the intestinal wall where they will be flushed before reaching a viable population level [30]. A probiotic may colonize the fish intestinal tract and prevent the proliferation of pathogenic or opportunistic bacteria if certain of its growth characteristics are superior to that of the pathogen. This experiment identified no bacteria grown in mucus with a RI greater than that of the two pathogens. Pathogens are usually fast-growing [31], r-selected strategists. Thus, a high starting concentration of probiotics may make them more competitive. This would favor the use of AP3, which showed relatively high inhibition potential. Knowing its growth pattern, the bacteria could be cultured in large numbers before addition to the aquaculture system. For example, as the probiont reaches its maximum specific growth rate μ, the pathogen, having a smaller population size, will only be reaching its own maximum μ at an overall smaller population size. Although the doubling time of some of the candidate probiotics may be longer than those of the pathogens, it must be noted that the concentration of probionts introduced into the water is likely to be much higher (from $10^5$ to $10^7$ cells ml$^{-1}$) than that of the pathogens. A slow rate of bacterial colonization has been shown to be beneficial to turbot larvae during the critical period of gut development [32], suggesting that an optimal dose exists that allows sufficient numbers of the probionts to compete with the pathogens.

Substrate availability is another factor to be considered when predicting the outcome of in vivo studies based on results obtained from in vitro experiments. The Monod relation [34] predicts bacterial growth rate (μ) as a function of substrate concentration. Mucus in the intestine is likely to be a replenishable growth substrate occurring at concentrations greater than that used in this experiment. Likewise, in vivo bacterial growth may be faster than that found in this experiment. Consequently, the RIs of some
bacteria may increase as $\lambda$ would remain constant while $\mu$ increases.

Furthermore, the effect of probiotics on previously established microflora and on the proliferation of opportunistic bacteria both in vitro and in vivo warrants further investigation using microbiologically matured seawater with its diverse microflora. Matured seawater is dominated by non-opportunistic (K-strategist) bacteria with a high substrate affinity and better competitive ability at low substrate supply as compared to opportunist (r-strategist) bacteria [31,35]. For example, microbial maturation of tank water has been shown to enhance larval survival and growth [35]. The bacterial concentration of seawater has been shown to range from $7 \times 10^3$ cells ml$^{-1}$ [36] to $3.7 \times 10^4$ cells ml$^{-1}$ [37] while microbiologically matured seawater contains approximately $7.5 \times 10^4$ bacteria ml$^{-1}$ [38] and relatively more K-selective species [27].

In conclusion, probiotics in vivo may become more competitive than predicted from experimental observations of their growth patterns or their starting population density as the production of antimicrobial metabolites or better attachment ability lead to inhibition in vitro. The production or effect of inhibitory metabolites depends on the media on which the probiotic is cultured [39]. Further tests examining the production of antimicrobial metabolites in fish mucus need to be carried out to verify preliminary conclusions from this study and account for the factors that affect the ranking of candidate probiotics in vivo. Furthermore, the methods developed in this study can help in the screening process of probiotics in human and terrestrial animal nutrition.

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