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Poly-β-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*

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Abstract The bacterial storage polymer poly-β-hydroxybutyrate (PHB) has the potential to be used as an alternative anti-infective strategy for aquaculture rearing. In this research, the effects of (partially) replacing the feed of European sea bass juveniles with PHB were investigated. During a 6-week trial period, the PHB showed the ability to act as an energy source for the fish. This indicated that PHB was degraded and used during gastrointestinal passage. The gut pH decreased from 7.7 to 7.2 suggesting that the presence of PHB in the gut led to the increased production of (short-chain fatty) acids. The diets supplemented with 2% and 5% PHB (*w*/*w*) induced a gain of the initial fish weight with a factor 2.4 and 2.7, respectively, relative to a factor 2.2 in the normal feed treatment. Simultaneously, these treatments showed the highest bacterial range-weighted richness in the fish intestine. Based on molecular analysis, higher dietary PHB levels induced larger changes in the bacterial community composition. From our results, it seems that PHB can have a beneficial effect on fish growth performance and that the intestinal bacterial community structure may be closely related to this phenomenon.

Keywords Prebiotics · Antibiotics · Infection · Growth promoting agent · Host–microbe interactions

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Introduction

The ban on the use of antibiotics in both aquaculture and terrestrial animal production has challenged researchers throughout the world to look for alternative biocontrol strategies (Karunasagar et al. 2007; Nicolas et al. 2007; Sapkota et al. 2008). Organic acids have been described to be capable of exhibiting bacteriostatic and bacteriocidal properties towards pathogenic bacteria (Thompson and Hinton 1996; Ricke 2003; Vazquez et al. 2005). In general, the mechanism is believed to be caused by the undissociated form of the acid which is able to penetrate through the bacterial cell wall. Once inside, the acid releases its protons (H^+) in the neutral cytoplasm and lowers the intracellular pH. The bacterium redirects its efforts towards the efflux of the excess protons, thereby exhausting the cell metabolism and leading to lower cell growth and even cell death (Goncalves et al. 1997; Hismiogullari et al. 2008).

The research concerning the application of organic acids in aquaculture is limited up to date, although their potential was shown by experiments with the aquaculture model organism *Artemia franciscana*. Upon challenge with *Vibrio campbellii*, several types of short-chain fatty acids (SCFA) dosed at ca. 2 g L^{-1} could double the survival of the test specimens (Defoirdt et al. 2006). However, the use of SCFA may not be as suitable for aquaculture since these

compounds are highly soluble in water. Even formulated within feed particles, part will be lost by diffusion resulting in a low uptake efficiency of the SCFA by the animals. This would require higher feed doses than theoretically necessary and result in an uncontrolled stimulation of microbial growth in the water. Therefore, an approach to effectively concentrate SCFA in the gastrointestinal tract of the fish or shrimp had to be developed.

A solution was found in the form of the bacterial storage polymer poly-β-hydroxybutyrate (PHB). This compound serves as an intracellular energy and carbon reserve for bacteria (Madison and Huisman 1999; Tokiwa and Calabia 2004). It is insoluble in water and has been shown to be biologically degradable into β-hydroxybutyric acid (Defoirdt et al. 2007a). The latter can exhibit growth inhibition towards certain pathogens and protect *Artemia* like other SCFA do (Defoirdt et al. 2007b). As such, if PHB is supplemented through the feed and subsequently degraded in the gastrointestinal tract of aquaculture organisms, the locally released SCFA or PHB oligomers may induce their beneficial effects. In several experiments with *A. fransiscana*, this approach increased the survival up to 73% upon infection with the pathogen *V. campbellii* (Defoirdt et al. 2007b; Halet et al. 2007).

Until now, no attempt has been made to use PHB in the feed of aquaculture animals like fish or shrimp. The goal of this research was to assess the effects of several dietary levels of PHB on the growth performance of juvenile European sea bass in combination with the effects on the gut bacterial community composition.

Materials and methods

Origin of fish and acclimatization conditions

One-month-old juvenile European sea bass (*Dicentrarchus labrax*) were purchased from Ecloserie Marine (Gravelines, France). Upon arrival, they were acclimatized in artificial seawater (Meersalz Professional Salt, 30 ppt salt) for 3 weeks in a climate chamber at 16–18 °C while they were fed formulated feed (Skretting, Boxmeer, The Netherlands) at 3% on wet body weight per day.

Experimental feed preparation

The experimental diets were prepared using formulated feed (Skretting, Boxmeer, The Netherlands) with a pellet size of 3 mm. For each diet, a determined fraction of the basic feed (*w*/*w*) was replaced with an accurately weighed amount of PHB. PHB particles (98% poly-β-hydroxybutyrate–2% poly-β-hydroxyvalerate) with an average size of 30 μm were purchased from Goodfellow (Huntingdon, England). To incorporate the PHB in the feed, the PHB particles were dissolved in a solution of chloroform and distilled water (80:20). The feed particles were coated with the PHB solution by spraying it homogeneously. The coated feed particles were air dried under ventilation for 2 days. In this way, four experimental diets were prepared: 0% PHB (in which no PHB was incorporated in the feed) was used as a control; 2% PHB (in which 2% of the feed was replaced with PHB); 5% PHB (in which 5% of the feed was replaced with PHB); and 10% PHB (in which 10% of the feed was replaced with PHB). The 100% PHB diet (in which all the feed was replaced with PHB) consisted of the PHB particles without any manipulation. The caloric value of each of the feeds was determined by means of IKA-calorimeter C7000 (Staufen, Germany).

Experimental design

The experiment consisted of six tanks (in duplicate) of 200 L placed in a climate chamber at a temperature of 16–18 °C. Each tank contained 160 L artificial Instant Ocean seawater (aerated and charcoal filtered for at least 24 h) and was stocked 2 weeks before the actual start of the experiment with 75 acclimatized fish at an initial weight of 1.2–1.4 g. The light regime was set at a fixed 14 h light and 10 h dark. Each tank was equipped with an individual filter system (Eheim, Deisizau, Germany) filled with activated charcoal (Calgon Carbon Corporation, Feluy, Belgium) and Rivalon synthetic filter wadding. The tanks were provided with constant aeration using a 3-mm-diameter plastic regulator and airstone. During 6 weeks, the fish in each of the 12 tanks were fed twice a day with a different experimental diet at a total of 3% on their wet body weight per day. This was weekly adjusted based on the weight of the fish at the beginning of each week and the number of fish in the tank. There were six different treatments: (1) a nonfed; (2) a 0% PHB; (3) a 2% PHB; (4) a 5% PHB; (5) a 10% PHB; and (6) a 100% PHB treatment. The water quality was analyzed on a regular basis for the concentration of total ammonia nitrogen, nitrite, and nitrate using Visocolor Test Kits (Marcherey-Nagel, Düren, Germany). When the levels of these exceeded 0.1, 1.0, and 20 mg L^{-1}, respectively, 50% of the water was replaced.

Measured parameters

The survival of the fish was determined weekly for each treatment as the ratio of the number of fish that survived each week over the total number of fish present in the tanks at the start of that week. For determination of the fish weight, every week, four fish were randomly sampled from each tank after 24 h without feeding, blotted dry, and weighed. Subsequently, the fish were dissected, and an

incision was made in the gut for measurement of the intestinal pH by means of a biotrode pH electrode (Hamilton, Switzerland). The average fish weight gain over 6 weeks for each treatment was calculated as follows: the weight of each of the eight fish sampled on the final day of the experiment was subtracted with the average weight of the fish as measured at the beginning of the experiment. This value was calculated as a percentage of the average initial weight what resulted for each of the eight sampled fish in the percentage fish weight gain. The average fish weight gain per treatment was then calculated as the average over these eight values. The same approach was used for the calculation of the average feed conversion ratio (FCR), expressed as the feed consumption (*g*) over the weight increase of the fish (*g*; Sammouth et al. 2009) per treatment.

Bacterial community analysis—DNA extraction and polymerase chain reaction

Samples for the extraction of the 16S rRNA genes from the intestinal microbial community were taken after 2 weeks (= 14 days) and 6 weeks (= 42 days) during the experiment. At both time points, three fish from each treatment were sampled at random, euthanized using an overdose of MS222 (50 mg L^{-1}), with sodium bicarbonate to maintain neutral pH, and dissected. The gut with content was removed and frozen at −20 °C until further analysis. After thawing, the intestinal matter from each gut sample was gently removed. The three intestinal matter samples were pooled and mixed in 1.4 mL of ASL buffer from a stool DNA extraction kit (UBI Life Sciences, Saskatoon, SK, Canada). The microbial DNA was further extracted according to the manufacturer's protocol. To study the bacterial community composition, a nested polymerase chain reaction approach was used (Boon et al. 2002). The first round made use of the bacterial primers P63f and R1378r, while for the second, these were the total bacterial primer PRBA338f-GC and the universal primer 518r.

Bacterial community analysis—denaturing gradient gel electrophoresis analysis

A Bio-Rad DGeneTM system (Hercules, CA, USA) was used to perform denaturing gradient gel electrophoresis (DGGE) analysis as described previously (Boon et al. 2002). The obtained DGGE patterns were subsequently analyzed using Bionumerics software version 2.0 (Applied Maths, Sint-Martens-Latem, Belgium). A matrix of similarities for the densiometric curves of the band patterns was calculated based on the Pearson product-moment correlation coefficient and dendrograms were created by using UPGMA linkage. A calculation of the range-weighted richness (Rr) for the DGGE band patterns was performed as described by Marzorati et al. (2008). The Rr was calculated according to the formula:

$$\mathrm{Rr} = \left(N^2 \times \mathrm{Dg}\right)$$

where N represents the total number of bands in the pattern and Dg the denaturing gradient of the DGGE gel (in percentage) comprised between the first band and the last band of the pattern. The higher the value of the Rr, the higher the bacterial richness in the sample could be considered based on the number of species and guanine–cytosine variability in the 16S rRNA genes.

Statistical analysis

Comparison of mean values was done by using one-way analysis of variance. Statistical Software SPSS 15 was used for this purpose. Grouping of treatments based on significant differences in mean values was done according to Student Newman Keuls or Tamhane T2 tests (0.05 level of confidence), depending on homoscedasticity results of the Levene test.

Results

Fish survival

A weekly survival of 96–100% was observed for the control treatment of 0% PHB (Fig. 1). Similar survivals could be observed for the treatments in which 2%, 5%, or 10% of the feed was replaced with PHB. For the fish fed with only PHB (100%), the survival was slightly lower, especially at the end of the experiment. The survival of the nonfed fish was

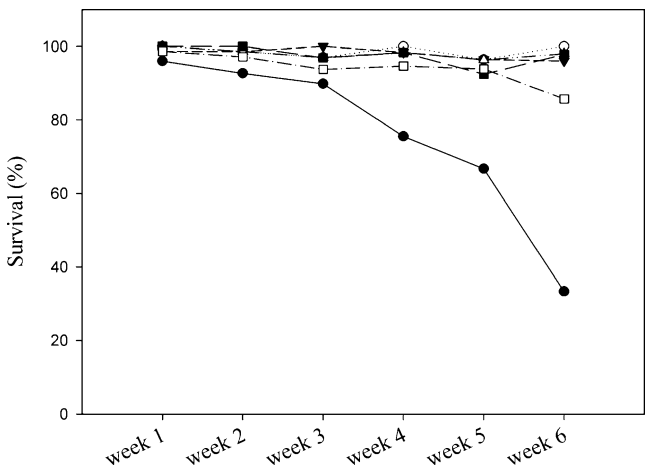

Fig. 1 Weekly percentage survival of juvenile European sea bass during a 6-week feeding trial with diets containing different levels of PHB (*w/w*): No feed (—●—), 0% PHB (··○··), 2% PHB (-▼-), 5% PHB (—△·—), 10% PHB (·■·), and 100% PHB (—□—)

clearly lower than that of the other treatments and decreased during the course of the experiment.

Fish growth

The final weight of the fish after 6 weeks of feeding and the resulting average fish weight gain during this experimental period were determined for all treatments. At the start of the experiment, the fish in all treatments weighed between 1.69 and 1.89 g. The fish grew to an average 5.83, 5.81, 6.50, and 5.83 g in the 0%, 2%, 5%, and 10% PHB treatments, respectively. The average weight of the fish in the nonfed treatment and 100% PHB treatment did not increase. When calculating the average fish weight gain, no significant difference could be observed between the 0% and the 10% PHB treatment (Fig. 2). The value for the 2% PHB treatment was significantly higher than both. However, it was still significantly lower than the highest value of 271% that was observed for the 5% PHB treatment group. Since no increase in the weight was observed for the nonfed treatment and the 100% PHB treatment, their respective weight gain values were significantly lower than the other treatments.

Caloric value of the feeds

The caloric value of each of the experimental diets was determined. For the 0%, 2%, 5%, 10%, and 100% PHB diet, this was 19.6, 19.7, 19.7, 19.9, and 22.4 kJ g^{-1}, respectively.

Feed conversion ratio

The FCR expresses the amount of feed dry matter needed per unit of fish weight gain. In this experiment, the feed dry matter dosed to the water was used for the calculation since only in a limited number of cases the fish did not eat all of the feed (except for the 100% PHB treatment, which was therefore omitted from calculation). The average FCR was 1.10 in case of the 0% PHB treatment group. A similar FCR of 1.09 was calculated for the sea bass fed with the 10% PHB diet. The supplementation of 2% or 5% PHB resulted in a significant decrease of the FCR value. This was translated in a value of 1.03 for the 2% PHB treatment, while the largest effect, although not significantly different from the latter, could be noted for the 5% PHB treatment with a FCR of 0.98. Based on the calculated FCR values, the economical feasibility of using PHB in the feed for sea bass could be determined (Table 1).

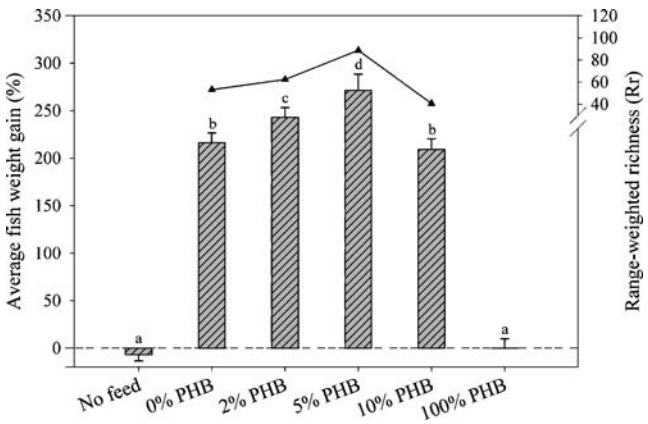

Fig. 2 Average fish weight gain of juvenile European sea bass (▨) and range-weighted richness of the gut microbial community (—▲—) after a 6-week feeding trial with diets containing different levels of PHB (n= 8 per treatment). Treatments indicated with a *different letter* show a significantly different average fish weight gain

Intestinal pH

A clear effect of the level of PHB in the diet on the intestinal pH could be observed (Fig. 3). As a general trend, a higher level of PHB resulted in a lower gut pH after 6 weeks. No significant decrease of the initial gut pH could be observed for the nonfed and 0% PHB treatment. For the treatments with PHB levels of 2%, 5%, 10%, and 100%, the decrease was significant with 0.18, 0.26, 0.41, and 0.43 units, respectively.

Bacterial community composition

A DGGE analysis on the intestinal bacterial community was performed after 2 and 6 weeks in the experimental period (Fig. 4). The bacterial community composition in the nonfed and the 100% PHB treatment were not analyzed since not enough intestinal matter could be collected. The samples from the 0%, 2%, 5%, and 10% PHB treatments clearly clustered together according to sampling date. For the samples taken on day 14, no clear trend of the PHB level on the similarity between the band patterns could be noted. After 6 weeks, it could be observed that a higher level of PHB in the experimental diet resulted in a larger change in the composition of the bacterial community residing the gastrointestinal tract. This was reflected in the lower similarities that were obtained when the band patterns for the 2%, 5%, and 10% PHB treatments were compared to the band pattern of the 0% PHB treatment. The range-weighted richness (Rr) of the bacterial communities calculated based on the DGGE band patterns from day 42 revealed a highly similar trend to the average fish weight gain with a Pearson correlation coefficient of 0.977 (Fig. 2).

Discussion

Antibiotics have for a long time been used not only as therapeutic agents but also as growth promoters in animal production (Acar et al. 2000). Lately, some researches have focused on the promoting effects that alternative biocontrol

Table 1 Economical analysis of the application of PHB in the feed of European sea bass juveniles based on the measured feed conversion ratio (FCR) of 1.1 g feed per gram weight gain for the 0% PHB treatment and 0.98 g feed per gram weight gain for the 5% PHB treatment

	0% PHB treatment	5% PHB treatment
basic feed + PHB needed to produce 1 kg of fish live weight (kg):	1.1 kg basic feed	0.931 kg basic feed (95%) + 0.049 kg PHB (5%)
Feed cost per kg fish live weight produced (€ kg^{-1}): (X = price kg^{-1} basic feed; Y = price kg^{-1} PHB)	1.1 X	0.931 X + 0.049 Y
To be economically feasible, the cost of the 5% PHB feed should be equal of lower than the cost of the 0% PHB feed:		
$1.1\ X \geq 0.931\ X + 0.049\ Y$ or $Y \leq 3.45\ X$		
This means that at a given price of the feed (X), the maximum price of the PHB (Y) can be determined (and vice versa) for these FCR values.		
For example, the price of PHB as mentioned by Listewnik et al. (2007) is 11.5 – 14.0 € kg^{-1} (depending on purity). As a result, the price of the feed should be at least 3.3 – 4.1 € kg^{-1}. At lower feed prices, the benefit of a lower FCR resulting from 5% PHB supplementation is nullified by the low feed price		

The calculations are made for a weight increase of 1 kg fish live weight

X price per kilogram basic feed, *Y* price per kilogram PHB

compounds may bring to the growth performance of aquaculture species (Mahious et al. 2006; Zhou et al. 2007). However, the amount of information that is available for aquaculture is limited up to date (Burr et al. 2005). In this study, the effect of the bacterial storage compound PHB on the growth performance and the intestinal bacterial community structure of juvenile European sea bass was investigated.

The supplementation of PHB in the feed at 2% and 5% (*w*/*w*) was observed to have a positive influence on the average weight gain of the sea bass juveniles. During the rearing period of 6 weeks, the average fish weight gain increased to 243% and 271%, respectively, relative to 216% for the 0% PHB treatment group. This resulted in a FCR that was significantly lower for the 2% and 5% PHB treatment than for the other diets. The absence of a growth promoting effect for the sea bass from the 10% PHB treatment could be caused by a shortage in essential nutrients. Alternatively, it could be indicative that the ecological conditions in the fish guts in the 2% and 5% PHB treatment were different from the other treatments and that these were required in order to reveal the positive effect of PHB. Growth promoting effects

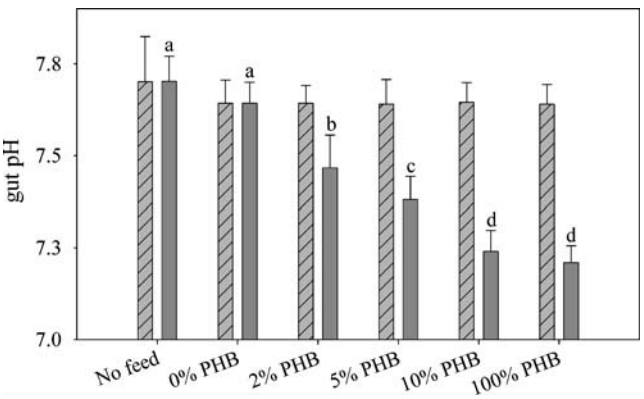

Fig. 3 Initial gut pH (▨) and final gut pH (▩) in juvenile European sea bass after a 6-week feeding trial with diets containing different levels of PHB (n = 8 per treatment). *Values* are represented as means±standard deviation. Treatments indicated with a *different letter* show a decrease in gut pH that is significantly different from each other

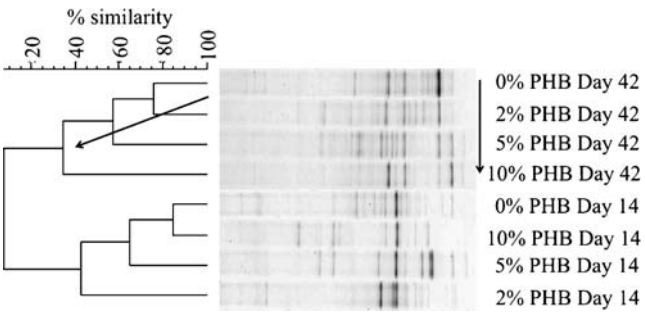

Fig. 4 DGGE band patterns based on the amplified bacterial DNA extracted from the intestinal matter of juvenile European sea bass fed with a diet containing 0% PHB, 2% PHB, 5% PHB, or 10% PHB. The gut was sampled on day 14 and on day 42. The *arrows* indicate the trend observed for the samples from day 42

have also been observed in the case of dietary inulin and oligosaccharides supplied to turbot larvae (Mahious et al. 2006) or Grobiotic™ AE supplied to juvenile hybrid striped bass (Li and Gatlin 2004). In contrast, no growth promoting effects could be observed for short-chain fructooligosaccharides fed to the white shrimp, *Litopenaeus vannamei* (Li et al. 2007). The previous compounds are classified as prebiotics, undigestable food ingredients that influence the growth and/or metabolism of one or a limited number of health-promoting bacteria in the intestinal tract and in that way stimulate the host in a beneficial way (Gibson and Roberfroid 1995). The beneficial effects of prebiotic compounds are mainly ascribed to an increased gastrointestinal health status (Scholz-Ahrens et al. 2007) or the stimulation of the intestinal microbial populations (Burr et al. 2005). Although PHB should not necessarily be considered a prebiotic, it is likely that similar effects occurred in this study.

It was observed that the PHB particles could act as an energy source for the fish. This was suggested by the higher survival of the fish that were fed only PHB as compared to the unfed sea bass (Fig. 1). These observations are in accordance with the results obtained by Defoirdt et al. (2007b) who observed that sterile *A. franciscana* could degrade and metabolize PHB for survival purposes. PHB is a fatty acid polymer and can thus be considered to be a typical source of energy (Azain 2004). This is also reflected in the caloric value of the PHB, which was slightly higher than that of the basic feed. The lack of weight increase for the fish from the 100% PHB treatment indicated that PHB could not be used as a source for growth. The decreasing trend in pH observed in the gut of the fish fed with PHB (Fig. 3) indicated that higher doses in PHB lead to higher concentrations in short-chain fatty acids like β-hyroxybutyric acid and/or its oligomers. Bongers and van den Heuvel (2003) described several mechanisms by which prebiotics can beneficially affect bioavailability of minerals and trace elements necessary for growth in the gastrointestinal tract of the host, including the production of SCFA and the resulting decrease in pH.

The composition of the bacterial community in the sea bass intestinal tract on day 42 was clearly different from day 14 (Fig. 4). After 2 weeks, it appeared to have developed in a random way. This can be interpreted as the requirement of a transition time to establish a stably functioning microbial community. After 6 weeks, a trend of lower band pattern similarity at higher levels of PHB in the feed could be observed. This indicated a dose effect of PHB and/or its degradation products on the changes in the bacterial community composition in the gastrointestinal tract of the sea bass. There is increasing interest in new approaches to study and describe host–microbe interactions in aquaculture (Rawls et al. 2004; Panigrahi and Azad 2007; Dierckens et al. 2009). In this view, the Rr can be used to study a microbial community based on the base pair composition of the DNA sequence—or its content of (guanine + cytosine) more specifically—and the percentage of denaturing gradient in the DGGE gel needed to describe the total diversity of the sample analyzed (Marzorati et al. 2008). The higher the Rr is, the higher the probability that the environment can host more different species with a higher genetic variability. The equal trends in Rr and average fish weight gain for the sea bass in this study indicated a relationship between the conditions for bacterial growth in the gastrointestinal tract and the growth performance on the fish. These results warrant further research on the possibility to use the (calculated) structure and functionality of the intestinal microbial community as an indicator for the growth performance or health status of aquaculture species and the changes therein.

It is not clear whether the change in the bacterial community is resulting from or causing the PHB degradation. Potentially, fish enzymes in the gastrointestinal tract (partially) degraded the PHB into β-hydroxybutyrate oligomers and monomers, which could be used as a growth source for the bacteria. This would mean that the change in the bacterial community is the result of the enzymatic PHB degradation. On the other hand, the presence of PHB may have stimulated the PHB degrading organisms. In this case, the adapting bacterial community would have caused the PHB degradation. Of course, a combination of both bacterial and fish enzymatic degradation of the PHB is also possible.

The partial substitution of the feed with PHB had no negative effect on the survival of the sea bass. However, even in the control treatment the overall mortality was rather high (ca. 10%). In other experiments using similar rearing tank set-ups, such high mortalities did not occur (De Schryver et al. unpublished). This could indicate that the batch of sea bass used in this experiment had a low health status, which possibly allowed the beneficial effect of PHB to occur stronger. However, the overall health status was quantified neither at the beginning nor at the end of the experiment. The increased survival of the fish fed with only PHB (100% PHB treatment) when compared to the nonfed treatment and the decreasing values in pH suggested that the PHB was at least partially degraded and absorbed during gastrointestinal passage in the fish. This was already hypothesized earlier by Defoirdt et al. (2007b). If PHB is not contained within a bacterial cell, it can be degraded by microbial extracellular hydrolytic enzymes in order to obtain carbon and energy (Gebauer and Jendrossek 2006). From various ecosystems, a high number of aerobic and anaerobic bacteria producing these extracellular PHB depolymerases have been isolated (Jendrossek and Handrick 2002; Tokiwa and Calabia 2007). The presence of such bacteria has until now not been shown in the gastrointestinal tract of animals. Efforts to isolate PHB degrading microorganisms from the gastrointestinal environment and apply these as probiotics in aquaculture production are currently being performed.

The use of PHB as a feed supplement does not only depend on the beneficial effects it bring to the aquaculture animals, but also on its cost. The context of its economic cost/benefit ratio should be considered carefully (Table 1). Therein, both the cost of the PHB and of the feed it is supposed to replace seems to be the determining factors.

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