Convergent dynamics of the juvenile European sea bass gut microbiota induced by poly-β-hydroxybutyrate

Peter De Schryver,[1] Kristof Dierckens,[2]
Quyen Quyen Bahn Thi,[2] Rezki Amalia,[2]
Massimo Marzorati,[1] Peter Bossier,[2] Nico Boon[1]
and Willy Verstraete[1]*

[1]*Laboratory of Microbial Ecology and Technology
(LabMET), Ghent University, Coupure Links 653, 9000
Gent, Belgium.*
[2]*Laboratory of Aquaculture & Artemia Reference Center,
Ghent University, Rozier 44, 9000 Gent, Belgium.*

Summary

**Poly-β-hydroxybutyrate (PHB) is a bacterial energy
and carbon storage compound which exhibits a con-
trolling effect on the gastrointestinal microbiota. Its
beneficial activities for aquaculture have already
been shown in terms of increased disease resistance
and growth performance in a number of studies.
However, the action of PHB on the intestinal microbial
community in the treated animals has not yet been
studied in depth. In this research, the effects of PHB
on the microbiota composition in the intestinal tract
of juvenile sea bass were examined. It was found that
fish cohabiting in the same tank were on average 87%
similar regarding the intestinal microbiota. When sub-
jected to the same treatment and environmental con-
ditions but reared in different tanks, the compositions
of the enteric communities diverged. The provision of
PHB overruled this tank effect by sustaining a micro-
bial core community in the gut that represented 60%
of the total bacterial diversity at the highest PHB level
of 10%. The microbial community compositions con-
verged between replicate tanks upon supplementa-
tion of PHB and were characterized by high dynamics
and increased evenness. The results are discussed in
the framework of hypotheses that try to relate the
intestinal microbial community composition to the
health status of the host organisms.**

Introduction

General consensus exists that the intestinal microbial com-
munity plays a key role in the health of the host (Reid, 2008;

Leser and Molbak, 2009). Hence in the aquaculture indus-
try, major efforts are directed towards manipulation of the
gut microbiota to enhance growth, feed digestion, immu-
nity and disease resistance of the host organism (Burr
et al., 2005). Most researches attempt to introduce benefi-
cial microbial species (= probiotics) or feed compounds
that stimulate beneficial species (= prebiotics) in the intes-
tinal tract (Gatesoupe, 1999; Verschuere *et al.*, 2000; Mac-
farlane *et al.*, 2006). Their effectiveness has been shown in
several researches (Li *et al.*, 2007; Aly *et al.*, 2008; Pieters
et al., 2008; Reyes-Becerril *et al.*, 2008; De Rodriganez
et al., 2009; Swain *et al.*, 2009). The potential beneficial
effects of the bacterial storage compound poly-β-
hydroxybutyrate (PHB) for aquaculture rearing were inves-
tigated on several occasions (Defoirdt *et al.*, 2007; Halet
et al., 2007; De Schryver *et al.*, 2010; Dinh *et al.*, 2010). In
the latter studies, not only an increased protection against
pathogenic infections but also increased growth perfor-
mance and increased larval survival have been observed
upon the use of PHB in the diet.

All of the previously mentioned studies focused prima-
rily on the positive effects at the level of the host. Only on
rare occasions, the effects on the gut microbiota were
described. Even then, observations often stayed limited to
increases or decreases in the viable count numbers or
verification of the presence/absence of the probiotic
species after treatment.

The benefits to the host resulting from a healthy and
functional intestinal microbial community have been
shown at several occasions for mammalians (Backhed
et al., 2004; Ley *et al.*, 2005; Robosky *et al.*, 2005; Turn-
baugh *et al.*, 2006). The evidence shows that growth per-
formance and disease occurrence can be related to the
quality and composition of the intestinal microbiota of an
individual (Thompson *et al.*, 2008). When focusing for
instance on host resistance towards infections – one
of the major problems in aquaculture (Vadstein *et al.*,
2004) – the intestinal bacterial community can induce
pathogenic colonization resistance (Chabrillon *et al.*,
2006; Sekirov and Finlay, 2009). Due to the close micro-
bial interactions, small shifts in these communities may
result in changes in metabolite production, changes in the
stimulation of the immune functions of the host or
changes in competition with the pathogen for sites of
attachment or nutrients, and thus induce considerable
alterations in the outcome of the host (Sekirov and Finlay,

2009; Lyte, 2010). A way of characterizing these shifts would be the determination of microbial resource management (MRM) parameters. From the denaturing gradient gel electrophoresis (DGGE) patterns of intestinal samples quantitative information can be obtained such as the number of bands, the intensity of the bands and the similarity between different band patterns. This can be translated into data on relative microbial abundances, changes in species richness and rates of appearance/disappearance of certain species as described by Marzorati and colleagues (2008). The relative abundances relate to the microbial community organization (Co). This MRM parameter gives an indication on how even a microbial community is or how dominant certain species are within the microbial community. The richness relates to the number of microorganisms present. When the genetic diversity is taken into account, the richness in species can be defined as the range-weighted richness (Rr). Finally, the appearance/disappearance of microorganisms gives an indication on how fast the microbial community is changing. In many cases this can be related to changing environmental conditions. The dynamics parameter (Dy) that describes microbial dynamics is expressed as the rate of change with the symbol Δt.

Culture-independent techniques such as DGGE or terminal restriction fragment length polymorphism (T-RFLP) are tools for studies on host–microbe interactions (Huber *et al.*, 2004; Hovda *et al.*, 2007; Li *et al.*, 2007; Navarrete *et al.*, 2010). However, the information on the intestinal microbial community composition and organization that has been obtained for fish using molecular fingerprinting techniques is still very limited at the moment (Ringø and Birkbeck, 1999; Gatesoupe, 2008).

In this study, PHB was used as a feed additive for juvenile European sea bass. The goal was threefold: (i) to assess the effect of PHB on the growth performance and the intestinal microbial community composition of the fish, (ii) to use the available molecular data set to characterize intra- and interhabitat differences in the intestinal gut microbiota of fish as these may be of importance in the steering towards a desired microbiota, and (iii) to introduce in aquaculture some new parameters, obtained by DGGE patterns, reflecting the composition of the intestinal microbial community.

Results

Experimental set-up

A conceptual representation of the experimental set-up and the sampling protocol is given in Fig. 1. Each tank containing 110 fish was considered to represent a different habitat. To avoid major stochastic inflow of water microorganisms into the intestinal microbial community, it was opted to use UV-treated seawater and operate the tanks in flow-through mode. This allowed for an optimal investigation of the effects of PHB on the microbial ecology in the intestinal environment of the sea bass. In a previous study performed by De Schryver and colleagues (2010) on PHB as feed additive for sea bass, it was observed that the PHB did not shape the microbial community after a feeding period of only 2 weeks. Therefore, the first faecal sampling in this experiment was performed at day 20 and subsequently every 10 days.

Survival, growth performance and gut pH

No mortalities were observed in any of the tanks during the course of the experiment. The initial average fish weight for all treatments was 1.82 ± 0.40 g and increased to 6.68 ± 0.95, 6.78 ± 1.03, 6.39 ± 0.78, 6.77 ± 1.21 and 5.96 ± 0.72 g for the 0%, 0.5%, 2%, 5% and 10% PHB treatment respectively. This corresponded to an average weight gain of $266 \pm 82\%$, $272 \pm 86\%$, $250 \pm 74\%$, $271 \pm 92\%$ and $227 \pm 68\%$ respectively. No significant effects on the weight gain of the fish could be observed resulting from the application of PHB in the feed. Upon measurement of the pH of the intestinal matter, no significant differences could be observed between the different treatments. Values ranged from 7.0 to 7.2 (data not shown).

Intrahabitat similarity in fish gut microbiota

The similarity between the faecal microbial communities of fish grouped in the same tank was assessed based on the comparison of the DGGE fingerprinting profiles. Among the 60 tanks from which three fish were sampled in the course of this experiment, 10 were randomly selected for the comparison analysis. This yielded 30 mutual comparison values, which were divided into similarity classes of 5% (Fig. 2). The average similarity within one tank ranged from $73.5 \pm 1.2\%$ to $97.1 \pm 0.9\%$ and the overall intrahabitat similarity was $87.0 \pm 8.5\%$.

Interhabitat similarity in fish gut microbiota

Since the lumen microbial community of three fish randomly sampled from the same tank was highly similar, further analyses were performed with one faecal sample representing each tank. In this way, three samples were analysed per treatment at each sampling date. The average similarity between faecal microbial communities of fish fed with the same diet but reared in a different tank was estimated. As visualized in Fig. 3, the lowest similarities between the three replicate tanks were observed on day 20 and 50 for the treatment with 0% PHB and on day 30 and 40 for the treatment with 0.5% PHB. On the other
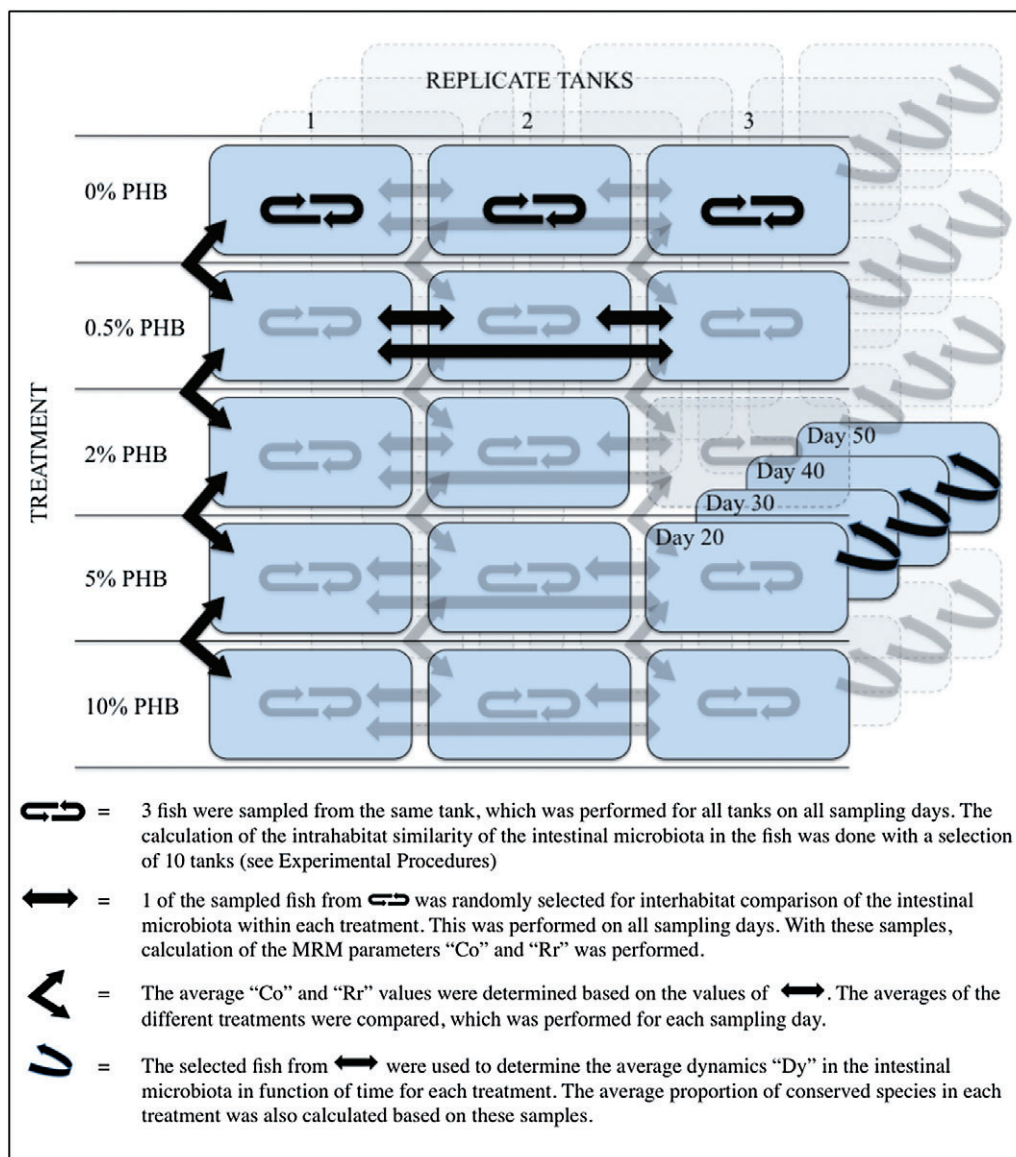
Fig. 1. Conceptual representation of the experimental set-up and faecal sampling protocol of a 50-day feeding trial on juvenile European sea bass fed with five diets containing different levels of PHB. All analyses were based on the DGGE patterns obtained from the sampled faecal matter. Some signs are drawn in light grey to keep a clear overview of the figure.

sampling days, the similarity for these treatments was considerably higher. At PHB levels of 2%, 5% and 10% in the feed, the similarities between the three replicate tanks were high and showed a constant value throughout the experiment.

Conservation of species in the fish gut during treatment

A deeper look into the fingerprinting profiles revealed that 49% of the bacterial genetic diversity present in the gut of the sea bass fed without PHB was conserved throughout the experiment (Fig. 4). This was significantly higher than the 0.5% PHB inclusion in the diet, in which a core com-

munity of conserved species representing 31% of the total diversity was sustained by the PHB. Although no significant differences could be observed, there seemed to exist a trend of larger core communities at higher levels of PHB in the diet. At 10% PHB, it represented 60% of the total diversity.

Dynamics of the intestinal microbial community upon the use of PHB

The rate at which an intestinal microbial community changes represents the stability in function of time and was assessed in this study for each treatment by a
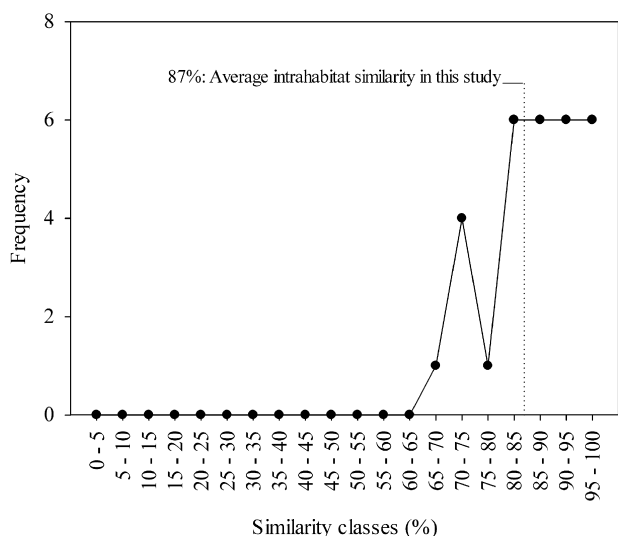
Fig. 2. Intrahabitat similarity of the gut microbial community of two sea bass originating from the same tank and grouped according to frequency into similarity classes. In the 10 tanks selected for this analysis, each time three fish were sampled for mutual comparison.
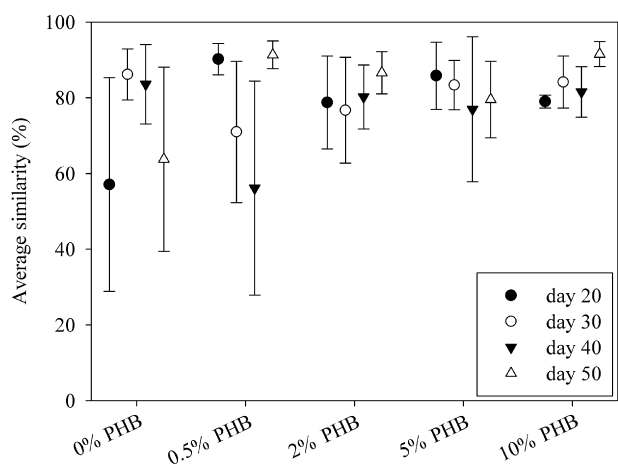

Fig. 3. Average interhabitat similarity between the intestinal microbial communities in juvenile sea bass fed with the same PHB diet but reared in different replicate tanks. Values represent means ± standard deviation of three fish reared in three different replicate tanks.

moving window analysis (Marzorati *et al.*, 2008). In this analysis, the change in the DGGE profile of a fish obtained at one sampling point is compared with that of a fish obtained from the same tank at the previous sampling point (10 days earlier). The average was calculated from the three replicate values obtained per treatment for each 10-day window (Table 1). The rate of change (Δt) in the intestinal microbial community was calculated for each treatment as the average of all moving window data points. For the 0% PHB treatment, this resulted in a Δt value that was significantly lower than the treatments with 0.5%, 2%, 5% and 10% PHB in the diet.

Community organization (Co) and range-weighted richness (Rr) in the microbial gut community

As PHB is a biodegradable compound, its presence can induce a selection in the intestinal microbial community. Therefore, the community organization (a parameter that show the degree of evenness) of the gut microbiota was determined. A value of 0 for the Co represents a totally even community in which each bacterial species is present at the same abundance. A value of 100 represents a totally uneven community with only one species present (= pure culture). In the course of the experiment the intestinal microbial community evolved to a higher evenness in all treatments. (Fig. 5). The higher the level of PHB was in the diet, the higher the evenness in the microbial community had become after 50 days although the difference was only significant between the 0% PHB and the 10% PHB treatment.

The Rr parameter was used to study the microbial community based on the base pair composition of the DNA sequence (its content of guanine + cytosine) and the percentage of denaturing gradient in a DGGE gel needed to describe the total diversity of the sample analysed. The higher Rr is, the higher the probability that the environment can host more different species with a higher genetic variability. Although Rr showed some variability in all treatments in the course of this experiment, no trends could be observed for this parameter (Fig. S1).

Discussion

Poly-β-hydroxybutyrate can induce increased growth performance and bring about increased protection against pathogenic infections for aquatic animals (Defoirdt *et al.*,
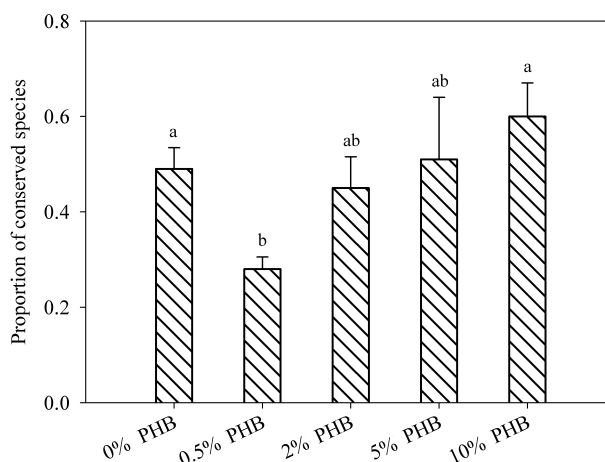

Fig. 4. Proportion of the bacterial diversity that is conserved in the intestine of juvenile sea bass fed with different PHB diets throughout a 50-day feeding trial (means ± standard deviation). Bars indicated with different letters are significantly different according to a two-tailed *t*-test ($P < 0.05$).

Table 1. Moving window analysis of the intestinal microbial community in juvenile sea bass fed with different PHB diets during a 50-day feeding trial.

	Day 30 versus day 20	Day 40 versus day 30	Day 50 versus day 40	Rate of change (Δt)
0% PHB	12.2 ± 2.1	19.5 ± 9.7	16.2 ± 10.3	16.0 ± 7.8[a]
0.5% PHB	42.8 ± 8.3	45.1 ± 2.4	53.3 ± 6.4	47.1 ± 7.2[b]
2% PHB	29.8 ± 12.0	39.4 ± 5.9	29.9 ± 8.5	33.0 ± 9.2[c]
5% PHB	32.3 ± 3.7	40.8 ± 5.5	40.6 ± 14.8	37.9 ± 9.1[b,c]
10% PHB	25.2 ± 4.3	37.6 ± 9.1	49.4 ± 16.1	37.4 ± 14.1[b,c]

The 10-day comparisons represent means ± standard deviation of three DGGE profile comparisons (= 1 per replicate tank). The rates of change indicated with a different letter are significantly different from each other according to a one-way ANOVA.

2007; Halet *et al.*, 2007; De Schryver *et al.*, 2010; Dinh *et al.*, 2010). Until now, no attempt has been made to study in depth the effect of PHB on the microbial community in the intestinal tract of the treated animals. In this study, an insight is provided on the effect that PHB has on the behaviour of the gut microbial community in juvenile European sea bass.

Cohabiting sea bass show a high similarity in gut microbial community

Curtis and Sloan (2004) postulated that the composition of microbial communities in physically identical environments will be different when they are formed from a very large reservoir of microbiota. The inflow of potential colonizers is an entirely random process that does not tend to lead to the same result. However, aquaculture animals are grown in a three-dimensional water matrix and by consequence are in stringent contact with each other and with their metabolic products. It can thus be expected that the intestinal microbial communities of cohabiting aquatic animals are more closely related than that of animals reared on land. The DGGE profile similarity for three fish originating from the same tank was on average 87.0 ± 8.5%. Such similarity is remarkably high. A previous study on rainbow trout reported a similar value of 82.8 ± 11.3% (Dimitroglou *et al.*, 2009). As a comparison with land-based species, a study by Thompson and colleagues (2008) investigated the similarity of the faecal microbial community from piglets and also observed a cohabitation effect; however, the similarities were lower with values ranging from 62.4 ± 18.8% up to 77.6 ± 23.4%. Moreover, both studies made use of a band based approach to determine the similarity between fingerprints, while this was a densitometric curve-based approach in the present study. As such, the obtained similarities are even more pronounced.

PHB induces convergence in the gut microbiota of non-cohabiting fish and steers towards a core community of microorganisms

When comparing the intestinal communities in fish that were treated with either 0% or 0.5% PHB in the diet but that were not cohabiting, the similarity varied between 56.1 ± 15.9% and 91.3 ± 3.4%, depending on the sampling date. This indicated a tank effect that induced stochastic variations in the intestinal tract environmental conditions between the fish sampled from different replicate tanks. At the higher PHB levels of 2%, 5% and 10%, the similarities between the replicate tanks varied between 76.7 ± 10.7% and 91.6 ± 3.0% (over all sampling dates). Although the tank effect was also observable here, it was overruled by a controlling effect of PHB. PHB can be seen as the deterministic factor for the environmental conditions in the intestines of the fish over all replicate tanks of a treatment. The supplementation of PHB acted as a steering factor and resulted at the level of 0.5% in a moderate selection towards a core group of gut microorganisms. The higher the level of PHB in the gut, the higher the steering effect observed. At the highest level, the core group made up 60% of the total diversity present in the gut. It should be noted that the core
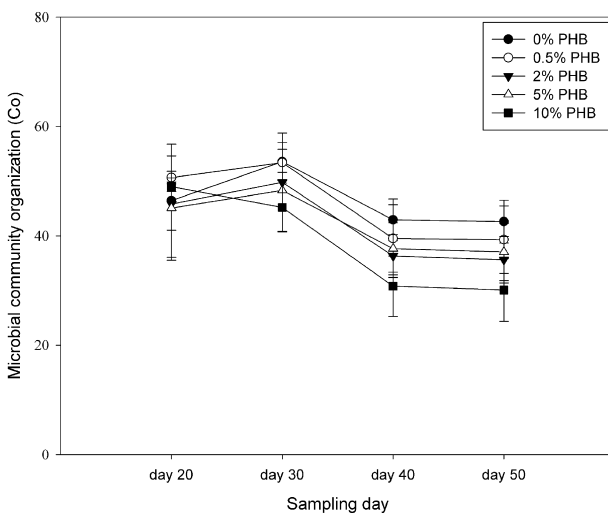

Fig. 5. Average intestinal microbial community organization (Co) as an indicator of the microbiota evenness in juvenile sea bass fed with different PHB diets during a 50-day feeding trial. Lower values indicate a higher evenness in the microbial community. Values represent means ± standard deviation of three fish reared in three treatment replicate tanks.

community size of the 0% PHB treatment was not significantly different from these in the > 0.5% PHB treatments. Although it was not verified in this work, the core communities in the PHB treatment are most likely made up out of species able to metabolize PHB and/or its degradation products and thus represent a different ecological niche relative to the core group of 49% experiencing no steering in the 0% PHB treatment. This should be further explored in follow-up experiments.

The use of PHB has earlier been reported to protect aquatic animals against *Vibrio* infections (Defoirdt *et al.*, 2007). Until now it has always been stated that this resulted from the antimicrobial effect of the β-hydroxybutyrate monomer (Defoirdt *et al.*, 2009). However, the role that the intestinal microbiota could play has never been assessed. It should be investigated if the development of a core group of bacteria feeding on PHB may contribute to increased resistance against infections, for example in challenge tests. If so, the converging effect of PHB on the microbial community can be seen as positive. Differences in the residing microbial community between individuals can lead to large variations in (intestinal) metabolic activity, disease resistance and nutritional health (Thompson *et al.*, 2008; Lyte, 2010). The use of PHB would result in the levelling of the susceptibility to pathogenic infections as all individuals in an aquaculture rearing system are more equally fit in perspective of the microbial community status.

PHB induces increased dynamics in the gut microbial community

When calculating the dynamics of the microbiota as the rate of change, a 16% change over a period of 10 days was found for the 0% PHB treatment. This indicated that the microbial community of the fish in each tank of the control treatment – although it differed between the tanks – was relatively stable in function of time. In fish at the larval stage, the community dynamics can be expected to be higher due to the interaction of the colonizing bacteria with an immune system that is still under full development (Rawls *et al.*, 2004). Indeed, Fjellheim and colleagues (2007) reported considerable differences in the gut microbiota of Atlantic cod larvae living in the same environment.

A moderate selective pressure of 0.5% PHB resulted in a high rate of change (47%) per 10 days. This does not necessarily have to be negative to the host as it was stated earlier that although a microbial community composition changes in function of time, it can still keep a high level of functionality (Fernandez *et al.*, 2000; Possemiers *et al.*, 2004). The lower dynamics of the gut microbiota at higher PHB levels are consistent with the development of a larger microbial core group. The high Δt values for these

treatments relative to the control treatment suggest that PHB and/or its degradations products induced an intestinal environment which was less accessible for the long-term establishment of invading microorganisms. It can be hypothesized that this will also contribute in the resistance against pathogenic infections. The interhabitat similarity data support the conclusion that PHB-treated fish have a more similar microbial composition, but the rates of change suggest that they are changing faster over time. It thus seems that PHB induces continuous changes to the gut microbial community that are reproducible and consistent between different tanks.

PHB induces a more even community composition

The moderate changes in the intestinal microbial community for the fish fed with the 0% PHB diet led to a more even distribution of the members as illustrated by the lowering in value for the microbial community organization throughout the experimental period. Although the difference in evenness on day 50 was only significant between the 0% PHB and the 10% PHB treatment there seemed to exist a trend effect that higher levels of PHB in the diet lead to a more even community. The PHB thus not only stimulated a core group of microorganisms, but also created more equal abundances within this group giving more members a chance to prominently take part in the microbial community. It can be hypothesized that this will also contribute to the well-being of the fish as it is more difficult for a pathogen to invade a community with several equal players relative to a situation with a few dominant species (Ley *et al.*, 2006).

PHB induced no growth-promoting effect in the sea bass

De Schryver and colleagues (2010) found an increased growth performance in juvenile European sea bass fed with diets containing PHB. In the current experiment, the use of PHB did not result in an increased growth performance of the juvenile fish. The choice for a feed ratio of 3% on body weight per day may have resulted – in this experiment – in food limitation to the fish. Since the growth source for the fish in all treatments would have been limiting in this case, the absence of differences in growth effects is a logic result. Alternatively, the absence of effects may have been related to the choice of using flow-through systems as housing for the fish in contrast to the recirculation systems in the former experiment. There seemed to be however an inverse relationship at higher PHB concentrations in the diets between the increased interhabitat microbiota similarity (averaged over all sampling days) and the decreased standard deviation on the average fish weight gain. The Pearson product–moment

correlation coefficient between the two was 0.97. The convergence in the gut microbiota composition at higher PHB levels thus seemed to result in a more consistent gain in the fish weight. This can have important practical implications during fish culture, as a more equal growth decreases the required frequency of laborious size grading to avoid for example cannibalism. Due to the lack of growth effects, the hypothesis by De Schryver and colleagues (2010) that an increased range-weighted richness in the intestinal microbial community may be related to an increased growth performance in juvenile European sea bass fed with PHB-containing diets could not be verified. The presence of PHB in the feed diets did not seem to have a significant trend effect on the range-weighted richness in this study.

In summary, this study showed that the presence of PHB in the diet of juvenile European sea bass induced not only an increased, but also a converging dynamics of the intestinal microbial community. The latter was the result of the emergence of an empowered core group of microorganisms that were more equally abundant at higher levels of PHB. Based on the levelling of the gut microbiota composition and relative abundances, PHB is a compound that warrants further attention to fight infections in aquaculture rearing.

Experimental procedures

Diet preparation

Five experimental diets were prepared containing different levels of PHB: a normal commercial diet 'Gemma' (Skretting, the Netherlands) containing no PHB (0% PHB) and a 0.5%, 2%, 5% and 10% replacement of the basal diet with PHB on a weight basis. The preparations were performed as described earlier (De Schryver *et al.*, 2010).

Experimental set-up and sampling (see also Fig. 1)

Juvenile European sea bass of *c*. 1.8 g live weight were obtained from Ecloserie Marine de Gravelines (Gravelines, France). Upon arrival, they were acclimatized in a 1 m³ tank supplied with UV-treated and charcoal-filtered North seawater at a daily replacement of 200%. The fish were fed approximately 3% on wet body weight day⁻¹ with the 0% PHB feed for 2 weeks. Thereafter, the fish were transferred into 15 rectangular tanks (five treatments in triplicate) containing 80 l of seawater (110 fish tank⁻¹). Each tank was operated in flow-through mode at 200% North seawater replacement day⁻¹ and supplied with continuous aeration by means of diffuser air stones, thereby ensuring a dissolved oxygen level of more than 5 mg l⁻¹. The water temperature was maintained at $18 \pm 2°C$ and a 12 h light and dark cycle was imposed. Each day after siphoning the excrements from the tanks, the fish were fed two times day⁻¹ with one of five experimental diets during 50 days. It was verified that all feed given, with a maximum of 3% on wet body weight day⁻¹, was eaten.

Every 10 days, three fish were sampled from all replicate tanks (= 9 per treatment) minimally 14 h after the last feeding for the determination of the wet weight and the gut pH. The sampled fish were euthanized using an overdose of benzocaine, blotted dry with a paper cloth and weighed. Afterwards, they were dissected and the gut content was removed and transferred into 1.5 ml Eppendorf vials. The pH was measured directly in the vials with a biotrode electrode (Hamilton, Switzerland). On the same day – starting from day 20 onwards – and 0.5 h after the last feeding, three individuals were randomly sampled from each tank and transferred into 2 l tanks containing 1.5 l of UV-treated seawater. The fish were left overnight and taken out of the experiment the next morning. The excreted faecal matter was collected in 2 ml screw cap Eppendorf vials and stored at −20°C until analysis.

Molecular fingerprinting of the faecal bacterial community

DNA extraction and PCR amplification. The DNA from the faecal pellets collected from the individual fish was extracted using an adjusted CTAB method, as described by Boon and colleagues (2003). To compare the microbial communities, the V3 segment of the 16S rRNA gene of all members of the bacteria present in the faeces was targeted by PCR using the primers P338F and P518r (Muyzer *et al.*, 1993). A GC clamp of 40 bp was added to the forward primer. The PCR mixtures were prepared with a Fermentas kit (Germany) and the PCR was performed with a GeneAmps PCR system 2700 thermal cycler (PE Applied Biosystems, the Netherlands) using the programme: 94°C for 5 min, 32 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 2 min and finally an extension period of 72°C for 10 min. Several samples were amplified and analysed two times to assess reproducibility.

Denaturing gradient gel electrophoresis (DGGE). DGGE analysis was performed using a Bio-Rad DGene™ system (Hercules, USA), as described by Boon and colleagues (2002). Staining and analysis of the gels were performed as described by the same authors. The reproducibility of the DGGE analyses was assessed by running duplicate samples once on the same gel and once on two different gels. On the same gels, this resulted in exactly the same patterns whereas the run on different gels resulted in the same band patterns but slightly different sample and background intensities. Therefore, samples that were used in one analysis were always run on the same gel.

Processing of microbial DGGE fingerprints

The processing of the DGGE fingerprinting patterns was performed with Bionumerics version 5.1 software (Applied Maths, Belgium). Here, it should be mentioned that DGGE visualizes only the most abundant populations in the faecal samples composing 1% or more of the microbial community (Hume *et al.*, 2003; Thompson *et al.*, 2008). During the processing, the different lanes were defined, background was subtracted, differences in the intensity of the lanes were compensated during normalization, and bands and band classes were detected. The calculation of the matrix of

similarities was based on the Pearson product–moment correlation coefficient and dendrograms were constructed by the unweighted pair group method, using arithmetic averages (UPGMA).

To determine the intrahabitat similarity, 10 tanks were randomly selected and taken into the analysis. These were from day 20: 0%PHB-r2; 5%PHB-r3, from day 30: 0%PHB-r2; 0.5%PHB-r1; 5%PHB-R2, from day 40: 0.5%PHB-r2; 2%PHB-r3; 10%PHB-r1, and from day 50: 2%PHB-r1; 10%PHB-r3 (r = replicate tank).

The proportion of conserved species during the experiment was determined for each treatment. For each tank, the number of bands that were common on the DGGE patterns obtained at day 20, 30, 40 and 50 were counted by categorizing them into band classes. Next, the ratio of the number of conserved bands over the total band number on each sampling day was calculated. These four values yielded the average number of conserved species in one tank. From the three replicate tanks, the average proportion of conserved species could be determined for each treatment.

The calculation of the MRM parameters – range-weighted richness (Rr) and dynamics of change (Dy) – was performed as described by Marzorati and colleagues (2008). To obtain the values for the MRM parameter microbial community organization (Co), the Gini coefficient described by Mertens and colleagues (2005) was multiplied with 100. To avoid band artefacts, only bands with a relative intensity of more than 0.5% were taken into the analyses.

Statistical analysis

Statistical analyses were carried out using the SPSS software (version 15). Data were compared with one-way ANOVA, followed by a Student Newman Keul's *post hoc* test or by a two-tailed *t*-test. For all statistical analyses, a 5% significance level was used.

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Range-weighted richness of the intestinal microbial community in juvenile sea bass fed with different PHB diets during a 50-day feeding trial. Values represent means ± standard deviation of three fish reared in three treatment replicate tanks.

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