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The Impact of Diet on the Gut Microbiota of Tasmanian Atlantic Salmon (*Salmo Salar* L.) Using a Semi-Continuous Fermenter Model

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Abstract Farmed Tasmanian Atlantic salmon in Australia may experience water temperatures as high as 20°C during summer, which may impact on health and mariculture productivity. In this study we investigated the impact of two commercial feed on the major bacterial population in the gut of Atlantic salmon using an anaerobic semi-continuous fermenter model set at 20°C. Fermentation was conducted in a 5L culture vessel with 100 rpm agitation under CO2. For each diet the hindgut contents of three farmed Tasmanian Atlantic salmon were collected, mixed and used as fermenter inocula. Samples were collected at day 0, 1, 6 and 12 and used for bacterial enumeration and measurement of the functional status of the gut microbiota as well as their metabolic capacity (MC) values. With diet A, Vibrio spp. and lactic acid bacteria (LAB) increased over the course of fermentation. In contrast, diet B did not support the growth of LAB and instead promoted the growth of Plesiomonasshigelloides. MC values of gut microbiota receiving either diet also increased over the course of fermentation, reaching the highest level on day 12. This was independent of the type of diet used as the functional status of the microbiota for both diets was highly similar at each sampling round. Our results indicate that at the temperature experienced by Tasmanian Atlantic salmon during warm season i.e. 20°C, the type of diet may select for the growth of specific species of bacteria.

Keywords: *gut microbiota, fermenter, semi-continuous, diet, Atlantic salmon*

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1. Introduction

The initial transient gut microbiota in fish originates after hatching from the surrounding environment and depending on the species develops into a stable community usually during or after the juvenile stages [1]. There are many factors which influence the gut microbial community in adult fish such as the bacteria present in the surrounding water during initial colonisation of the gut, the nutritional status and the diet provided as well as the age of the fish [2,3,4,5]. It has been shown that other environmental conditions such as geographical location also effect microbial gut composition [3,6-10]. Limited studies have assessed the effect of diet on the gut microbiota of salmonids however it has been found that the use of alternate dietary protein sources greatly influence the smaller subsets of bacteria in the GI tract of Atlantic salmon [11] a result which was not observed in Rainbow trout [12]. The abundance of LAB within the GI tract of Rainbow trout is however greatly affected by diet with an increase observed in fish receiving plant-based feed [13,14]. In Tasmanian Atlantic salmon, temperature rather than diet is the main effector of the major bacterial genera in the gut, which include *Vibrio*, *Pseudomonas*, *Aeromonas* and lactic acid bacteria [15].

There are currently several different fermentation models to study the gut microbiota, however, the use of either a semi-continuous or a continuous culture is shown to better simulate the gastrointestinal system [16]. The functional status of the whole gut microbiota to break down and ferment substrates can also be used to measure the effect of various diet components [17]. Semi-continuous and continuous fermenter models have been used in the past to assess the effect of lactic acid bacteria (LAB) [18], diet components [19] or the effect of both pre- and probiotics on the gut microbiota of humans [20]. Other studies have focused on other mammals such as rabbits [21] and cows [22], however, no studies have investigated the impact of diet on the gut microbiota of fish,and in particular Atlantic salmon, in a semi-continous fermenter model.

Farmed Tasmanian Atlantic salmon often experience high water temperatures during summer, peaking at 20°C. As diet components are known to be an important factor for the development and/or shift in the gut microbiota in fish, the aim of this study was to investigate the impact of two commercial feed on the major bacterial populations in the gut of Atlantic salmon using a semi-continuous fermentation model.

2. Materials and Methods

2.1. Fermenter Media and Gut Microbiota Samples

Twenty litres of seawater were collected in sterile containers and transported on ice to the laboratory and sterilised via autoclaving after addition of fish feed. Two commercially available fish feeds (A and B) with different protein: fat ratios suitable for the different growing seasons (Table 1) were used to prepare a 1% (w/v) solution in 2L of seawater and autoclaved to serve as the working medium for the fermenter.

Table 1. Composition of the fish feed used as the sole source of energy to assess the impact of diet on growth of gut microbiota of the Atlantic salmon *in vitro*

Composition	Diet A (% composition)	Diet B (% composition)
Total protein	45	25
Fat	25	30
Vegetable protein	26	35
Fishmeal	25	20
Land animal protein	22	9
Fish oil: poultry oil	50:50	25:75
Other	2	6
DP:DE	19.6	16.3
Digestible energy (MJ)	20.1	20.05

Atlantic salmon (S*almo salar* L.) (n=3) (average pen weight 2.8 kg), reared in sea cages in a commercial fish farm south of Hobart, Tasmania as previously described [15], were caught at random via netting, from a pen stocked with 70,000 female smolt. The fish were sacrificed by an overdose of the anaestheticisoeugenol (Aqui-S®) according to the commercial fish farms procedure by the staff veterinarian (ethics number A12001). The hindgut was excised and the entire faecal content of the hindgut was squeezed into sterile 50 mL centrifuge tubes [15]. Samples were pooled in order to minimise the inter-individual differences [23] allowing the shifts in the major bacterial populations in response to the feed to be observed. In order to test the reproducibility of the fermentation system, a different feed was used. To achieve that, fresh faecal samples were collected from the same cage of the fish farm one month later and were used as inocula in the same manner as the previous sample. At each occasion, the hindgut of each of the fish was removed and transported on ice to the laboratory and the contents were used within eight hours of harvest as fermenter inocula. Anaerobic semi-continuous culture fermentation was conducted in a 5L culture vessel of a laboratory fermenter (BioFlo/CelliGen 115 Fermenter/ Bioreactor) by continually flushing the headspace of the culture vessel using CO_2 gas as previously described [24]. No enzymes were used in this study and the fermenter pH remained stable throughout the experiment and it was not necessary to buffer the solution as it was maintained at 7.00. The hindgut contents of the fish were mixed and were directly inoculated into the vessel through the inoculation port. The fermenter was set at 100 rpm agitation throughout the experiment. The anaerobic environment was monitored and maintained throughout the experiment by gas flow controllers. The experiment was repeated at 20°C for each diet.

As a negative control, conical flasks containing a 1% (w/v) solution of fish feed in sterile seawater were used. The negative controls were routinely sampled and grown on Tryptone Soya Agar (TSA) (Difco) without dilution. At the time of both hindgut collections, the water temperature ranged between 11-13°C.

2.1. Feeding Regime and Sampling

Once inoculated, the culture was grown under batch conditions for 24 h after which it was switched to semi-continuous culture [25]. Based on the feeding routine and subsequent faecal excretion of the farmed Atlantic salmon, as shown previously [26], it was calculated that the volume of feed and sample to be replaced each day was 31.8 mL per fish. As our working volume in the culture vessel was 2L, which roughly equated to the hindgut contents of 20 Atlantic salmon (on average 100 mL per sampled fish), the total medium to be replaced each day was calculated to be 636 mL [26]. Samples were collected on days 0, 1, 6 and 12 by use of the sterile collection port, immediately prior to feeding with the same amount of medium for bacteriological analysis.

2.2. Bacterial Enumeration

The hindgut samples were initially diluted (1:1 w/v) followed by serial dilution in sterile phosphate buffered saline (PBS) (pH 7.4) and 100 µL of each dilution was spread on TSA to obtain a total count of bacteria, Herellea agar [27] for isolation of *Acinetobacter* spp., *Pseudomonas* agar (Difco) for isolation of *Pseudomonas* spp., Thiosulfate-citrate-bile salts- sucrose agar (TCBS) (Oxoid) for isolation of *Vibrio* spp., de Man, Rogosa and Sharpeagar (MRS) (Oxoid) for isolation of LAB, and inositol brilliant green bile salts agar (IBBS) (HiMedia Laboratories) for isolation of *P. shigelloides*. Plates were incubated for up to 48 h at 20°C. This served to enumerate the major bacterial populations in the hindgut of Tasmanian Atlantic salmon [15,23] and was used as the baseline counts of all bacterial species for further experiments.

2.3. Functional Status and Metabolic Capacity of the Faecal Biota

For each sample collected from the fermenter, the bacterial microbiota were tested for their functional status using the PhPlate-48 generalised microplate system (PhPlate AB, Stockholm, Sweden) as described previously [28]. The PhP-medium comprised of 0.011% (w/v) bromothymol blue and 1% (w/v) proteose peptone. Samples from the fermenter (5 mL) were centrifuged at 1258 × g for 10 minutes and the bacterial pellet was resuspended in 15 mL of PhP medium and 150 µL of the suspension was inoculated in each well of the PhP-48 plates using a multi-channel pipette. The PhP-system is

based on measuring the kinetics of bacterial growth in liquid medium and establishes a correlation coefficient between all tested samples to show the degree of similarities amongst them and therefore neither the number of bacteria nor the dry weight affects the end results [29]. Plates were then incubated at 20°C and changes of the reaction were measured at 4, 16, 24, 48 and 72 h by scanning the plate images using a HP Scanjet 4890 desktop scanner. At the end of the experiment, images were imported into the PhP- software that converted the absorbance of each substrate into a numerical value ranging from 0 to 30. The mean absorbance value from all individual readings was calculated for each substrate giving 48 numerical values ranging between 0 (full utilisation of the substrate) and 30 (no utilisation of the substrate). The similarity between the values of substrate utilisation (48 values for each sample) was calculated after a pair-wise comparison and expressed as the correlation coefficient and clustered according to the unweighted pair group method with arithmetic averages (UPGMA) [30] to yield a dendrogram. In this assay the kinetics of substrate utilization was obtained, instead of an absolute 'positive' or 'negative' value as is resultant in conventional assays, and reflected the overall functional status of the microbiota [31]. The 48 substrates used in the PhP-48 plates are provided (Table 2).

Table 2. Degree of substrate utilisation, as shown by the number of + sign,by the gut microbiota subjected to either diet A or diet B at each sample point over the course of 12 days of fermentation

	Diet A				Diet B			
Substrate	Day 0	Day 1	Day 6	Day 12	Day 0	Day 1	Day 6	Day 12
Mannonic acid lactone	-	+	+	+	-	-	+	-
L-Arabinose	-	+	+++	++	-	+	++	++
D- Xylose	-	+	++	++	-	+	+++	+++
Galactose	-	-	++	++	-	-	+++	+++
Maltose	-	-	+++	+++	-	-	+++	+++
Cellobiose	-	-	+++	+++	-	-	+++	+++
Trehalose	-	+	+++	+++	-	-	+++	+++
Palatinose	-	+	+++	++	-	-	++	++
Sucrose	-	-	+++	+++	-	+	+++	+++
Lactose	-	-	++	+	-	-	++	+
Melibiose	-	-	+++	+++	-	-	+++	+++
Lactulose	-	+	+++	++	-	+	++	++
Gentobiose	-	+	+++	+++	-	+	+++	+++
Melezitose	-	-	+	+	-	-	++	++
Raffinose	-	-	+++	+++	-	-	+++	+++
Inosine	-	+	++	++	-	+	+	+
Adonitol	-	-	+	-	-	+	+	+
Inositol	-	-	+	-	-	-	+	+
D-Arabitol	-	-	+++	++	-	-	++	++
Glycerol	-	+	+++	++	-	+	++	++
Maltitol	-	-	+	+	-	-	+	+
Sorbitol	-	-	+	+	-	-	++	++
Dulcitol	-	-	-	-	-	-	+	+
Sorbose	-	+	++	+	-	+	+	-
Deoxy-glucose	-	-	+	+	-	-	+	-
Deoxy- ribose	-	-	+	+	-	-	+	+
Rhamnose	-	-	+	+	-	-	++	++
D-Fucose	-	+	+	+	-	+	++	+
L-Fucose	-	+	+	+	-	+	+	+
Tagatose	-	+	+++	++	-	+	++	++
Amygdalin	-	-	+++	+++	-	-	++	++
Arbutin	-	-	+++	+++	-	-	+++	+++
β -Methyl-glucoside	-	-	+++	+++	-	-	+++	+++
5- Keto-gluconate	-	+	+	+	-	-	+	+
Gluconate	-	-	+	+	-	-	+	-
Melbionate	-	-	+	+	-	+	+	+
Galacturonic acid	-	-	-	-	-	-	-	+
Salicin	-	-	+++	+++	-	+	+++	+++
Citrate	+++	++++	+++	+++	++	+++	++	++
Fumarate	+	++++	+++	++	++	+++	++	++
L- Malate	-	++++	++	+++	++	+++	++	++
Malonate	+	++++	+++	+++	+++	+++	++	+++
Pyruvate	+	+++	+++	+++	+	+++	+	++
L-Tartarate	+	++++	+++	+++	+	+++	++	++
Urea	+	++++	+++	++	-	+++	+++	+++
Ornithine	+	++++	++++	+++	+	+++	+++	++

The ability of bacterial biota of each sample to utilise various carbon sources was expressed as the metabolic capability (MC) of that sample and calculated as described before [28,29]. A high MC value (maximum =1) for the sample indicates that all carbon sources have been metabolised by members of the biota while a low MC value (minimum =0) indicates that no substrates have been utilized [28,29]. All data handling, including importing and conversion of images, calculations of correlations and coefficients, MC values as well as clustering and printing dendrograms were performed using the PhP Win software version 4 (PhPlate AB, Stockholm, Sweden).

3. Results

3.1. Bacteriological Analysis

An initial decrease in the total number of culture able bacteria was observed in samples subjected to feed A from

2.5 x 10^{11} to 3 x 10^{5} CFU/mL after day one and thereafter stabilising at 7.5 x 10^{8} CFU/mL for the rest of the experiment (Figure 1a). This was associated with an increase in the diversity of the major bacterial populations tested over the period of the experiment. Similarity among the metabolic profile of the bacterial biota, which was very low during the first 24 hours, increased and samples collected on days 6 and 12 showed a highly similar metabolic profile, which was an indication of a similar bacterial biota. There was an increase in the number of certain bacterial species, including *Vibrio*, *Pseudomonas*, *Acinetobacter* and LAB in samples collected after day 6 (Figure 1a).

The number of bacteria in the faecal sample used for diet B was much lower than in samples collected for the first fermenter study (Figure 1b). However, the metabolic profile of the faecal biota within the 12 days of fermentation process was highly similar to that obtained with diet A (Figure 1b). Despite that, there were some differences between the number and presence of different bacterial species between the two diets when subjected to fermentation. This was mainly due to the increase in the number of *P. shigelloides* and the absence of LAB in the faecal sample collected for this round of fermentation (Figure 1b).

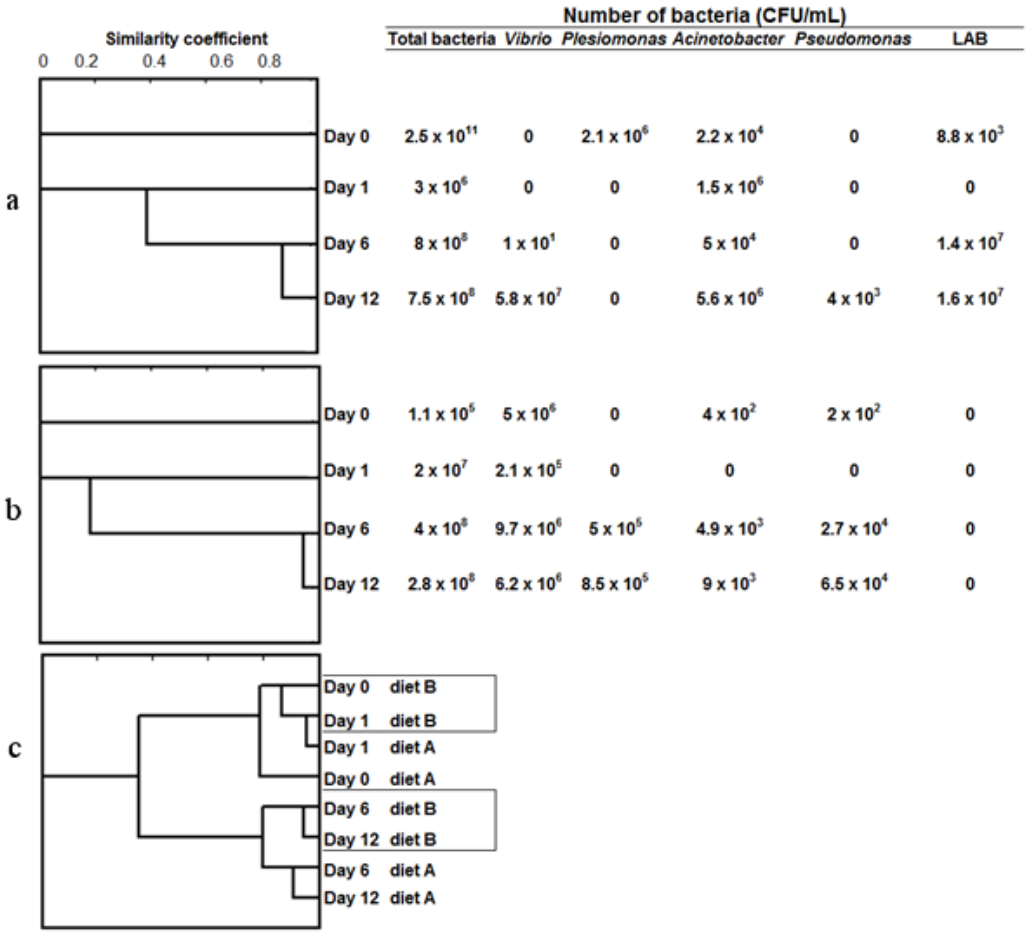

Figure 1. Number of bacteria (CFU/g of faecal material of Atlantic salmon and per mL of the fermenter culture) and metabolic profile over 12 days of the study using feed A (Figure 1a) or B (Figure 1b). Figure 1c shows similarity between the functional status of the gut microbiota receiving diets A or B during the 12 days of sampling

3.2. Substrate Utilisation and MC Value

The pattern of substrate utilization of faecal microbiota subjected to both diets showed an increase in the overall similarity of the microbiota between samples collected on days 6 and 12. During this period the metabolic profile of bacterial microbiota subjected to both diets showed a high similarity throughout the study with the maximum similarity obtained between day 6 and 12 (Figure 1a and Figure 1b). Comparison of metabolic profile of microbiota subjected to differing diets showed that there were high similarities between the faecal microbiota of each sample collected over 12 days (Figure 1c). This was associated with an increase in the MC values of both faecal microbiota at the temperature tested (Figure 2).

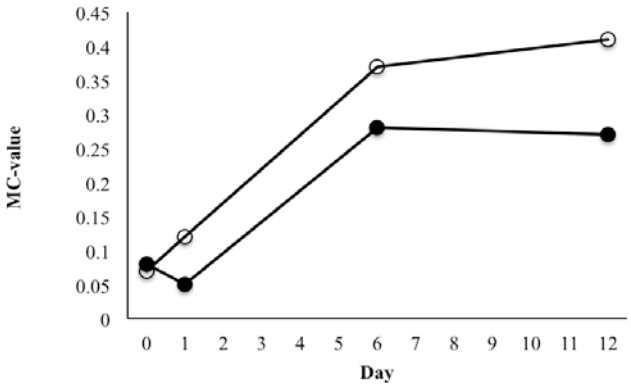

Figure 2. Changes in the Metabolic capacity (MC)-value of the gut microbiota of Tasmanian Atlantic salmon fed diet A (●) or B (○) over 12 days of fermentation

Whilst some of the 46 carbon substrates were utilised by the faecal bacteria receiving either diets, the overall pattern and the number of substrates utilised varied between the two diets with diet A (Summer diet) utilising more substrates at a higher rate at one time point(16 substrates) than diet B (winter diet) (10 substrates). However, overall the degree of substrate utilisation by bacteria receiving diet A was lower for most substrates yielding a lower MC value for this microbiota (Table 2).

4. Discussion

Diet components have been shown to be an important factor for the development and/or shift in the gut microbiota in fish [32,33,34]. In this study we assessed the impact of two commercially available feed, i.e. summer and winter diets, on the culture ablegut microbiota of Tasmanian Atlantic salmon targeting the major populations observed in our previous studies [15,23]. Diet A (summer diet) appeared to sustain LAB populations and was also associated with the growth of *Vibrio* spp. in the fermenter vessel. This increase in the number of *Vibrio* spp. in the hindgut of Atlantic salmon was previously shown when the water temperature increased to 20°C [15] and also occurred with both feed in the fermenter. The first faecal samples used as inocula were collected during the cooler months and contained a small population of *Vibrio* spp. similar to our previous study [15] when the temperature was low. Based on that observation, the high temperature of water, rather than the feed impacted the growth of *Vibrio* spp. in the gut of farmed Atlantic salmon. Changes in the gut microbiota of Atlantic salmon exposed to feed B, unlike with diet A, were subtle. It has to be noted that the initial faecal sample used for testing the impact of this feed contained a high number of *Vibrio* spp. and therefore this population did not increase dramatically at the temperature tested.

A large decline in the total number of bacteria was observed in the fermenter subjected to diet A. This however is not uncommon as the fermenter model itself can lead to the rapid loss of less competitive bacteria due to the presence of unprotected free cells in the microbiota rather than their natural state of being in faecal matrix within the gut [35,36,37,38]. Interestingly this phenomenon was not observed for the microbiota subjected to diet B where the number of bacteria in the inoculum was low i.e. 10^6 from the beginning. An alternate explanation could be that faecal samples collected for trial B had already experienced a natural bacterial washout of less competitive species due to higher temperatures experienced by the sampled fish.

Variability among the number of bacterial populations belonging to smaller groups was observed with a decrease in the number of *P. shigelloides* subjected to diet A and an increase in the microbiota subjected to feed B. The lack of growth of LAB in response to diet B indicates that either these bacteria require a specific diet component similar to what was found in feed A or that these bacteria were not present in the initial faecal sample. Alternatively, it is possible that LAB were present at undetectable levels over the course of fermentation due to the overgrowth of other bacterial species such as *Vibrio* spp.

The MC value used in this study is an indication of the ability of gut microbiota to utilise different substrates as a community. The MC value is high when one or few bacterial species present in the community have a high metabolic function. Alternatively, it can be due to the high diversity of bacterial species in the community each contributing to the utilisation of different substrates. Therefore, the results of MC values should be evaluated in conjunction with the functional status of the microbiota. In this study, the low MC value of the early gut microbiota sample could be attributed to the initial shift of the hindgut microbiota from the gut environment to the fermenter where the only source of food is the fish feed. This may require a period of adaptation for the microbiota and probably the production of required enzymes. Over the period of this study the pattern of substrate utilization of the community shifted in response to changes in the gut microbiota and this was associated with an increase in MC values. The higher MC values observed after 12 days could possibly be due to the higher number of metabolically active bacteria receiving in particular feed B. The high presence of LAB in samples receiving feed A could explain the lower MC value as these bacteria are not as metabolically active as most of the Gram-negative species found in the microbiota found in feed B.

An interesting observation of this study was the fact that despite the differences in the composition of the feed, the response of the gut microbiota to either feed showed a high similarity at every sampling round. We postulated that this could in part be due to the fact that the fermenter model used in this study did not select for the growth of specific bacteria, rather the community as a whole, making the semi-continuous fermenter model used in this study a valuable tool to assess the impact of diets on the composition of gut microbiota. The suitability of the fermenter model to assess the changes in metabolic function of the gut microbiota has also been reported by others [25]. However, the use of this model to evaluate the impact of different feeding regimes on the development of gut microbiota of fish and in particular Atlantic salmon has not been previously reported.

It has been suggested that pooled faecal samples may suffice the need for replication, however this may also lead to inter-microbiota interactions favouring the growth of certain bacterial species and therefore leading to an increase in unrepresented microbiota [39]. This could be counteracted by use of the same inoculum from several fish [10], which would provide enough inoculum to avoid consecutive sampling, as this has been shown to temporally alter the microbiota [40]. Due to logistical constraints we were not able to use the same inocula to test the two diets simultaneously. However, the consistency of the data obtained from the MC value and the level of substrate utilisation as well as the overall increase in the number of certain species i.e. *Vibrio* species, implies that the inocula collected at different time intervals had little effect on the overall results obtained.

Whilst the use of culture based techniques to investigate the impact of diet on the gut microbiota of fish can provide a valuable insight into bacterial population dynamics, the use of molecular techniques may provide additional information on small changes occurring in a fermenter study such as this one. On the other hand, the present study provided information on the functional

status and MC of the gut microbiota of fish over time, an approach which has not been utilised to its full extent in other studies. We would also like to emphasis that in our fermenter model the impact of the gut physiology of fish has not been taken into account and therefore data obtained from this fermenter study should be interpreted with care.

In conclusion, this study showed that the semi-continuous fermenter model can be used as a reproducible and valuable tool for assessing the impact of feed as well as factors such as fluctuation in the environmental temperature on the hindgut microbial populations of fish. Further refinement however, is needed to capture the true potential of this system for such studies.

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Conflict of Interest

The authors declare that there is no conflict of interest with the organization that sponsored this research and publications arising from this research.

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