# THE USE OF TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (TRFLP) ANALYSIS TO MONITOR BACTERIAL POPULATION DYNAMICS IN RAINBOW TROUT (*Oncorhynchus mykiss*)

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## Introduction

Although there have been a number of studies of the intestinal microflora of rainbow trout, most have relied on standard bacteriological techniques for the isolation and subsequent characterisation of bacterial species. However, it is widely accepted that many species of bacteria are non-culturable and, since many of the species bacteria that can be cultured are morphologically similar, the effort involved in identifying representative colonies can be almost insurmountable.

In recent years, culture-independent molecular methods have been developed which may provide more detailed information on such complex communities. Genetic fingerprinting methods, many based on the use of 16s rRNA primers, have been used to determine dominant populations from amplified DNA products. Such methods can reduce workloads and increase the reliability of data.

We have investigated the use of terminal restriction fragment length polymorphism (tRFLP) analysis to determine dominant bacteria present in the intestines of rainbow trout. Although it does not provide taxonomic information, it is a rapid, semi-quantitative assay lending itself to the comparison of large sample numbers, and has an advantage over traditional culture methods in that population dynamics can also be assessed more readily.

We assessed the feasibility of using the tRFLP approach to monitor changes to the bacterial population present in the intestine of rainbow trout, as a result of feeding a prebiotic supplemented diet. A comparison of the results obtained using both the traditional culture and tRFLP approaches will be presented.

## Materials and Methods

### *In vivo* study

- Two groups of 15 fish (each weighing approximately 150g) and previously acclimated, were transferred to 300litre tanks containing dechlorinated freshwater at 15°C.
- One group was offered a modified diet consisting of standard trout pellets coated with a potential prebiotic compound (1g/kg BW/day), using vegetable oil. A control diet of standard trout pellets was offered to the other group.
- Both groups were fed at a 1% feeding rate for 10 days.
- A single fish from each tank was sampled on days 1 (prior to first feed), 3, 5, 7 and 10.

### Sample Processing

- A transverse section of the distal intestine (as illustrated in Figure 1), approximately 1cm anterior to the anus, was sampled and processed as follows. Intestinal content was removed and 75mg intestinal tissue was homogenised in 1.5ml of peptone-saline diluent, using sterile glass beads and a vortex, prior to bacteriological and molecular analysis.
- Samples for molecular analysis (0.5ml of gut homogenate) were microcentrifuged at 10000g for 2mins and pellets frozen at -20°C until required.

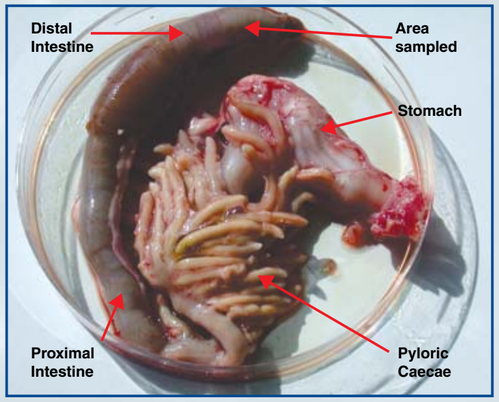


*Figure 1: Digestive system of a rainbow trout*

### Conventional Culture Techniques

- Samples were serially diluted and duplicate spread plates prepared on Tryptone Soy Agar (TSA), incubated aerobically at 22°C. Preliminary tests on a range of media, under different incubation conditions, indicated that these parameters were satisfactory.
- Dominant bacteria (morphologically identical) were counted and identified using the BIOLOG system and 16srRNA sequencing and stored on Microbank™ beads (Pro-Lab Diagnostics) at -80°C.

### Molecular Analysis (tRFLP)

T-RFLP analysis was carried out using a modified version of the method described by Osborne *et al* (1). All reagents and equipment were UV sterilised, where possible, to avoid contamination.

- Total bacterial DNA was extracted from gut homogenate using a Proteinase K and Phenol/Chloroform/Isoamyl alcohol extraction method.
- PCR products of approximately 1324bp were generated using 16srRNA fluorescent-labelled primers (63FFAM, 1387 RHEX).
- PCR products were run on a 1% gel containing Ethidium bromide and visualised under UV light.
- Excised PCR products were purified using the Geneclean II® kit as per the manufacturers instructions.
- Restriction digests were carried out using Alu I and Hha I.
- Digests were run on the ABI PRISM® Genetic Analyser using Genescan™ software.

## Results and Discussion

### Conventional Culture Techniques

- The total intestinal microflora of fish fed the modified test diet increased at day 5 and remained at this level until day 10 (Table 1).
- The total intestinal microflora of fish fed the control diet did not change significantly during the study with few or no bacteria isolated (Table 1).
- Two dominating bacteria could be isolated and identified:
  - *Aeromonas* sp. (not *A. salmonicida*)
  - *Carnobacterium piscicola*

*Table 1: Relative numbers of total bacteria cultured using conventional techniques*

| | Day 1 | Day 3 | Day 5 | Day 7 | Day 10 |
|---|---|---|---|---|---|
| **Modified diet** | + / - | 0 | ++ / +++ | +++ | +++ |
| **Control diet** | 0 | + / - | 0 | 0 | + / - |

### Molecular Analysis (tRFLP)

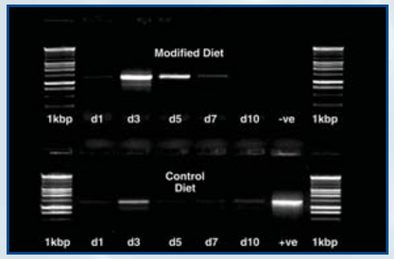


*Figure 2: 1% agarose gel showing 1324bp fragments of the 16S rRNA gene, amplified from total intestinal DNA extracted from the gut homogenates of fish fed either a modified or control diet, over a 10 day period*

- PCR products were generated for all samples but these were generally weak. In fish fed the modified diet, PCR products of increased intensity were observed in samples taken at day 3 and 5 (figure 2).

  When the samples were analysed using GeneScan™ software (figure 3) it appeared that the total intestinal microflora of fish fed the modified test diet increased at day 3 and then decreased up to day 10, in contrast to data obtained by culture methods. It is likely however, that the lack of PCR products in this study is due to inhibition of the PCR as the number of bacteria increased; conventional isolation methods showing a dramatic increase in bacterial counts from day 5 to 10. This problem with amplification could be overcome in future experiments by diluting the DNA extract prior to amplification.
- The total intestinal microflora of fish fed the control diet did not change significantly during the study with few or no bacteria detected (figure 4) corresponding to results obtained using conventional tests.
- Two dominant products were observed in samples from day 3 and day 5 (modified diet), at 50bp and 200bp, suggesting two dominating bacterial groups. This is consistent with the results obtained when using conventional culture methods and suggests that no significant numbers of non-culturable bacteria had been stimulated by the modified feed. However, as this assay does not provide taxonomic data, RFLP analysis of cloned bacterial DNA and the subsequent sequencing of representative groups will confirm whether these are the same groups of bacteria as those identified by conventional culture methods or whether these are additional bacterial species that are non-culturable.

  The generation of amplicons at day 3, in the absence of bacterial growth, may reflect differences in the sensitivity of the two assays rather than the presence of non-culturable bacteria. This can also be confirmed by RFLP and sequence analysis.

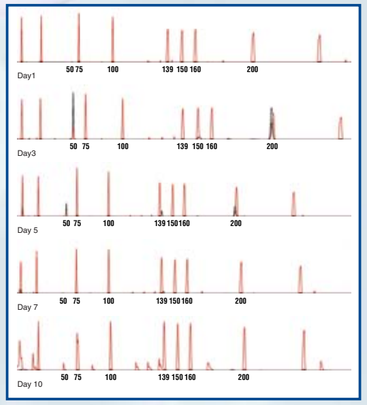


*Figure 3: **Modified diet**, days 1-10, Alu I (Peaks representing bacteria=black, Rox500 size standard=red, x-axis=restriction fragment size (bp))*

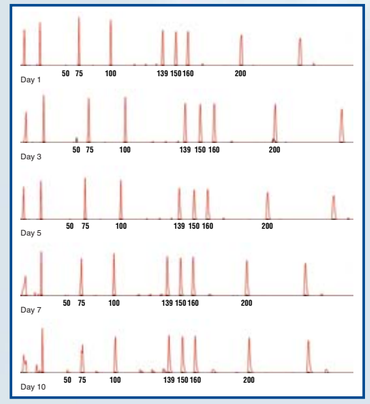


*Figure 4: **Control diet**, days 1-10, Alu I (Peaks representing bacteria=black, Rox500 size standard=red, x-axis=restriction fragment size (bp))*

## Conclusions

- TRFLP analysis is rapid, semi-quantitative and lends itself to the comparison of large sample numbers. Therefore, it is useful in the investigation of:
  - Dominant species
  - Population dynamics
  - Mechanisms of infection
- In combination with the RFLP analysis of cloned bacterial DNA and the subsequent sequencing of representative groups, taxonomic data can be achieved and non-culturable bacteria determined.
- The tested prebiotic compound increased the growth of certain intestinal bacteria. Further studies will be carried out to determine whether this may affect the resistance of rainbow trout to disease.
- Further optimisation of the protocol is required to increase sensitivity and peak size, in order to obtain visible TRFLP profiles in all samples. It can then be established if the growth of all, or of only selected, bacterial populations are stimulated during treatment.

## Acknowledgements

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## References

(1) Osborn, A.M., Moore, E.R.B., Timmis, K.M. (2000). An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ Microbiol*, 2(1), 39-50.

(2) Vergin, K.L., Rappé, M.S., Giovannoni, S.J. (2001). Streamlined method to analyse 16S rRNA gene clone libraries. *BioTechniques*, 30:938-944.

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