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Organic acid feed supplementation and *Yersinia ruckeri* infection affect gut microbiota of rainbow trout, *Oncorhynchus mykiss* (Walbaum)

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Abstract

Gut microbiota (GM) composition of vertebrates are considered to influence health parameters including immunological parameters in the host but knowledge of these issues in fish are sparse. We have performed a 45 day experimental study elucidating effects of dietary supplementation of organic acids (Mera™ Cid) and immunostimulants on the GM composition of rainbow trout *Oncorhynchus mykiss* (Walbaum) and investigated its association with blood parameters and disease resistance (against enteric red mouth disease (ERM) caused the bacterium *Yersinia ruckeri*). A total of 640 fish received either 1) basic fish feed without organic acids or immunostimulants, 2) fish feed with immunostimulants (β -glucans, MOS, nucleotides, lactic acid bacteria, vitamin C and E) but no organic acids, 3) fish feed with immunostimulants and organic acids and 4) fish feed without immunostimulants but with organic acids. Half of the fish (16 groups) were then *Y. ruckeri* challenged on day 21 and 28 of feeding. Haemoglobin, lysozyme and gut microbiota composition (16S rRNA gene based denaturing gradient gel electrophoresis DGGE) were determined at days 14, 30 and 45. Gut microbiota analyses showed that challenge of fish with *Y. ruckeri* and feeding with Mera™ Cid affected the intestinal content.

Keywords: Rainbow trout, *Yersinia ruckeri*, Mera™ Cid, Gut Microbiota, DGGE

Short running title: Gut microbiota in trout

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Introduction

Relatively few studies investigating the role of gut microbiotas (GM) in health and disease of fish have been conducted. This basic parameter may none-the-less be of crucial importance also to fish health because intestinal bacteria contribute to digestion of food, provision of essential nutrients, gut development, gut maturation, regulation of the immune system and may prevent pathogen colonization of the gut (Xu *et al.* 2003; Macpherson *et al.* 2004; Nicholson *et al.* 2005; Bates *et al.* 2006; Ringø *et al.* 2007). The gastrointestinal tract is composed of a complex and heterogeneous microbial ecosystem consisting of several hundred bacterial species (Ley *et al.* 2006) which colonize the intestinal lumen shortly after birth in a way dependent on genetic, nutritional, and environmental factors (Savage 1977; Eckburg *et al.* 2005; Austin 2006; Gómez & Balcázar 2007; Kim *et al.* 2007).

One of the most economically important diseases in rainbow trout farms (Enteric redmouth disease – ERM) is caused by the gram-negative enterobacterium *Yersinia ruckeri* (Fernandez *et al.* 2007; Tobback *et al.* 2007) and it can partly be controlled by vaccination (Raida & Buchmann 2008; Ispir & Dorucu 2010; Deshmukh *et al.* 2012) but still a significant use of antibiotics is necessary to secure freedom of ERM in modern trout farms. Alternative preventive practices that may help to maintain high animal welfare standards and in this regard immunostimulants and other feed additives are accepted by fish farmers to provide some protection (Navarre & Halver 1989; Siwicki *et al.* 1994; Kim & Austin 2006; Misra *et al.* 2006; Xueqin *et al.* 2012). A new approach is the use of Mera™ Cid, a special blend of formic and propionic acid for inclusion in aquaculture feeds (NOVUS 2011). It is not known if this feed additive may act as immunostimulant and/or affect the autochthonous gut microbiota in fish. Analysis of gastrointestinal composition has relied traditionally on culture-dependent techniques (Sugita *et al.* 1995; González *et al.* 1999) including cultivation on selective or non-selective media followed by characterisation using phenotypic and biochemical key characteristics. However, the classification by culture-based methods can often be

difficult, laborious and not suited for analysis of multiple samples. In addition, it has been shown that many species that exist are non-culturable (Fry 2000; Spanggaard *et al.* 2000; Huber *et al.* 2004). Therefore, culture-independent techniques have been developed to explore the microbial diversity and to analyse the structure of intestinal microbial communities. Denaturing Gradient Gel Electrophoresis (DGGE) is a culture independent fingerprinting technique that previously has been used to investigate the GM diversity of mammals and fish (Huber *et al.* 2004; Hovda *et al.* 2007; Fushuku & Fukuda 2008; Liu *et al.* 2008; Hufeldt *et al.* 2010a,b). The aim of the present study was to investigate the influence of including organic acids (Mera Cid) and/or immunostimulants in fish feed on resistance to ERM and GM composition as determined by DGGE. Further these effects were compared to the influence of including commonly used immunostimulants in commercial fish feed.

Materials and Methods

Fish and experimental design

Rainbow trout *Oncorhynchus mykiss* (Walbaum) with an initial mean body weight of 3 to 4 g were obtained from the Bornholm salmon hatchery (Nexø, Denmark) where they had been hatched and reared under pathogen-free conditions. They were then (three months post-hatch) brought to the experimental facilities at the University of Copenhagen. The fish were acclimated in our fish keeping facility in 300 L tanks with municipal freshwater recirculated by internal filters (Eheim GmbH & Co KG, Deizisau, Germany) at 11-13 °C for 1 week under a constant 12:12 h light-dark cycle. Fish were during the acclimatization fed with a basic non-supplemented pelleted trout feed (as outlined below) at the rate of 1% of their biomass per day. Water (municipal water) was replenished daily and concentrations of nitrite, nitrate, ammonia and pH-levels were measured on a regular basis (Merck Aquacant, Germany) ($\text{NO}_3^- < 10 \text{ mg/l}$, $\text{NO}_2^- < 1 \text{ mg/l}$, NH_3 (not detectable), and pH 7.2). No mortality was observed during the rearing period.

Fish (total 640) were divided randomly into 32 fish tanks (20 L aquaria containing 17 L water and 20 fish) (quadruplicate groups) with four subgroups received either 1) basic fish feed without organic acids or immunostimulants, 2) fish feed with immunostimulants (β -glucans, MOS, nucleotides, lactic acid bacteria, vitamin C and E) but no organic acids, 3) fish feed with immunostimulants and organic acids and 4) fish feed without immunostimulants but with organic acids as outlined below. Half of the fish (16 groups) were exposed to a low dosage of bacteria (*Yersinia ruckeri*) after 21 and 28 days of experimental feeding. Sampling was conducted every 14

days, where two fish were sampled from each single aquarium at day 14 and 30, and three fish from each single aquarium at day 45.

Experimental fish diet

The basic control diet was 1.5 mm pelleted feed based on fish-meal and fish-oil (protein 46%, fat 28%, carbohydrate 16%, and ash 7%) provided by BioMar (Brande, Denmark). Another feed type was similarly composed but enriched with immunostimulants and probiotic bacteria. It was composed by β -glucan from yeast (*Saccharomyces cerevisiae*), mannan-oligosaccharides (MOS), nucleotides, vitamin E and C and lactic acid bacteria (*Pediococcus acidilactici* 18/5M as 1×10^9 CFU kg^{-1} feed). This combination was based on reports on immune promoting effects of glucans (Bagni *et al.* 2005), of MOS (Dimitroglou *et al.* 2009, Torrecillas *et al.* 2012), and vitamin C and E (Ortuno *et al.* 2001) and these compounds in mixed formulations (Xueqin *et al.* 2012). Two additional feed codes were produced by adding organic acid (Mera™ Cid) to each of the described feed types by top-coating Mera™ Cid onto feed to a concentration of 0.6%. This product contains propionic acid up to 30%, formic acid up to 20% and silicon dioxide up to 35% (NOVUS Deutschland GmbH, Gudensberg, Germany). The product was added to pelleted feed during continuous stirring and sealing by spraying with fish oil (25 ml kg^{-1} feed). Control feed was similarly oil sprayed but without adding Mera™ Cid (Ortuño *et al.* 2001). Feed was stored at 10-12 °C during the experimental period. All diets were fed at a rate of 2% of body weight per day for 45 consecutive days. Thus, four different feeding-groups were represented by four replicate tanks with 20 fish.

Challenge with *Yersinia ruckeri*

Fish were challenged with *Yersinia ruckeri* serotype O1, biotype 1 (strain 392/2003) (Fouz *et al.* 2006) on day 21 and 28 of feeding. The bacterial culture was prepared by inoculating *Y. ruckeri* into LB medium with incubation on a plate shaker (100 rpm) at 20 °C for 48 hours. Fish with body weights between 8.4 g and 8.8 g were challenged with 7.3×10^6 CFU added to 17 L water (4.3×10^5 CFU L^{-1}) by adding bacteria into each individual aquarium with 17 L water containing 20 fish. Challenge and post-challenge maintenance were performed at 15 °C in a temperature controlled room.

Isolation of pathogen in fish following challenge

Mortality was checked continuously with few hours interval for 25 days and moribund fish were removed and euthanized. To confirm that mortalities were caused by *Yersinia ruckeri*, samples of head kidney from dead fish were cultured on blood agar plates at 20 °C for 48 hours in order to re-isolate *Y. ruckeri*. In addition, further diagnoses were performed using a specific plate agglutination kit containing rabbit anti *Y. ruckeri* serum raised against serotype O1 (Mono Yr test[®], Bionor lab, Norway).

Blood sampling

Blood samples for haemoglobin and plasma lysozyme activity were taken from the caudal vessel by using Na-heparinised 10 µl, 25 µl and 50 µl micro pipettes (Hirschmann[®] Laborgerate, Germany). The blood was used immediately for haemoglobin analysis or for lysozyme measurement centrifuged at 4 °C, 6000 RPM (3000 G), 10 min and the plasma was stored at -20 °C until use.

Haemoglobin

Blood samples were analysed immediately for their haemoglobin content by use of blood analysis device CR 3000 (Medital Nordic, Copenhagen, Denmark).

Lysozyme

Serum lysozyme activity was analysed by the turbidimetric assay described by Skov *et al.* (2012). Briefly, a suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich, Denmark) was prepared by dissolving 0.2 mg ml⁻¹ in sodium phosphate buffer (SPB, 0.06 M, pH 6.2). Plasma was three-fold diluted in phosphate-buffer sodium (PBS, 0.15 M, pH 7.4) (KU-Life Pharmacy University of Copenhagen, Frederiksberg, Denmark). Flat bottomed MaxiSorp[™] 96 wells microtiter plates (WVR, Copenhagen, Denmark) were used in which 190 µl of the suspension was mixed with 10 µl of diluted plasma samples in individual wells. Samples were analysed in duplicate wells. A reference sample was included in all plates within the same experiment to keep track of plate-to-plate variations. A microplate spectrophotometer reader (Epoch, Biotek, Holm & Halby, Copenhagen, Denmark) measured the reduction in absorbance at 450 nm at 1 min and 10 min. One unit was defined as 1% decrease of absorbance during an incubation time of 9 min: (1 % unit = $((OD^{1 \text{ min}} - OD^{10 \text{ min}}) / OD^{1 \text{ min}}) * 100$).

Gut microbiota (DNA extraction, PCR amplification and DGGE)

Intestinal content of fish were collected by dissection and removal of the intestine. By applying a gentle pressure on the intestine the content was recovered for faecal sampling. Samples were stored at -80 °C until DNA extraction. Total bacterial DNA was extracted from the faecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN, GmbH, Germany) according to the manufacturer's instructions but with the addition of an initial bead beating step using a FastPrep apparatus (FP120 Cell disrupter, QBiogen, MP Biomedicals, France) at speed 5.5 for 3x30 sec. The quality of the purified DNA was assessed visually by 1.5% agarose gel electrophoresis. The V3 region of the bacterial 16S rRNA gene was amplified by PCR by using the universal primer set PRBA338f (5'C GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGGACT CCT ACG GGA GGC AGC AG'3, TAG, Copenhagen, Denmark) and PRUN518r (ATT ACC GCG GCT GCT GG'3, TAG, Copenhagen, Denmark). All reactions were carried out in a 50 µl volume containing 30 µl RNase free water (Sigma-Aldrich, Denmark), 5 µl dNTPs (10 µM) (50 mM), 5 µl 10xbuffer, 1.5 µl MgCl₂ (Sigma-Aldrich, Brøndby, Denmark), 1 µl primer F (10µM), 1 µl primer R (10 µM), 0.5 µl BioTaq™ DNA Polymerase (Bioline) (5 unit µl⁻¹), 0.5 µl bovine serum albumin 0.1% w/v (Sigma-Aldrich, Denmark), 0.5 µl formamide 1% v/v, and 5 µl template. The PCR reaction was performed on a T3-Thermoblock, Biometra (Stratagene, Aarhus, Denmark). Initial denaturation was performed at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 40 s; with a final elongation step at 72 °C for 10 min. Denaturing Gradient Gel Electrophoresis (DGGE) was performed as described by Hufeldt *et al.* (2010a) on a polyacrylamide gel with a 35-65% gradient (where 100% correspond to 40% formamide and 7M urea).

Data analysis

Normality Test (Shapiro-Wilk) was used for evaluation of the normal distribution of lysozyme and haemoglobin data within each group. Then, data were compared between experimental fish and control fish (days 14, 30, and 45) using a Student's *t*-test. Data were displayed as mean ± standard error of means (SEM). Survival of fish in various groups was compared using Kaplan-Meier survival plots and log rank test. BioNumerics version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyse the 16S rRNA gene amplicon-based profiles obtained by DGGE. Levels of similarity between DGGE profiles were calculated according to the Dice similarity coefficient (composition and position tolerance 1%) followed by tree construction using optimization and 1% position tolerance using the un-weighted pair group method with arithmetic

averages (UPGMA) and principal component analysis (PCA). Student's *t*-test was used to detect differences between groups based on Principal Component (PC) values of the first 3 PCs. In all cases, data were considered significantly different when $P < 0.05$.

Results

Survival post-challenge

The survival of rainbow trout fed different experimental diets for 45 consecutive days and exposed to challenge on day 21 and 28 was followed. No significant differences were seen between any groups but the highest survival rate was found in fish fed dietary immunostimulants without Mera™ Cid (Group 2, 84.5% survival). The fish fed Mera™ Cid without immunostimulants performed the second best (Group 4, 78.8% survival). Survival rate was 75.5% for fish fed with both organic acids and immunostimulants (Group 3). Trout fed basic feed without additives (Group 1) showed a 73% survival (Fig. 1).

Haemoglobin

Haemoglobin levels varied somewhat during the experiment but a significant increase was merely seen in fish fed with Mera™ Cid (Group 4) at day 14. Following *Y. ruckeri* challenge some variation appeared in all groups but no clear trend was observed (Fig. 2).

Lysozyme activity in plasma

Lysozyme activity was slightly increased at day 14, 30 and 45 in non-infected groups supplemented with immunostimulants and Mera™ Cid compared fish on the control diet. A significant decrease was seen following infection at day 30 and 45 in groups receiving challenge, whereas non-supplemented fish were un-affected (Fig. 3).

DGGE analysis of gut microbiota

The PCR-DGGE technique was employed to evaluate the association between feed additives, *Yersinia* infection and gut microbiota (GM). Sampling of the intestinal content was conducted at days 14, 30, and 45.

Day 14. The GM of the different groups varied with an overall similarity index of 53% in the cluster analysis, but in general no clear visual association between diet and GM was observed (Fig. 4a). However, PCA analysis revealed that the GM of fish fed Mera™ Cid supplemented feed was significantly different from fish fed non-supplemented feed (Fig. 5a). Variance analysis based on

PC1, PC2 and PC3 revealed a significant difference ($P<0.03$) in PC2 (explaining 19.46% of the variance) values between these two groups.

Day 30. After 30 days two major clusters were seen (Fig. 4b), with *Y. ruckeri* exposure being a driving force in the separation of the different profiles, as seen from Fig. 5b (combined PC1, PC2 and PC3-model, $P=0.05$). No clear patterns between diet and GM were observed.

Day 45.

After 45 days on experimental diets trout GM differed widely between samples as seen from Fig. 4c. Fish receiving Mera™ Cid in their diet could be clearly separated from those not receiving this feed additive (Fig. 5c) ($P=0.007$ on PC1). Moreover, variance analysis based on the first (X), second (Y) and third (Z) principal component (PC) revealed a significant difference ($P<0.007$) in PC1 (explaining 56.73% of the variance) values between these two groups. It should be noted that the immunostimulant supplemented groups did not cluster at any time point.

Discussion

Despite a trend for an increased survival of rainbow trout fed organic acid and immunostimulants following challenge no significant effect on susceptibility of rainbow trout towards the bacterium *Yersinia ruckeri* was observed. The survival rate was high in all groups probably due to the low challenge dosage applied. Using higher dosages (2.3×10^8 CFU mL⁻¹) Skov *et al.* (2012) provoked higher mortality in trout fry but the low dosage used here was based on our wish to mimic a farm situation with a low and consistent exposure. The trend for lower mortality in immunostimulant-supplemented fish may be explained by the fact that these compounds in fish can modulate non-specific immune responses and enhance resistance of various fish towards pathogenic microorganisms (Yano *et al.* 1989; Siwicki *et al.* 1994; Samuel *et al.* 1996; Misra *et al.* 2006; Jeney *et al.* 1997; Selvaraj *et al.* 2005; Ai *et al.* 2007; Xueqin *et al.* 2012). Mera™ Cid fed fish performed as the second best group but the background is still unknown. Pacific White Shrimp *Litopenaeus vannamei* (weight 3 to 4 g) fed 0.5% Mera™ Cid for 60 days and challenged with bacteria *Vibrio harveyi* (10^3 , 10^4 , and 10^5 CFU mL⁻¹) exhibited reduced *Vibrio* spp. counts in the gut associated with increased survival rates (NOVUS 2011). It would therefore be relevant to investigate if Mera™ Cid directly can affect *Y. ruckeri* counts in the gut or indirectly control pathogenic bacteria by enhancing growth of other microorganisms competing with *Y. ruckeri*. Alternatively, the acid action of Mera™ Cid may, according to suggestions by Biziulevicius (2004), induce lysis of commensal bacteria in the intestine and thereby liberate immunostimulatory elements such as LPS and flagellin – molecules which are potent inducers of innate immunity in trout (Chettri *et al.* 2011).

Haemoglobin levels were slightly higher in groups fed immunostimulants and Mera™ Cid compared to control fish receiving basic feed but significant differences were merely seen in some groups at some sampling time points. This suggests that effects of the applied feed additives on haematopoiesis should be further investigated. Plasma lysozyme activity of unchallenged fish showed a clear trend for an increase after feeding for 14, 30 and 45 days in fish groups fed dietary immunostimulants and Mera™ Cid compared to control fish. Several authors have reported enhancement of lysozyme activity following administration of various immunostimulants including β -glucans (Engstad *et al.* 1992; Matsuyama *et al.* 1992; Paulsen *et al.* 2003; Misra *et al.* 2004; Bagni *et al.* 2005; Misra *et al.* 2006; Ai *et al.* 2007; Xueqin *et al.* 2012), but the lysozyme activity variation found in this study indicates that this parameter may be regulated in a more indirect way. Various components were combined in the immunostimulant containing feed code applied in this work and interactions between these elements are at present unknown. Thus, a significantly increased level of lysozyme activity was solely seen in group 4 (\div immunostimulants, + Mera™ Cid) at day 45 of feeding. Fish challenged and infected exhibited a decrease of lysozyme activity after 30 and 45 days. It may therefore be suggested that the slightly increased survival in challenged fish is associated with usage and finally exhaustion of the plasma lysozyme in fish blood which was initially increased due to feed supplementation.

Gut microbiota composition of mammals and fish depend on genetic, nutritional, and environmental factors (Savage 1977; Eckburg *et al.* 2005; Austin 2006; Gómez & Balcázar 2007; Hufeldt *et al.* 2010b). Mera™ Cid fed fish for 14 and 45 days had significantly different intestinal microbiota compared to non-Mera™ Cid fed fish indicating an effect of organic acids on the intestinal flora. When evaluated together our DGGE analysis of bacterial community profiles showed that fish challenged with *Y. ruckeri* and also fed different experimental diets had different DGGE patterns. This suggests that Mera™ Cid may influence colonization of the host intestine also with regard to pathogenic bacteria. Manipulation of the intestinal microbial composition through dietary supplementation has previously been shown feasible in mammals, broiler, and fish (Broom *et al.* 2006; Gunal *et al.* 2006; Licht *et al.* 2006; Bakke-McKellep *et al.* 2007; Fujiwara *et al.* 2008; Dimitroglou *et al.* 2009; Merrifield *et al.* 2009). In our study we showed that Mera™ Cid fed fish had different DGGE patterns (different GM composition), but we did not specifically show if the feed ingredients directly depressed some potentially harmful species or stimulated some potentially beneficial bacteria. Inclusion of dietary immunostimulants did not demonstrate a similar effect as Mera™ Cid on GM composition which is

in line with Liu *et al.* (2008) revealing that the intestinal microflora was not affected by intraperitoneal glucan-injection of Atlantic salmon (*Salmo salar* L.). However, trout GM modulation following MOS exposure was shown by Dimitroglou *et al.* (2009) and Balcázar *et al.* (2007) found that oral exposure of brown trout to *Lactobacillus sakei* modified the intestinal flora of the host. Therefore combination of feed ingredients for oral stimulation of fish may show different effects compared to exposure of fish to single compounds – an effect which should be further investigated.

In conclusion, the results of the present study showed that different experimental diets merely induced a slight protection of rainbow trout towards *Y. ruckeri*. Haemoglobin levels were higher in a number of groups fed with immunostimulants and Mera™ Cid when compared to control fish. The plasma lysozyme activity showed increases in unchallenged fish fed experimental diets and significant decreases in supplemented but challenged fish. Furthermore, DGGE analysis of bacterial community profiles revealed that fish fed Mera™ for 14 and 45 days obtained a significantly different intestinal microbiota compared to non-Mera™ Cid fed fish. In addition, these changes in GM of Mera™ Cid fed fish coincided with increased plasma lysozyme activity at day 45 and haemoglobin increases at day 14 in blood.

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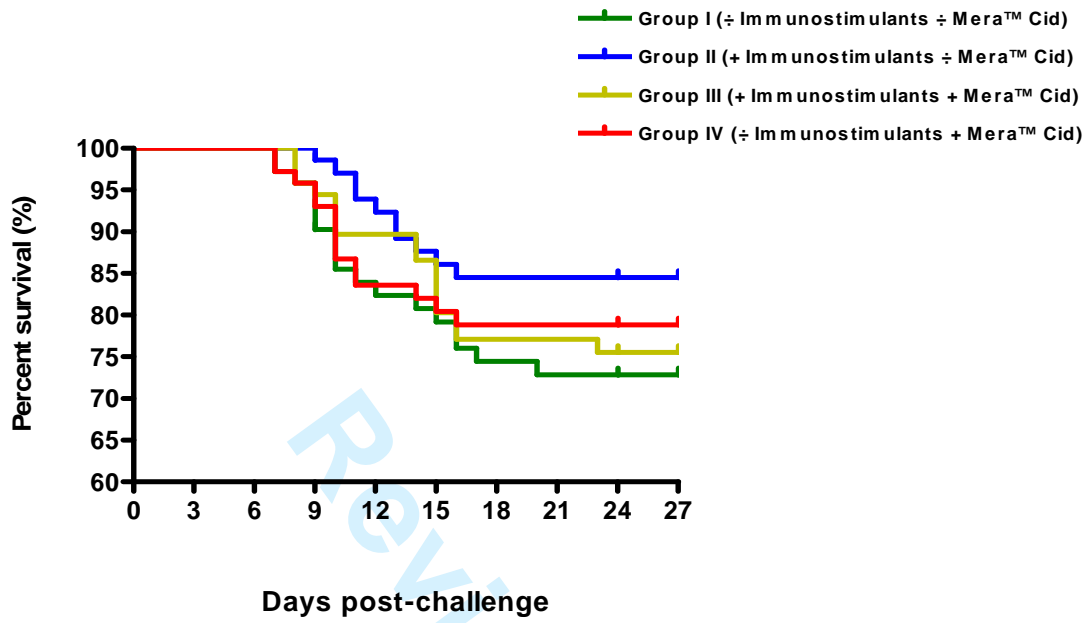
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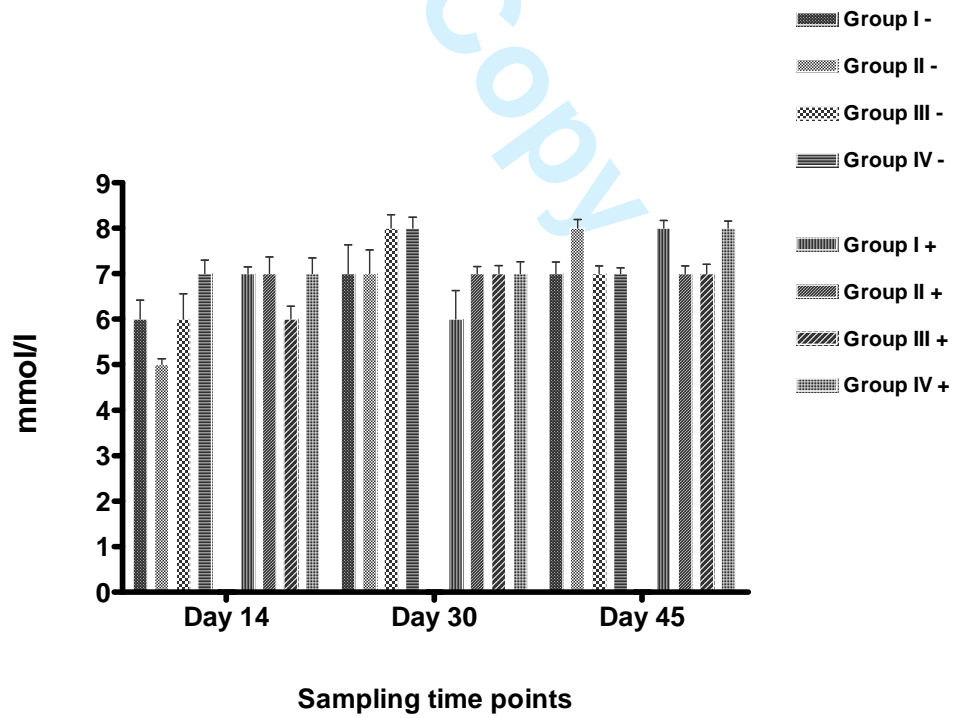
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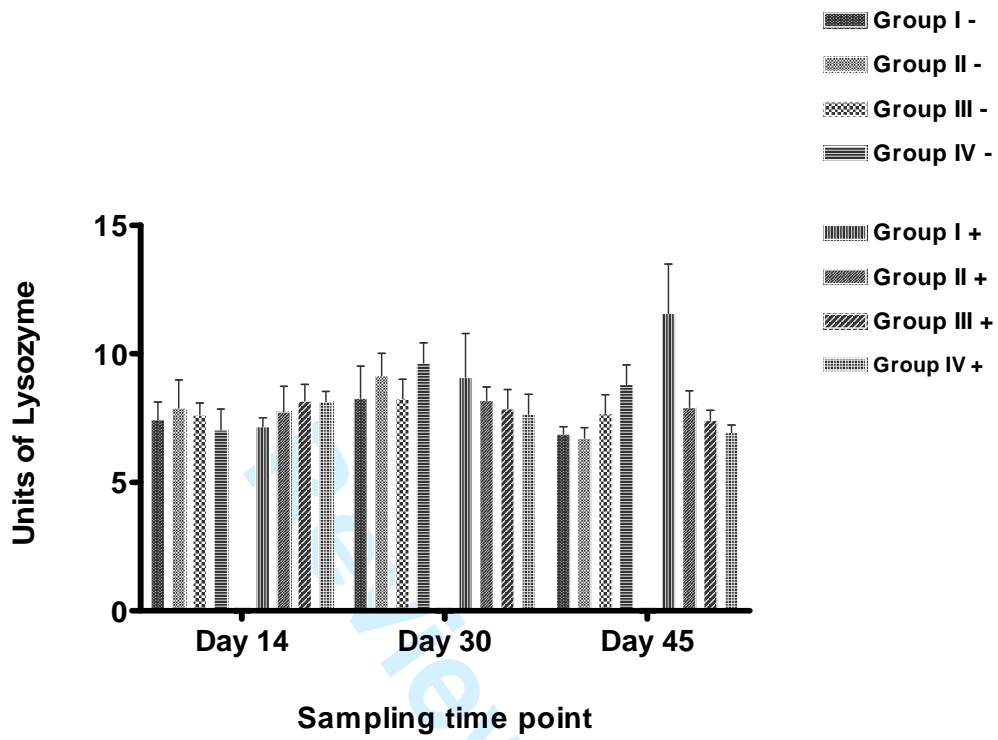
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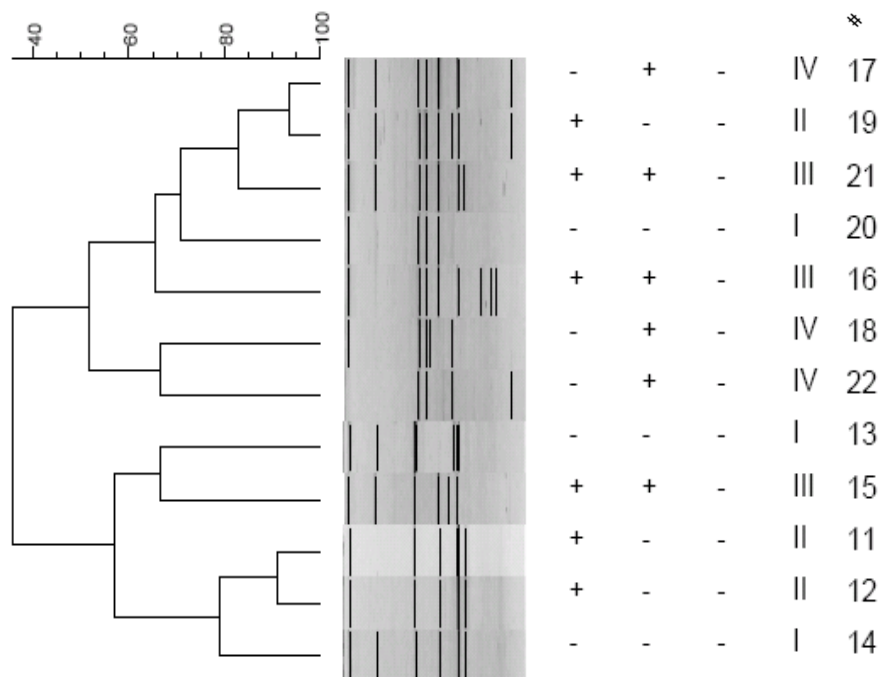
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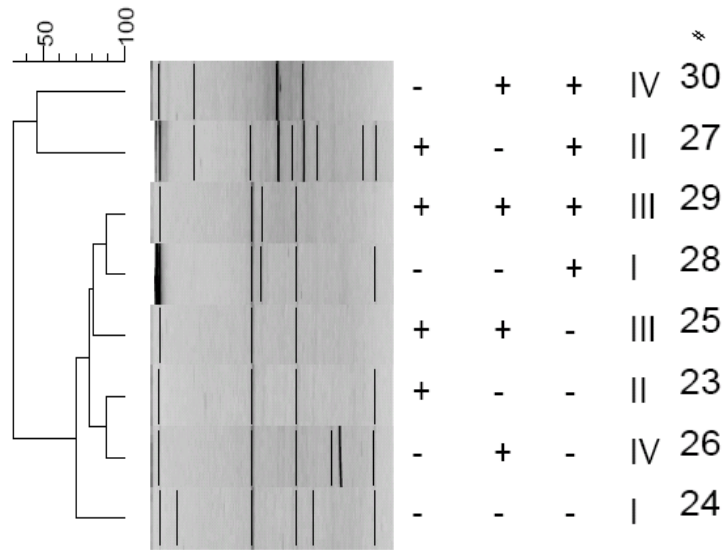
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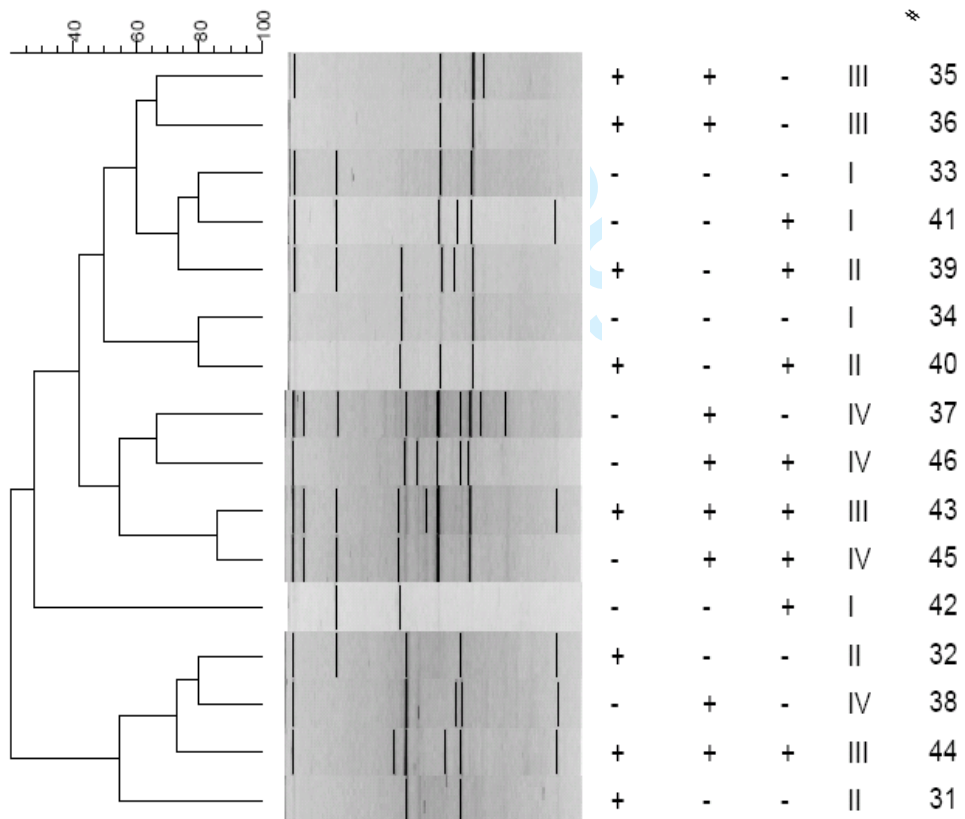
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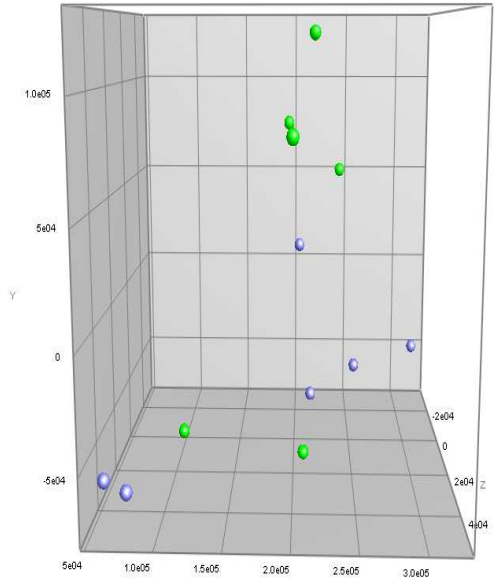
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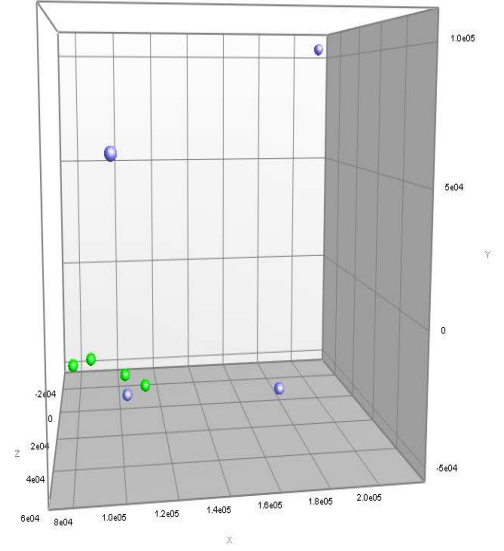
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(5b)



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