Development and application of novel measures of foregut lamina propria in Atlantic salmon given 5 different diets

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01.03.2021

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Abstract:
The trial was composed of five diets: a fishmeal/fish oil diet, a 20% soybean meal/30% fishmeal & fish oil diet, a fishmeal/rapeseed oil diet, plant protein concentrates/fish oil diet, plant protein ingredients/mix of rapeseed oil & fish oil diet. Atlantic salmon (*Salmo salar*) was used as experiment species. Traditional histological approaches are not comparable between different organs and/or treatments, mucosal mapping using universally applicable measures is standardised and can compare different organs and treatments in an unbiased manner. Sectional orientation is of great importance for traditional histological approaches, for mucosal mapping the orientation has no effect.
Bases for lamina propria tissue being an aid as an indicator for determination of physical health were found. LPr showed higher correlations with other measures than LPwdth. The relationships shown through LPr were more conclusive and explanatory where LPwdth acted as a supportive measure. In addition, the LPr seems better suited for integration with the mucosal mapping technique than LPwdth, since LPwdth seems more sensitive to sectional direction. For that reason, LPr will create a more well balanced digitalised and automated system than LPwdth would produce.

Keywords: Lamina propria, density, ratio, width, diet.

Acknowledgement

I would like to offer my gratitude to my supervisor Karin Pittman for her knowledge, guidance and wisdom but most of all for her positive spirit and enthusiastic personality. Mearge Okubamichael has been of great support and help and has impressed with his statistical knowhow and his everlasting willingness to extend a helping hand. I would like to express to Embla Øye how nice it was to have her next to me performing similar work and supplying a certain comradery. Last but not least I thank my husband Rune Gullaksen for his endless support and encouragement throughout this process.

Writing a master thesis entails many different learning goals and opportunities, like working with colleagues on a day-to-day basis, extracting knowledge and knowhow from your supervisor and all in all developing into a well-rounded individual ready to enter the work environment.

The COVID-pandemic has definitely not made it easy to go about this thesis traditionally but the many people within Quantidoc AS have created a great learning- and fun working environment.

Introduction

Fish health in relation to the gastrointestinal tract

Determining fish health is often assessed through fish morphology like biometrics and/or histology (biological tissue level) or immune response (biological cell and gene level) (Gatlin, 2007). Mucosal barriers, inflammatory and gene response are part of the innate immune system of fish (Masso-Silva & Diamond, 2014). The mucosa serves as a first defence barrier against pathogens or other stressors (Roberts, 2012; Lazado & Caipang, 2014; Dash et al., 2018; Cabillon & Lazado, 2019). The gastrointestinal tract has shown to respond to dietary stressors affecting the health of the fish (Van den Ingh et al., 1991).

Tissue level

The general build-up of the digestive system includes serosa, muscularis, submucosa and mucosa. Mucosa features two tissue types, epithelia and lamina propria. The epithelial tissue includes the lining of the gastrointestinal tract and is protected by a thin layer of gel-like mucous. Mucous is produced by mucous cells laying close to the epithelial surface. When the gastrointestinal tract is agitated mucous cell size and density ratios increases and shortening/flattening of the intestinal folds can occur (Baeverfjord & Krogdahl 1996).

Lamina propria is a loose connective tissue underlaying the epithelial tissue. In healthy conditions the tissue is thin and elongated but changes morphologically when the gastrointestinal tract is agitated (Ross & Pawlina, 2006). These changes have been described as widening of the lamina propria tissue (Baeverfjord & Krogdahl 1996). However, these are often described through histological observations and lack a consistent quantitative approach (Penn et al., 2011).

Morphological changes to the mucosa as a whole, and the lamina propria within, can already be seen after 2 days of gastrointestinal agitation. Even though these changes were supported later on by actual onset of enteritis, they were too non-specific to be used as a precursor in traditional qualitative histological manners (Baeverfjord & Krogdahl 1996). However, there are indications that distortion of lamina propria tissue happens over a longer period of time and indicated that lamina propria as a tissue undergoes a longer response cascade than discrete acute responses like inflammatory cells (Van der Sluis et al., 2006).

Cell level

The lamina propria is often mentioned in relation to gastrointestinal dysfunction and especially linked to inflammatory diseases (Van der Sluis et al., 2006). Lamina propria in relation to release of inflammatory cell responses has been well documented, with leucocytes (mainly lymphocytes) featuring (Baeverfjord & Krogdahl 1996). Teleost showed to have gut-associated lymphoid tissue (GALT) that dispersed immune cells (Zapata & Amemiya, 2000) including lymphocytes (Hellberg et al., 2013; Salinas et al., 2011). As a response to an infection lymphocyte levels would increase (Abós et al., 2015). Levels of lymphocytes actually showed a decline for Atlantic salmon when suffering from soybean

induced enteritis (Romarheim et al., 2013). Phagocytes provide an immune response by enveloping pathogens and increases in levels as well when immune response is activated (Featherstone & Elliss, 1995).

Gene level

The relative expression of certain genes has been proven to be of aid in the maintenance or defence of the mucous layer and the underlying mucosa. Mucin is gradually released from mucous cells (also referred to as goblet cells) to maintain the mucous layer (Ellis., 2001; Olafsen, 2001). When the gastrointestinal tract is agitated mucin levels are elevated either through increase and/or acceleration of production (Torrecillas et al., 2011; Schroers et al., 2009; Plaisancié et al., 1998). Cathelicidin and defensin are anti-microbial peptides (AMP's). Immune response for the relative expression of these genes is triggered by several stressors of which inflammation is one (Chang et al., 2006).

Dietary component impacts on gut measures

Many dietary ingredients have been included in fish feed over the years to substitute different fish derived components. Various neutral, positive or negative effects have been documented.

The effect of plant-based oils is still debatable. Replacing fish oil with sunflower oil indicated a decrease in immune system function for Atlantic salmon (Thompson et al., 1996). Rapeseed oil shows high similarity to fish oil and is expected to not have detrimental effects on fish (Bell et al., 2003 ;Ackman, 1990). Dietary treatments containing high rapeseed oil levels (19 gm) showed heavier weights for Atlantic salmon then treatment with lower levels of rapeseed oil. However, when compared to fish fed marine fish oil no differences in weight were found. Indicating rapeseed oil does not seem to have detrimental effects. Marine fish meal and fish oils, being the salmons' natural diet, had positive effects on fish health (Bell et al., 2003). In addition, marine fish meal and fish oil showed to aid phagocytic ability and phagocytic capacity (Sørensen et al., 2020).

Corn gluten affects the appetite of the fish and results in reduced weight (Fauconneau, 1988; Cowey & Cho, 1992).

Pea protein concentrate caused Atlantic salmon to experience a decreased nutrient digestibility and had detrimental effects on growth and intestinal morphology presenting shortened mucosal folds and an increase in lamina propria widths (Penn et al., 2011)

Soybean meal causes enteritis, and morphological changes to the gastrointestinal tract can already occur as early as 3 weeks into soybean meal exposure (Baeverfjord & Krogdahl 1996). These effects have been shown to diminish the immune system's ability to respond to dietary agitation or pathogens (Baeverfjord & Krogdahl, 1996; Torrecillas et al., 2015). Soybean has also shown to contain antinutrients (Liener & Kakade, 1980).

Antinutrients from plant sources are compounds produced by the plant as a protective measure against grazing from animals and can cause detrimental effects when consumed. These compounds can hinder uptake of essential nutrients and decrease digestibility for lipids. In addition, enteritis, diarrhoea and neoplasia can occur (Krogdahl et al., 2010; Iwashita et al., 2008).

Available methodology for tissue analyses

Histology examines the structure and function of separate tissues through the use of 2D sections, where the sectional orientation is of great influence on the qualitative evaluation (Ross & Pawlina, 2006). A newly developed technique to analyse histological samples is mucosal mapping obtained from design-based stereology. It represents recreation of 3D structures from 2D sections where directional sectioning is not of importance increasing practical and *in vivo* application (Pittman et al., 2011, 2013; Torrecillas et al., 2015; Dang et al., 2019, 2020).

Comparing histological qualification of mucous cells to mucosal mapping several differences emerged. Samples taken for histology need to precisely run the length of the epithelia to produce a viable section of 1-2 mm thick slices. Whereas for mucosal mapping these samples just had to represent 1-2 cm^2 of surface area. Units of measure for histological samples were relative to other structures in the represented tissue. Mucosal mapping uses universally applicable units of measure. This means that traditional histological approaches are not comparable between different organs and/or treatments, mucosal mapping using universally applicable measures is standardised and can compare different organs and treatments in an unbiased manner. As mentioned before sectional orientation was of great importance for traditional histological approaches, for mucosal mapping the orientation has no effect (Dang et al., 2020).

Gastrointestinal tract responses are complex. The mucosal mapping strategy has given rise to standardised, scalable analyses of barrier tissues, including the gastrointestinal tract. No standardized method exists to measure and/or analyse lamina propria. Measuring lamina propria tissue easily subjects to many different unbiased approaches that are unsuited for large-scale bases. To generate neutral, accurate and well-balanced results over a large dataset the following protocol, based on mucosal mapping, was adopted for pilot trial and main trial application.

The aim of this trial is to investigate how lamina propria, as a tissue, reacts to different stressors delivered through dietary ingredients.

It is hypothesised that if fish are treated with various dietary ingredients different reactional patterns will emerge and when subjected to detrimental ingredients an increase in lamina propria volumetric density and tissue width will be seen. These detrimental ingredients are mostly represented by the negative control treatment group.

Material and method

The feed trial was approved by National Animal Research Authority (Mattilsynet), Norway (FOTS-ID 14983). Animal husbandry was performed in accordance to approved protocols.

For this study Atlantic salmon (*Salmo salar*) post-smolts were attained from Cermaq, Hopen, Bodø, Norway (Aquagen strain, Aquagen AS, Trondheim, Norway) and kept at the Research Station, Nord University, Bodø, Norway.

Datasets

This trial developed and implemented novel measures for lamina propria on foregut samples taken from the main trial carried out by Sørensen. *et al*., 2020. Analyses of foregut mucous cells has been carried out by Øyen, 2020 containing data reflecting mucous cell sizes and defence activity. These two datasets were matched to an individual level (fish ID's).

In addition, data for inflammatory cell responses was provided as well by Sørensen. *et al*., 2020, containing data concerning phagocytic ability, phagocytic capacity and lymphocytes. Phagocytic ability relates to number of cells with the capacity to absorb at least one particle and phagocytic capacity is a measure of how many particles the cells are able to absorb. For this dataset different fish were sampled. The data for the inflammatory response was merged per coinciding tank.

Data for genetic responses was also supplied by Sørensen. *et al*., 2020 containing data concerning cathelicidin (CATH1), defensin (DEF3) and mucin (MUC2) relative gene expression. For this dataset different fish were sampled as well. The data for the genetic response was merged per coinciding treatment group.

Experimental diets

The trial was composed of five diets: BG1, a fishmeal/fish oil diet mimicking the natural diet (positive control); BG2, a 20% soybean meal/30% fishmeal & fish oil diet (negative control); BG3, a fishmeal/rapeseed oil diet; BG4, plant protein concentrates/fish oil diet; BG5, plant protein ingredients/mix of rapeseed oil & fish oil diet which resembled commercial diet (Fig. 1).

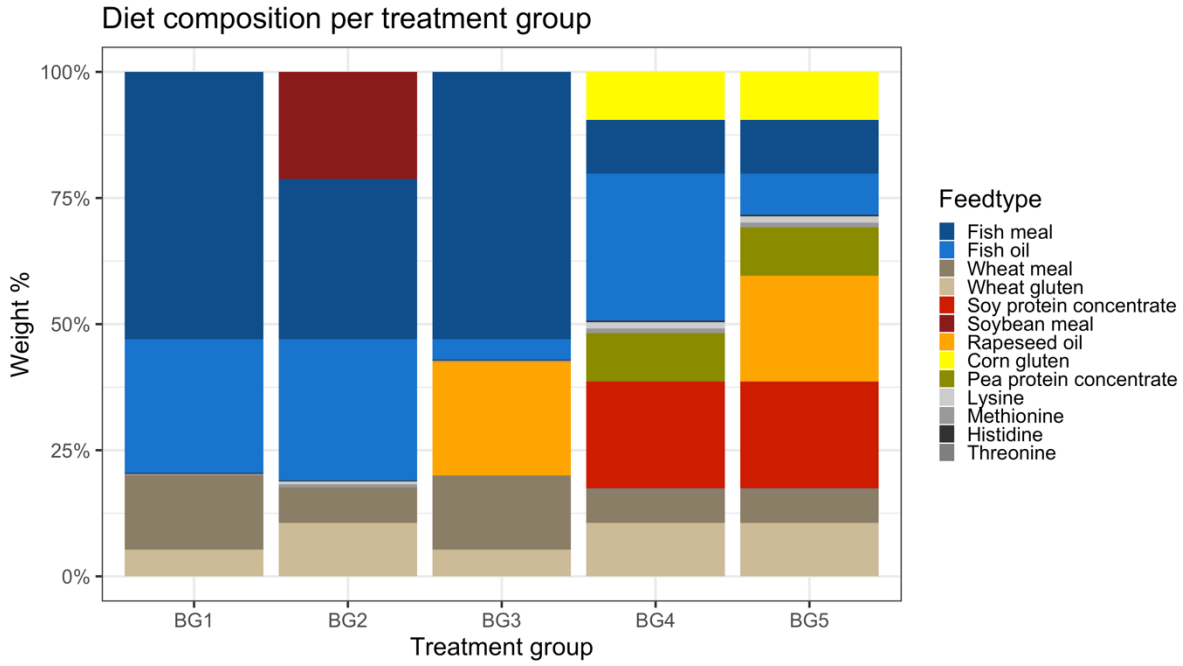


Fig.1. Diet composition per treatment with weight given in percentages. BG1, fishmeal/fish oil diet (positive control) mimicking natural diet; BG2, 20% soybean meal/30% fishmeal & fish oil diet (negative control); BG3, fishmeal/rapeseed oil diet; BG4, plant protein concentrates (main protein source)/fish oil diet; BG5, plant protein ingredients/mix of rapeseed oil & fish oil diet (commercial resembling diet). Several ingredients meant for other purposes are excluded.

All diets contained supplements with crystalline amino acids (lysine, histidine, methionine and threonine) and inorganic phosphate. In addition, yttrium oxide was added to assess digestibility (Table 1).

Table 1. Diet composition including all ingredients. Units of measure in gm. Each ingredient is given with coinciding contribution for each treatment group. All diets contained supplements with crystalline amino acids (lysine, histidine, methionine and threonine) and inorganic phosphate. Yttrium oxide added for digestibility assessment.

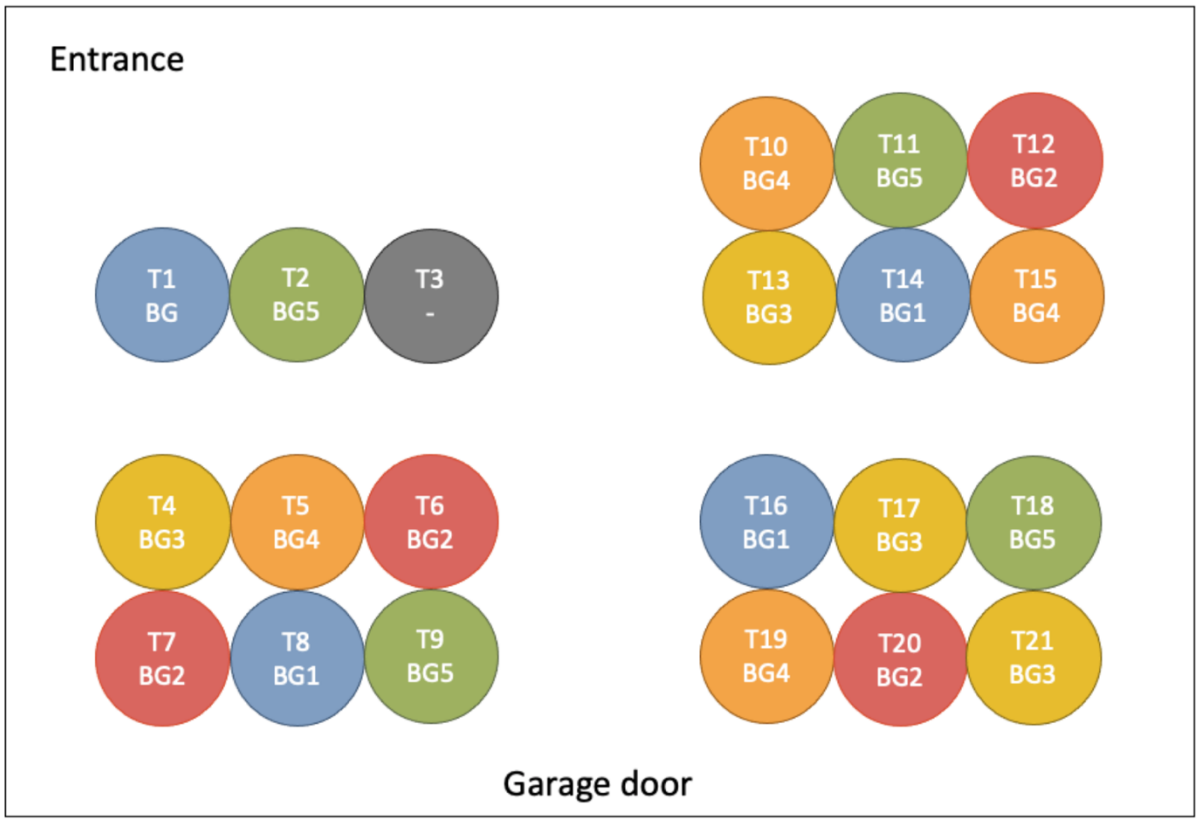
	BG1	*BG2*	*BG3*	*BG4*	*BG5*
Fishmeal	50	30	50	10	10
Wheat meal	13.85	6.55	13.85	6.05	6.05
Wheat gluten	5	10	5	10	10
Soy protein concentrate	0	0	0	20	20
Soybean meal	0	20	0	0	0
Corn gluten	0	0	0	9	9
Pea protein concentrate	0	0	0	9	9
Fish oil	25	26	3.8	27.5	7.7
Rapeseed oil	0	0	21.2	0	19.8
Mineral premix	0.59	0.59	0.59	0.59	0.59
Vitamin premix	2	2	2	2	2
Monosodium phosphate	2.5	2.5	2.5	2.5	2.5
Carop. Pink (10% Astax)	0.05	0.05	0.05	0.05	0.05
Yttrium oxide	0.01	0.01	0.01	0.01	0.01
Choline	0.5	0.5	0.5	0.5	0.5
Methionine	0.3	0.6	0.3	0.9	0.9
Lysine	0	0.5	0	1.2	1.2
Threonine	0	0.1	0	0.4	0.4
Histidine	0.2	0.2	0.2	0.3	0.3

Each diet was prepared as described in Sørensen. *et al.*, 2020.

Experimental set-up

The trial was deployed in a flow-through system containing 1100 fish in total (initial weight 72.7 ± 1.4 g) (mean ± SD). These were divided over 20 circular fiberglass tanks with 4 tanks per treatment group, resulting in 220 fish per diet (Fig. 2). For this study 4 fish from 3 tanks per treatment group were sampled resulting in 12 samples per diet (n=60).

Fig. 2. The experimental lab set-up comprised of 20 circular fiberglass tanks, with 4 tanks per treatment group, 5 treatment groups in total (n=1100). BG1: blue, BG2: red, BG3: yellow, BG4:orange, BG5: green. For this study 4 fish from 3 tanks per treatment group were sampled resulting in 12 samples per diet (n=60). For treatment group BG1 tanks T1, T8 & T14 were used. For treatment group BG2 tanks T6, T7 & T12 were used. For treatment group BG3 tanks T4, T13 & T17 were used. For treatment group BG4 tanks T5, T10 & T15 were used. For treatment group BG5 tanks T2, T9 & T11 were used. Experiment species: Atlantic salmon (*Salmo salar*).



Fish rearing conditions and environment

Environmental parameters were monitored with a flow rate of 1000L/h, and an average of 7.6 T, with a salinity of 35 ‰ respectively during a 24-h photoperiod throughout the experimental trial (65-d). Oxygen saturation was maintained above 85% (measured at water outlet). Water supply initiated from 250 m depth, Saltenfjord. Feeding regimes were ad libitum and automated (Arvo Tech, Finland) with a timeframe of 7 timeslots per 12-h (08:00-10:00, 10:00-12:00, 12:00-14;00, 14:00-16:00, 16:00-18:00, 18:00-19:00 and 19:00-20:00) (Sørensen *et al.*, 2020).

295 ## Biometric measures
296 All fish were individually weighed (W), and length measured (L), both at 1-d and
297 65-d, after being anesthetized using tricainemethanesulfonate (MS 222, 140
298 mg/L). From L and W condition factors (K) were calculated using the Fulton
299 formula $K=100 \cdot W/L^3$ (Nash et al., 2006).

Trial methodology

General trial methodology

Analysis of the epithelia and the lamina propria was done through the use of VIS, a histopathological image analysis software for diagnostics (Version 3.6.5.0; Visiopharm Integrator System), in combination with modified mucosal mapping as described by Dang et al., 2020.

Regions of interest (RIO's) were manually drawn on an image following the mucosal folds. Then counter frames (CF) were randomly deployed over the image. Each CF contains two types of stereological probes representing different volumes. Large probes (1890.41 µm2) represent an area four times the volume of small probes (472.6 µm2). Since epithelial tissue is present in a much higher volume then lamina propria tissue, only large probes were used indicating epithelial tissue and small probes were used indicating lamina propria tissue.

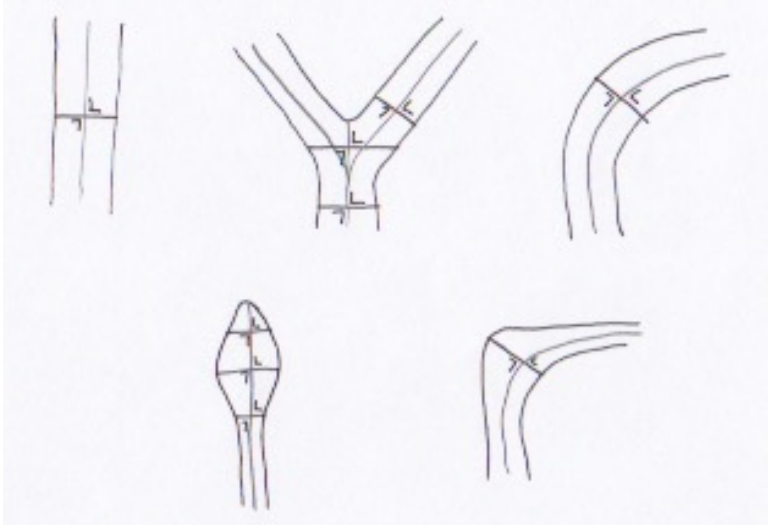
Lamina propria density ratio

The first method generated lamina propria density ratio (LPr) through registering epithelial and lamina propria tissue. Epithelial volume (Ev) and lamina propria volume (LPv) were determined through counts of the particular tissue and the coinciding probe volume (LPv=LP count*small probe volume/ Ev=E count*large probe volume). These variables were used to show the proportion of LPv in relation to Ev calculated through LPr=LPv/Ev which was used for further analyses.

Lamina propria width

The second method generated lamina propria width (LPwdth). Subsequently to registering epithelial and lamina propria tissue, lamina propria tissue was measured in a perpendicular manner to the midline there where a small probe crossed lamina propria generating LPwdth (Fig. 3).

Fig. 3. For this trial a method for measuring lamina propria width was developed that measured lamina propria tissue in a perpendicular manner to the imaginative midline there where a small probe crossed lamina propria (LPwdth). This midline decided how the 90°-degree angles were positioned in relation to the direction of the lamina propria.

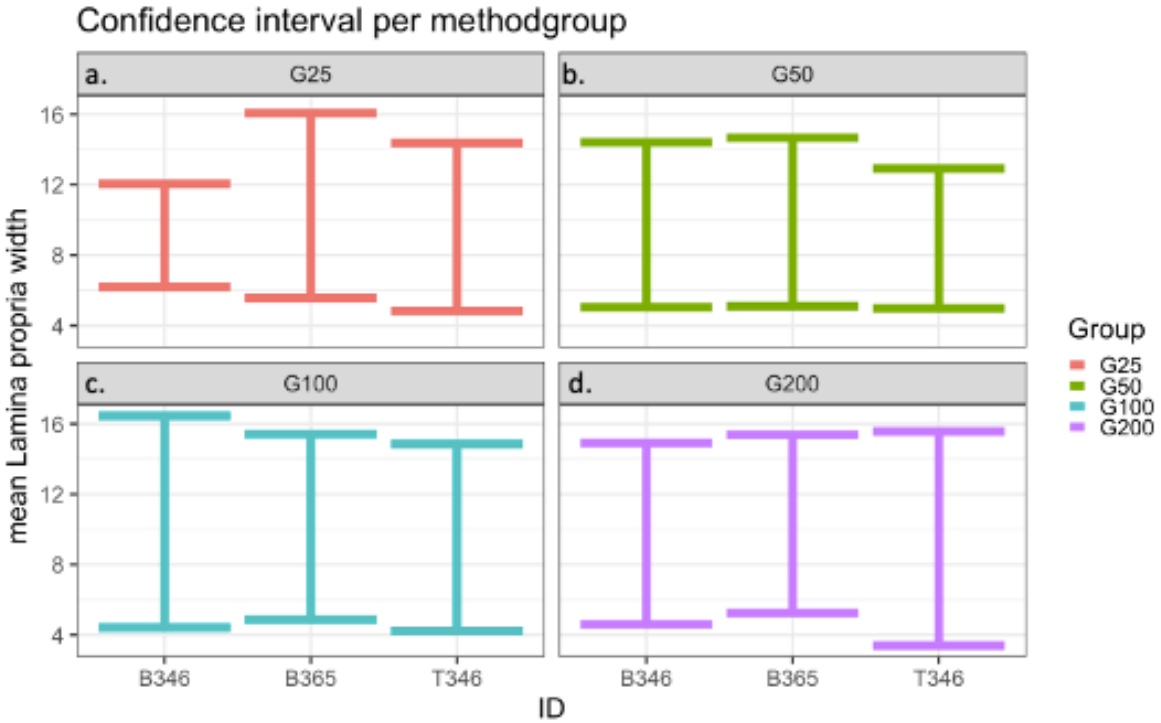


Pilot trial

The pilot trial used three randomly selected sections of foregut and applied the stereology methods to measure the mean LPwdth. The pilot results were checked for reproducibility of mean results, normal distribution and confidence intervals.

Approximately 200 LPwdth's were measured per section. From these 200 measurements, three additional groups were created (100-, 50- and 25-measurement groups), to analyse how many measurements were needed per section. Four totals, of selected unbiased sites for obtaining measures, were plotted to show a point of stabilisation where confidence intervals would represent similar results from group to group. The 50-, 100- and 200-groups showed similar distributions whereas the 25-group showed higher variation between samples (Fig. 4).

Fig. 4. Samples were chosen in a blind set-up (n=3). For each sample regions of interest were manually drawn, and counter frames (CF) were randomly deployed. Per CF epithelial tissue and lamina propria tissue were registered, registered lamina propria were also width-measured in a perpendicular manner to a midline. Approximately 200 measurements were attained per sample and divided in four count groups. These count groups represented 25 measurements (a), 50 measurements (b), 100 measurements (c) and 200 measurements (d). Confidence intervals were produced per group for assessment of point of stabilisation of the data determining the number of measures and counting frames needed for statistical accuracy. Experiment species: Atlantic salmon (*Salmo salar*).



Since stabilisation started from the 50-group a conservative approach was chosen, due to the novelty of the methods. The 100-group was taken as a baseline since the 200-group proved time consuming. Average no. measurements per CF was 1 meaning to attain approximately 100 LPwdth's per section a 100 CF were needed.

Main trial

The methodology developed in the pilot trial was implemented on the main trial on all samples (n=60). This was done blind where all information was withheld except for ID's.

Statistical data analysis

Weight was tested through a one-way ANOVA test (Linear mixed effect model with single categorical predictor; lme)

Normality was tested by the use of Q-Q plots applied to base-variables L, W, K, Ecount, EVcount, Ev, LPv, LPwdth and LPratio (Appendix A)

Significant differences between treatment groups for variables L, W, K, LPr and LPwdth were identified via independently run clustered linear mixed effect models (nlme) with a pre-set $p < 0.05$.

Correlations between K, LPr, LPwdth and other variables were tested by linear mixed effect models (nlme) with a pre-set $p < 0.05$.

All tests are performed through R studio version '1.3.959' (RStudio Team, 2020).

Results

Biometric measures

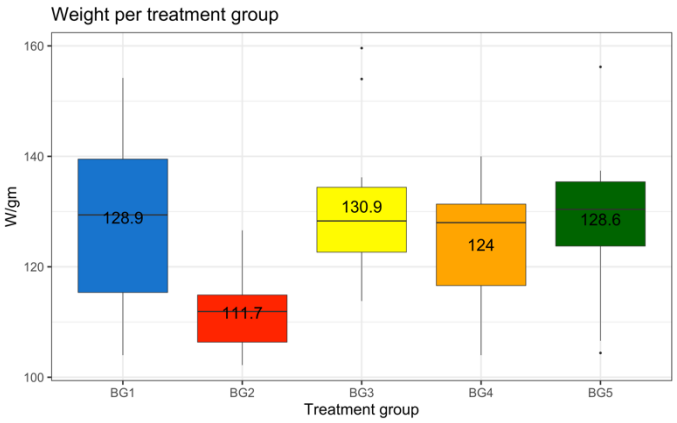
The post-smolts had an initial mean W of 72.7 ± 1.4 g (mean ± SD) and a final mean W of 124.8 ± 14.5 g after a 65-day trial. Treatment group BG2 was significantly different from all other treatment groups with a lower mean W of 111.7 g (P<.05) (Fig 5.a).

Mean final L for all treatment groups was 21.4 ± 0.8 cm. Treatment group BG2 had a shorter mean L of 20.9 cm when compared to other treatment groups (P<.068). Treatment groups did not display significant differences (Fig. 5.b).

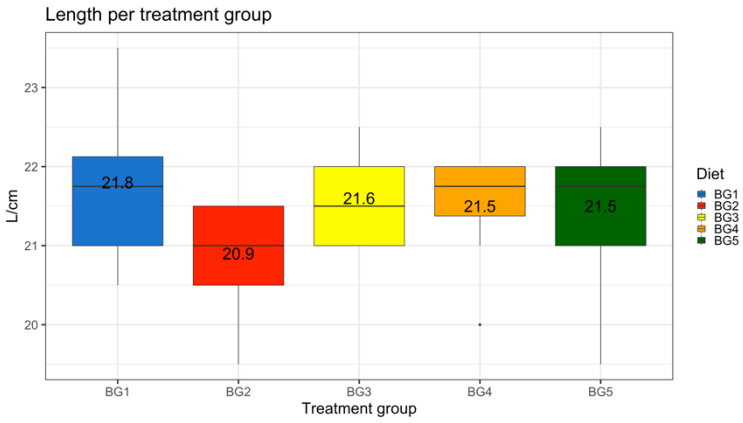
Due to linear relationship between L and W (P<.001), K was chosen for further analyses. Mean K for all treatment groups was 1.26 ± 0.08 with BG2 representing lowest mean value of 1.23. (Fig 5.c). Treatment groups did not display significant differences.

Fig. 5. At the end of the 65-d trial post-smolt were weighed (W) and lengths measured (L). Condition factor (K=100·W/L3) was attained though weights and lengths and given per treatment group. For W treatment group BG2 was significantly different from all other treatment groups (P<.05). Experiment species: Atlantic salmon (Salmo salar) (n=60).

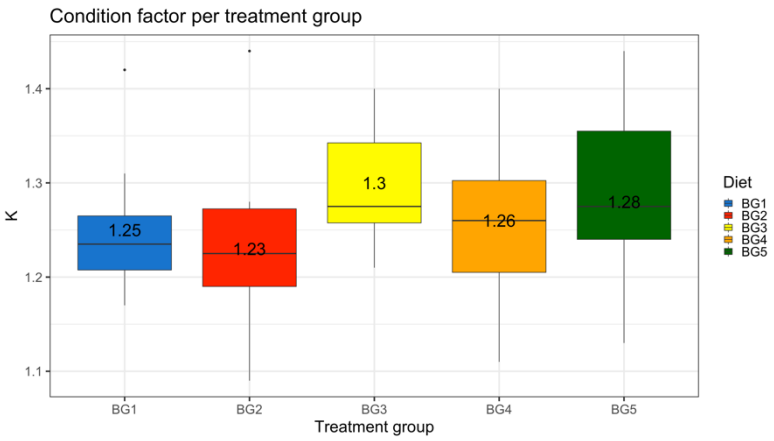
a.



b.



c.

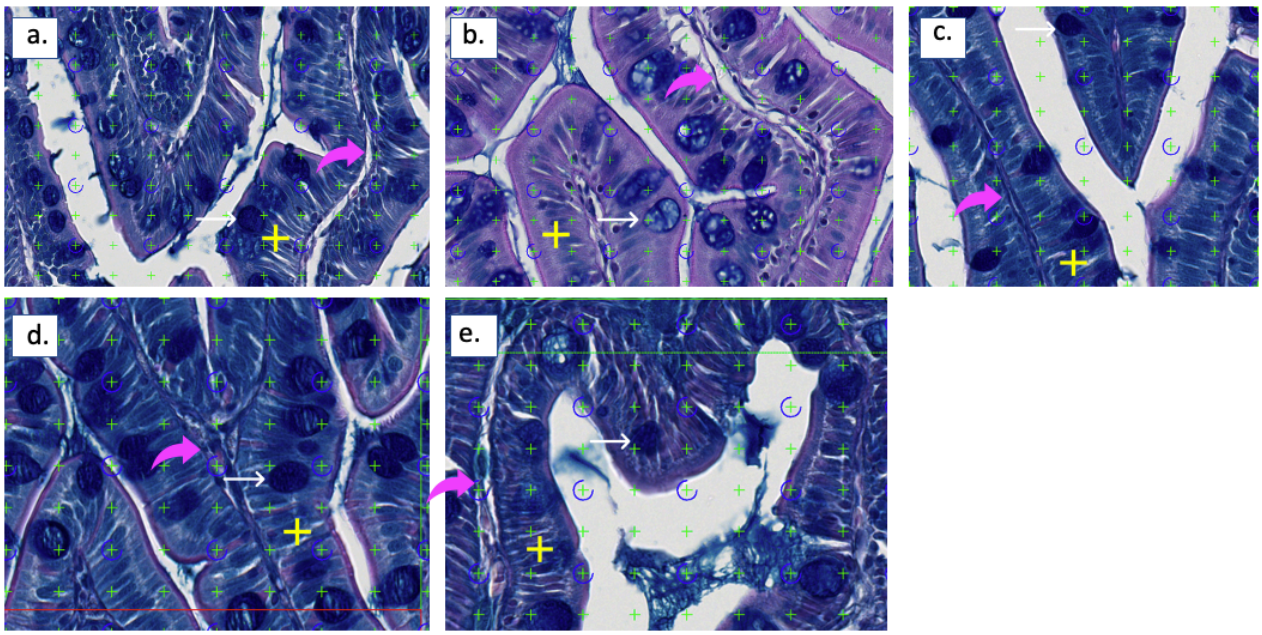


Morphological evaluation

The trial images all presented intestinal folds containing epithelia (yellow cross), lamina propria (pink thick arrow) and mucous cells (thin white arrow). A second reason for providing these images is to serve as an aid to understand upcoming Ev, LPv, LPr, LPwdth and mucous cell size variables.

Traditional histological observation shows that treatment group BG3 (Fig. 6.c.) presents the thinnest lamina propria tissue with little expansion. Expansion is indicated by white areas appearing in the lamina propria tissue or overall thickening of the tissue. Treatment group BG2 (Fig. 6.b.) and BG5 (Fig. 6.e.) show the most expansion indicating an increase in LPwdth. Treatment group BG1 (Fig. 6.a.) and BG4 (Fig. 6.d.) show an increase in LPwdth more than BG3 but less than BG2 and BG5. LPwdth in traditional histological approaches is where the eye is drawn and seems to easily indicate swelling which explains why, when referred to lamina propria tissue reactions in the literature, LPwdth is brought forward as a variable of interest for further investigation. LPr is unable to establish through this qualitative approach and for that reason most likely has not received more attention within the available literature.

Fig. 6. Reference images for each treatment group were all extracted from the VIS analysis software with a setting of 40x magnification The trial images all presented intestinal folds containing epithelia (yellow cross), lamina propria (pink thick arrow) and mucous cells (thin white arrow). a. BG2, b. BG2, c. BG3, d. BG4, e. BG5.



Lamina propria

Base measures

This section will focus on the distribution of Ev, LPv, LPr and LPwdth in relation to the different treatment groups.

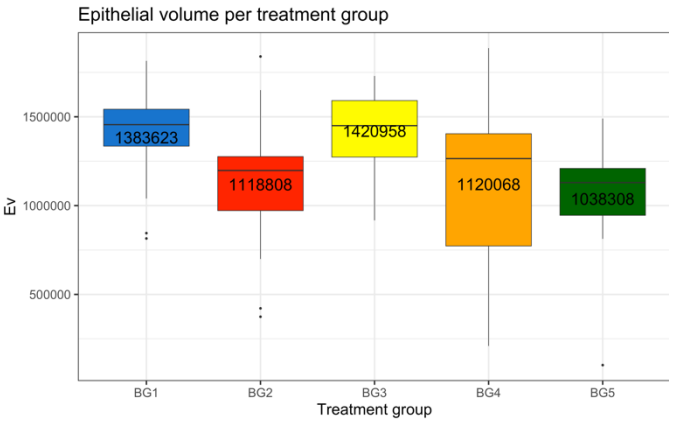
Mean Ev for all treatment groups was 1216353 ± 40074 µm2. Ev showed significant differences between treatment groups BG3 & BG5 (P<.05), BG1 approached significant difference in relation to treatment group BG5 (P<.075). Treatment group BG3 showed highest level of Ev and BG5 showed lowest level of Ev (Fig 7.a).

Mean LPv for all treatment groups was 78609 ± 26638 µm2. LPv showed no significant differences between treatment groups but did show a similar trend, to that observed for epithelial volume, with treatment group BG3 approaching significant difference from BG5 (P<.095). Treatment group BG3 showed highest level of LPv and BG5 showed lowest level of LPv (Fig 7.b).

All treatment groups showed linear relationships between LPv and Ev (P<.001) (Fig 7.c). Due to this similarity, further analyses were performed used LPr.

Fig. 7. Ev=E count*large probe volume (1890.41 µm2). For Ev BG3 & BG5 were significantly different (P<.05). LPv=LP count*small probe volume (472.6 µm2). A linear relationships was found between LPv and Ev (P<.001) (R^2: 0.79).Experiment species: Atlantic salmon (Salmo salar) (n=60).

a.



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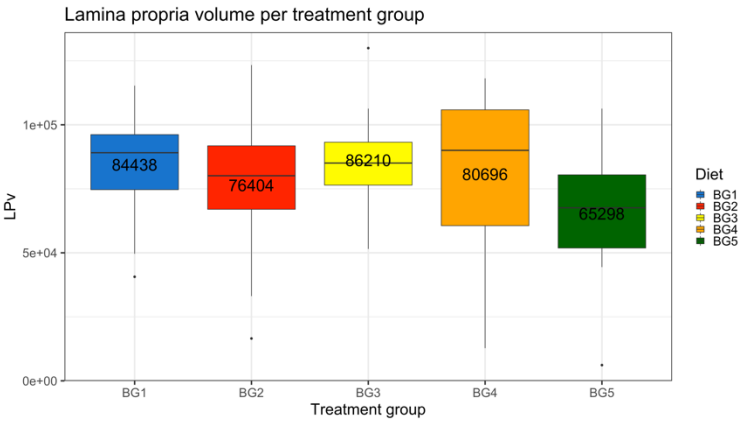

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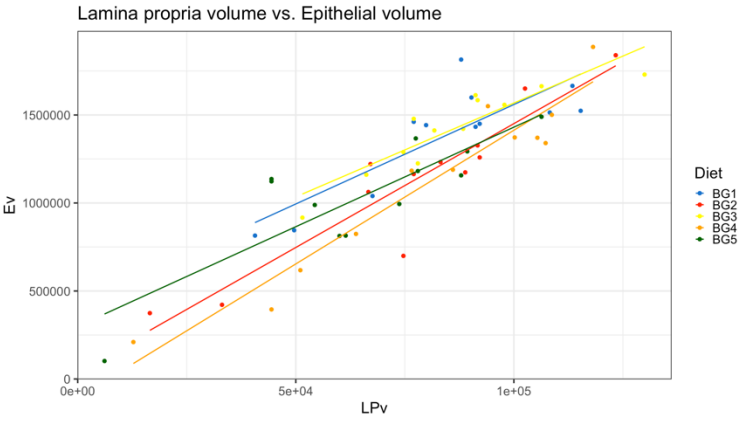


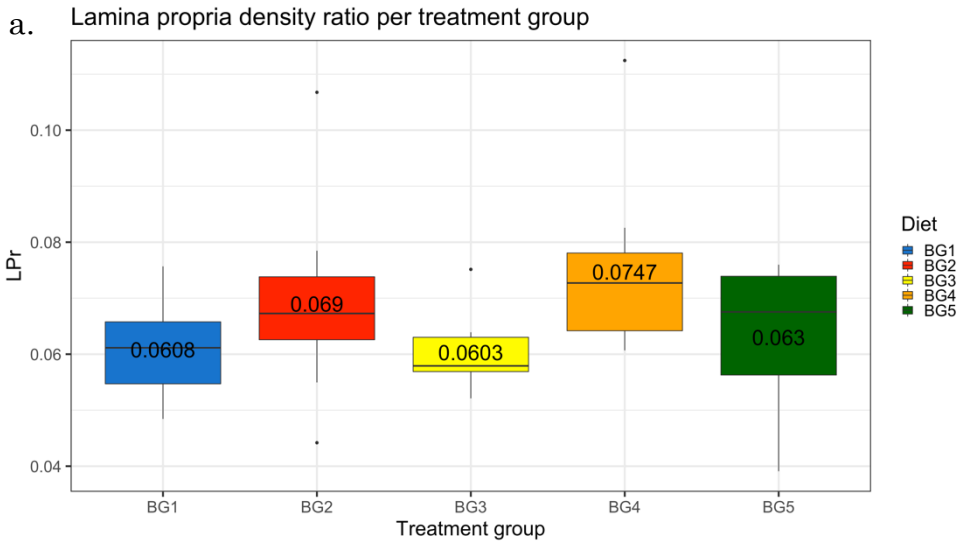
Mean LPr for all treatment groups was 0.0655 ± 0.0127. LPr showed significant difference between treatment groups BG3 & BG4 (P<.05), treatment groups BG1 & BG4 showed approaching significant difference (P<.065). Treatment group BG3 represented lowest LPr, meaning proportion of lamina propria tissue was smallest in relation to epithelia. Treatment group BG4 represented highest LPr meaning this treatment group represented the highest proportion of lamina propria tissue in relation to epithelia (Fig. 8.a).

Mean LPwdth for all treatment groups was 9.4 ± 1.7 µm. LPwdth showed no significant differences between treatment groups but approached significant difference between BG3 & BG5 (P<.082). Treatment group BG3 represented thinnest LPwdth and treatment group BG5 represented broadest LPwdth (Fig. 8.b).

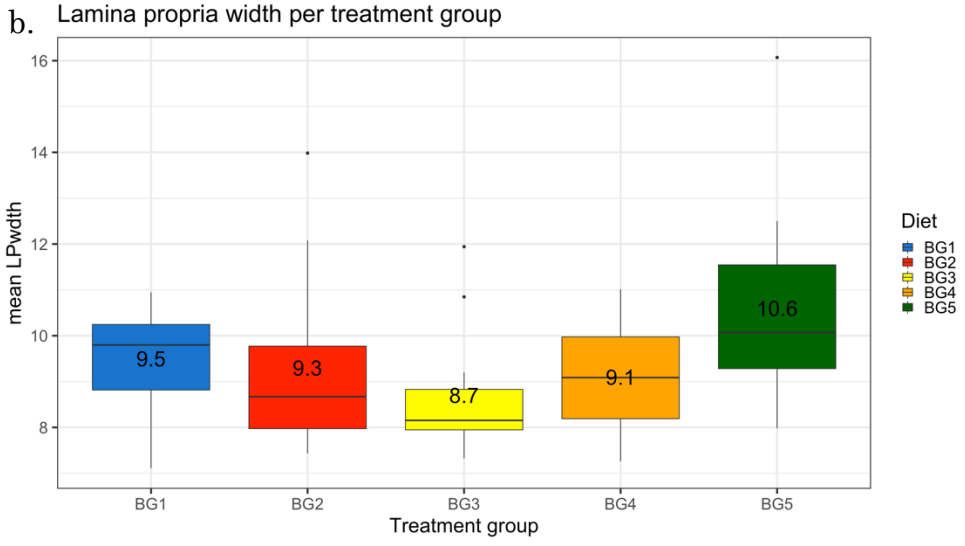
It is of concern that treatment group BG5, resembling wide-scale commercially used diets, features elevated LPr and the broadest LPwdth.

Fig. 8. LPr represents the proportion of lamina propria in relation to epithelia. LPr=LPv/Ev. For LPr there was significant difference between treatment groups BG3 & BG4 (P<.05). Lamina propria width represents the mean widths measured for each treatment group (µm). Experiment species: Atlantic salmon (Salmo salar) (n=60).

a.



b.



Lamina propria methodology correlations

Lamina propria density ratio

This section will focus on the correlations found between LPr and variables within the biological level of tissue, cell and genes.

Tissue level correlations

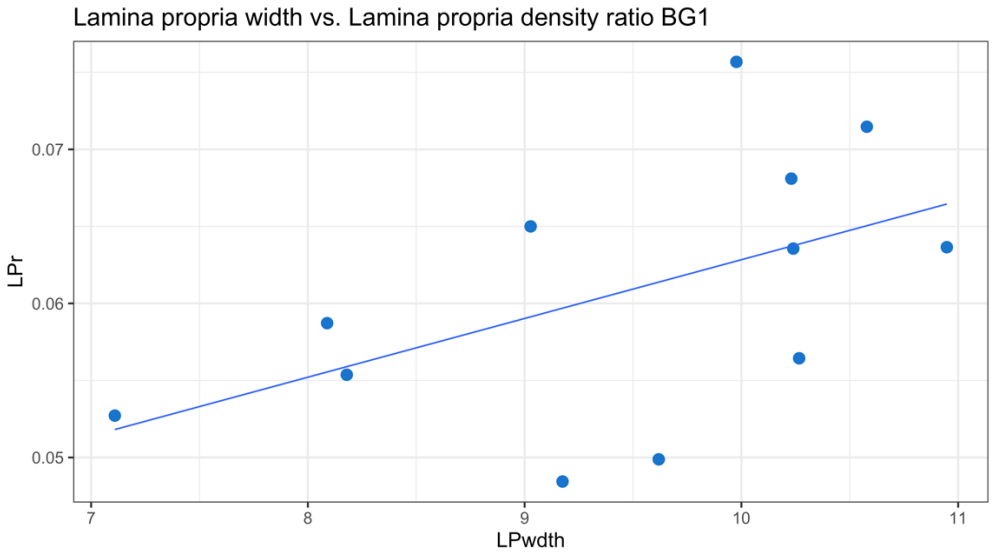
On tissue level correlations were found between LPr and LPwdth and LPr and mucous cell size.

Lamina propria density ratio in relation to lamina propria width

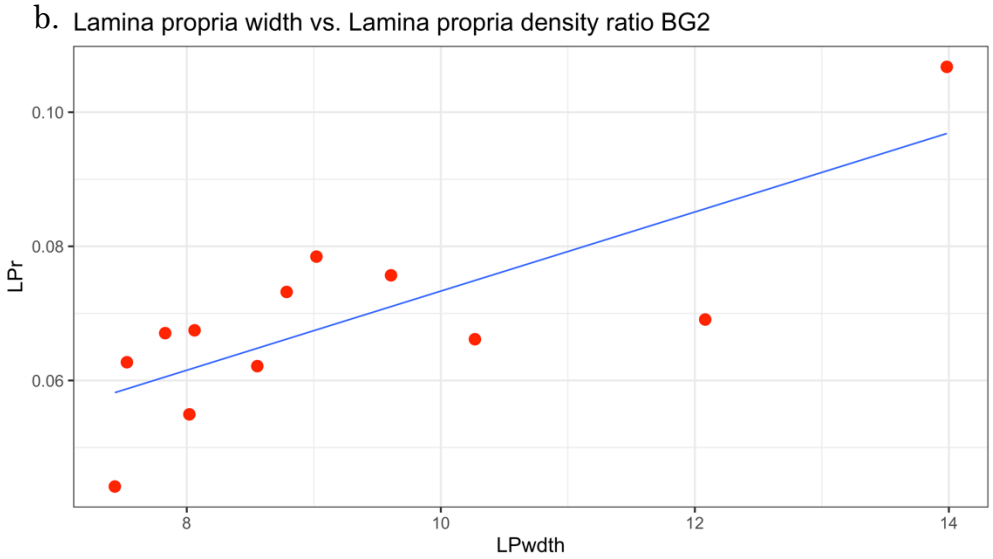
A linear relationship was found between LPr and LPwdth, meaning LPr increases when LPwdth increases. This relationship was approaching significance for treatment group BG1 (P<.086) (Fig. 9.a) and showed significance for treatment group BG2 (Fig. 9.b) and for all treatment groups when bundled (P<.05) (R^2: 0.07).

Fig. 9. A linear relationship was found between LPwdth and LPr. This relationship was approaching significance for treatment group BG1 (P<.086) (R^2: 0.27) (n=12) and showed significance for treatment group BG2 (P<.05) (BG2 R^2:0.61, n=12) and for all treatment groups when bundled (P<.05) (All R^2: 0.07, n=60). Atlantic salmon (Salmo salar).

a.



b.



Lamina propria density ratio in relation to mucous cell size

Mean mucous cell size for all treatment groups was 202 ± 67 µm. Mucous cell size showed significant difference between treatment groups BG2 & BG5 (P<.05), BG2 approached significant difference in relation to BG1 (P<.072) (Table 2) (Appendix C1) (Sørensen et al., 2020).

LPr in relation to mucous cell size showed a linear relationship for treatment group BG3 (P<.05) (Fig. 10.). Thus, for this treatment group, LPr increases as mucous cell size increases. This could mean that a healthy immune system in reaction to stressors increases in proportion of lamina propria tissue while simultaneously enlarging mucous cell sizes.

Fig. 10. A linear relationship was found between LPr and mucous cell size (µm) for treatment group BG3 (P<.05) (R^2: 0.35). Mucous cell size showed significant difference between treatment groups BG2 & BG5 (P<.05). Atlantic salmon (Salmo salar). n=12.

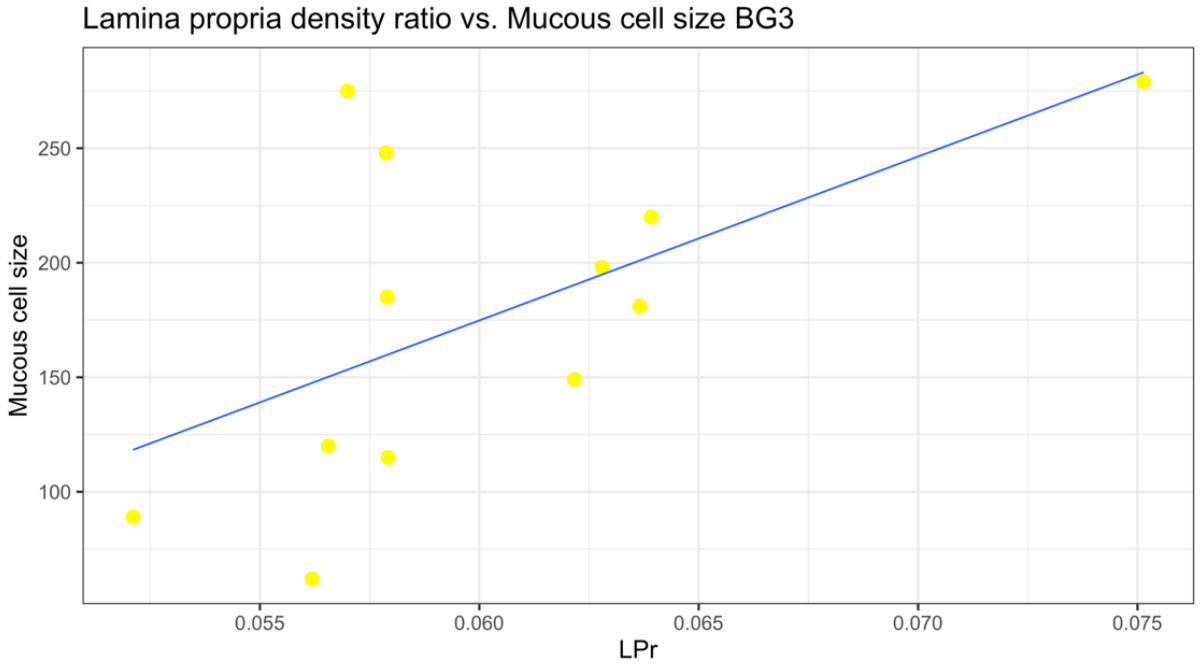


Table 2.
Significant difference per treatment group for all datasets, lamina propria, mucous, inflammatory and gene datasets.

Superscripts display significant differences between treatment groups with single characters marking origin, meaning that most differences are related to that particular treatment group. Double characters mark the origin treatment group with the following character marking the treatment group that is significantly different from the origin treatment group.

Diet *Mean*

	Lamina propria variables			Mucous variables		Head kidney variables			Genetic variables		
	Lamina propria width	Lamina propria ratio	Condition Factor	Mucous cell size	Defence activity	Phagocytic ability	Phagocytic capacity	Lymphocytes	Cathelicidin Gene expression	Mucin Gene expression	Defensin Gene expression
BG1	9.45	0.0608^{c}	1.25	220^{ab*}	0.53^{ab}	13.61^{a}	2.06^{a}	38.50^{ac}	0.032^{a}	0.978^{ab}	0.946^{ab}
BG2	9.26	0.0690	1.23	150^{a}	0.44^{a}	4.94^{ab}	1.73^{ab}	30.22	0.173	0.345^{a}	0.131^{a}
BG3	8.66^{a}	0.0603^{a}	1.30	177	0.47	5.53^{ac}	1.75^{ac}	37.54^{ab}	0.250^{ab*}	0.742^{ac}	0.784^{ac*}
BG4	9.06	0.0747^{ab}	1.26	225	0.66^{ac}	5.39^{ad}	1.78^{ad}	31.47	0.110	0.782^{ad}	0.281^{bd*}
BG5	10.64^{ab*}	0.0630^{cd*}	1.28	240^{ac}	0.65^{ad}	5.76^{ae}	1.69^{ae}	23.76^{a}	0.166^{ac*}	0.765^{ae}	0.691^{ae}

Cell level correlations

On cell level correlations were found between LPr and phagocytic ability and LPr and lymphocytes.

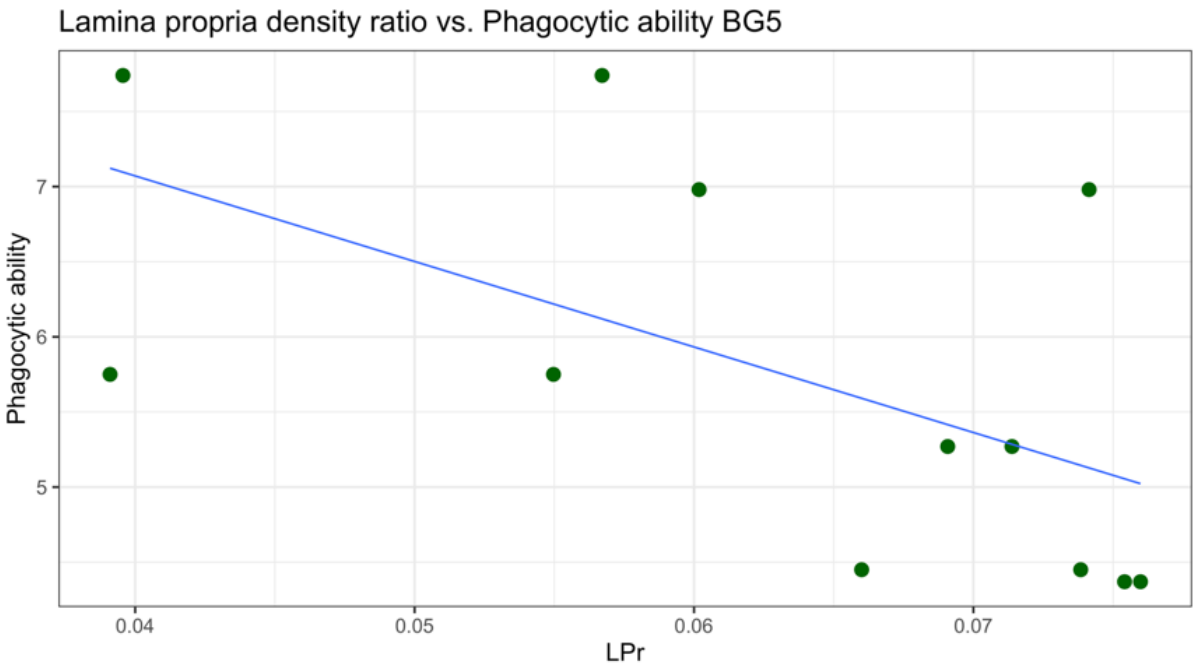
Lamina propria density ratio in relation to phagocytic ability

Mean phagocytic ability for all treatment groups was 7 ± 4. Phagocytic ability showed significant difference between treatment group BG1 and all other treatment groups (P<.05). Treatment group BG1 presented with much higher phagocytic ability levels then other treatment groups. The other treatment groups all represented much lower but similar phagocytic ability levels (Table 2: Appendix E1) (Sørensen et al., 2020).

It was shown that fish with high dietary fishmeal/fish oil percentages had better phagocytic ability and phagocytic capacity properties (Sørensen et al., 2020). Treatment group BG1, containing the highest proportion of fishmeal/fish oil, represented significantly higher phagocytic ability and phagocytic capacity than all other treatment groups (Appendix E1, E4) (Sørensen et al., 2020).

An inverse relationship was found between LPr and phagocytic ability for treatment group BG5 (P<.05) (Fig. 11.). Meaning, for this treatment group, that when LPr increases phagocytic ability decreases.

Fig. 11. An inverse relationship was found between LPr and phagocytic ability for treatment group BG5 (P<.05) (R^2: 0.33). Phagocytic ability showed significant difference between treatment group BG1 and all other treatment groups (P<.05). Atlantic salmon (Salmo salar). n=12.

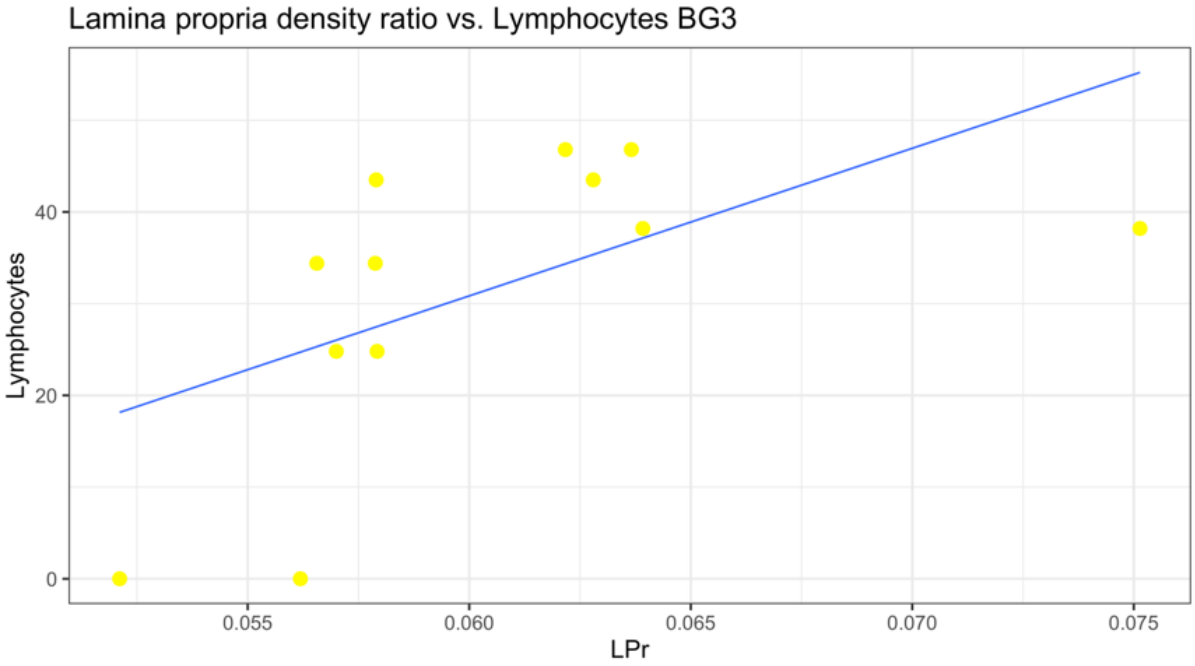


Lamina propria density ratio in relation to lymphocytes

Mean lymphocytes for all treatment groups was 32.4 ± 8.6. Lymphocytes showed significant difference between treatment groups BG1 & BG5 and BG3 & BG5 (P<.05) with treatment group BG3 representing the highest levels of lymphocytes (Table 2; Appendix E7) (Sørensen et al., 2020).

A linear relationship was found between LPr & lymphocytes for treatment group BG3 (P<.05) (Fig. 12.). Meaning, for this treatment group, that LPr increases as lymphocytes increases.

Fig. 12. A linear relationship was found between LPr and lymphocytes for treatment group BG3 (P<.05) (R^2: 0.33). Lymphocytes showed significant difference between treatment groups BG1 & BG5 and BG3 & BG5 (P<.05). Atlantic salmon (Salmo salar). n=12



Gene level correlations

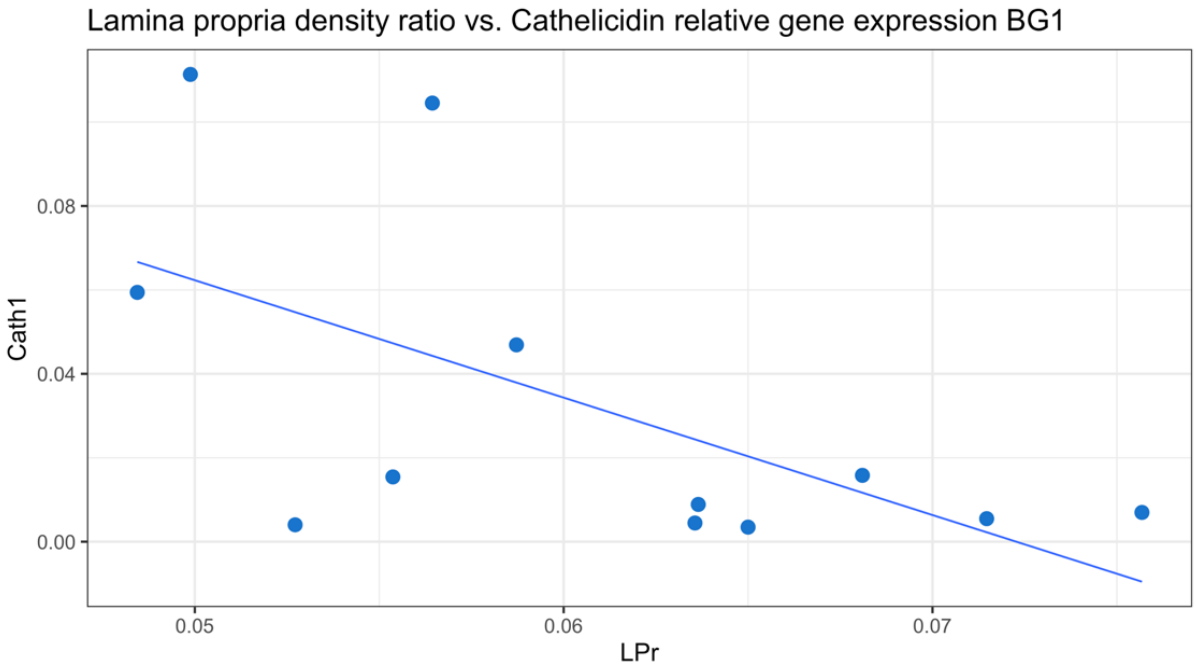
On gene level correlations were found between LPr & CATH1 and LPr & DEF3.

Lamina propria density ratio in relation to cathelicidin gene expression

Mean CATH1 for all treatment groups was 0.1463 ± 0.2. CATH1 showed no significant difference between treatment groups but did show approaching significant difference between treatment groups BG1 & BG3 (P<.066) and BG1 & BG5 (P<.056) (Table 2: Appendix G1). Treatment group BG3 presented highest variation (Sørensen et al., 2020).

An inverse relationship was found between LPr and CATH1 for treatment group BG1 (P.<05) (R^2: 0.37) (Fig. 13.). Meaning, for this treatment group, that when LPr increases CATH1 decreases.

Fig. 13. An inverse relationship was found between LPr and cathelicidin relative gene expression (CATH1) for treatment group BG1 (P<.05) (R^2: 0.37). CATH1 showed no significant difference between treatment groups. Atlantic salmon (Salmo salar). n=12.

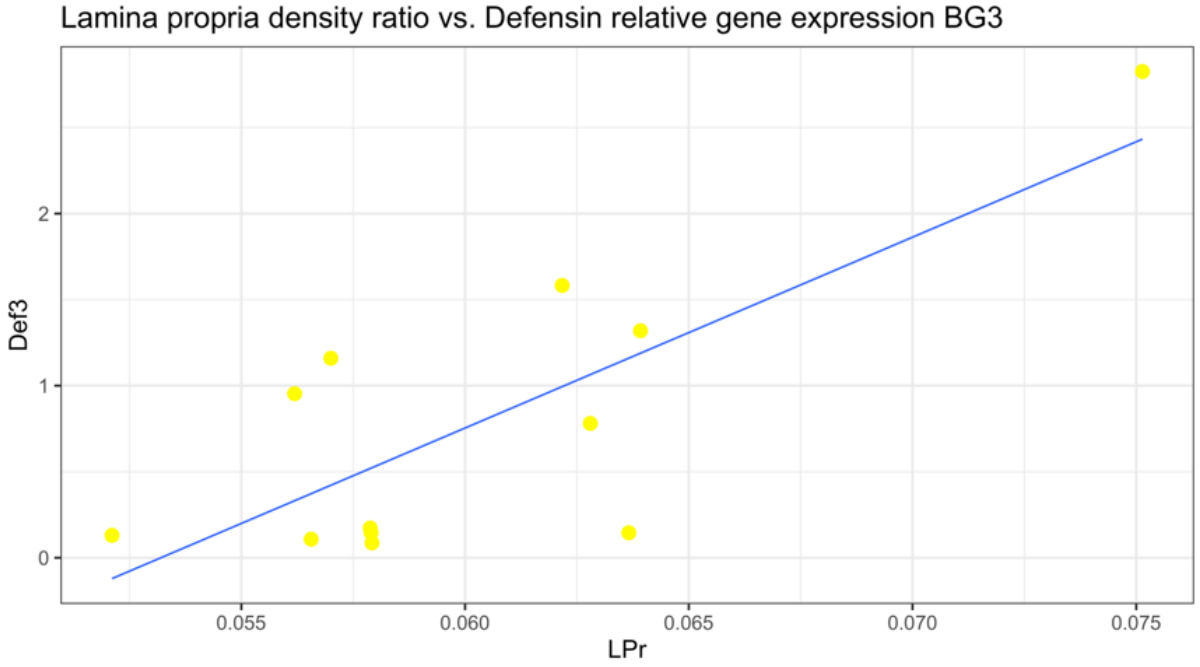


Lamina propria density ratio in relation to defensin gene expression

Mean DEF3 for all treatment groups was 0.5 ± 0.6. DEF3 showed significant difference between treatment groups BG1 & BG2 and BG2 & BG5 (P<.05). In addition, close significance was found for BG1 & BG4 (P<.058) and BG2 & BG3 (P<.089) (Table 2; Appendix G7).

A linear relationship was found between LPr and DEF3 for treatment group BG3 (P.<05) (R^2: 0.60) (Fig. 14.). Meaning, for this treatment group, that LPr increases as DEF3 increases.

Fig. 14. A linear relationship was found between LPr and defensin relative gene expression (DEF3) for treatment group BG3 (P<.05) (R^2: 0.60). DEF3 showed significant difference between treatment groups BG1 & BG2 and BG2 & BG5 (P<.05). Atlantic salmon (Salmo salar). n=12



Lamina propria width

This section will focus on the correlations found between LPwdth and variables within the biological level of tissue, in this case mucous cell size. In addition, correlations were found for gene level between LPwdth and CATH1

Tissue level correlations

On tissue level correlations were found between LPr and LPwdth and LPr and mucous cell size

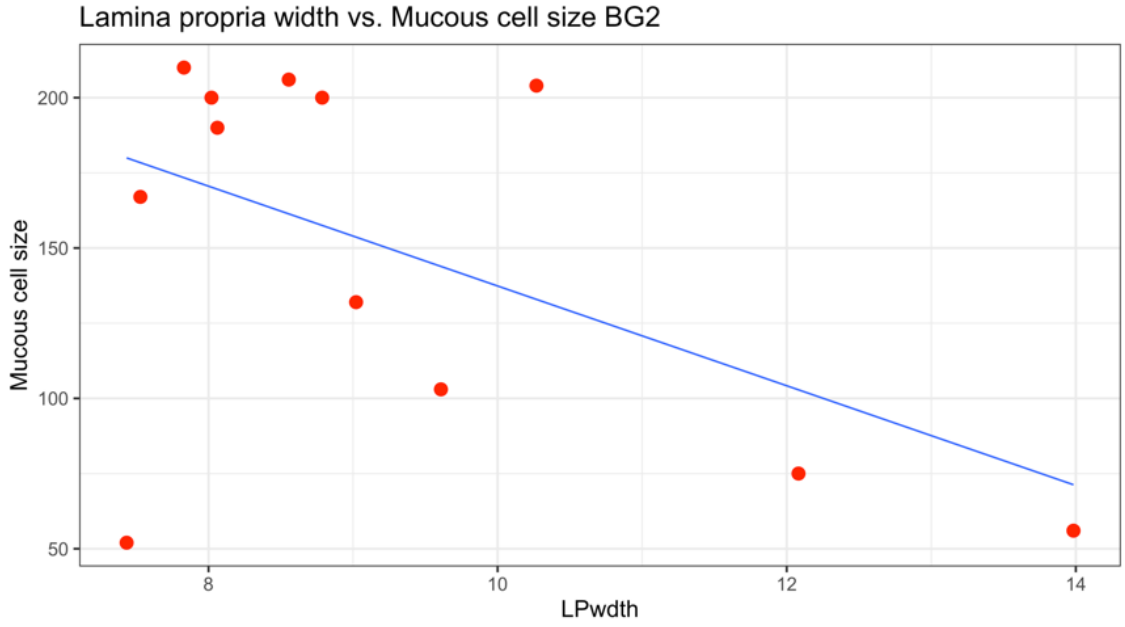
Lamina propria width in relation to mucous cell size

LPwdth in relation to mucous cell size showed an inverse relationship with approaching significance for treatment group BG2 ($P<.078$) (R^2: 0.28). Meaning, for this treatment group, that when LPwdth increases mucous cell size decreases (Fig. 15.).

Previous relationship found for treatment group BG3 showed that mucous cell size increased with LPr. It was shown as well that for several treatment groups LPr and LPwdth increase simultaneously. This means that the inverse relationship for treatment group BG2, showing a decrease in mucous cell size as LPwdth increases, is opposite to the mucous cell reaction for treatment group BG3.

This could indicate, since BG2 was meant to apply pressure to the immune system and create inflammation, that when the immune system has met a threshold that mucous cell defence is depleted and that mucous cell size decreases resulting in inflammation, expansion of LPwdth and LPr and ends with a compromised mucous layer. Treatment group BG2 was known to cause inflammation and poor health, which was reflected in lowest K and elevated LPr and LPwdth values.

Fig. 15. A non-significant but close inverse relationship was found between LPwdth (µm) and mucous cell size for treatment group BG2 ($P<.05$) (R^2: 0.28). Mucous cell size showed significant difference between treatment groups BG2 & BG5 ($P<.05$). Atlantic salmon (Salmo salar). n=12.



Gene level correlations

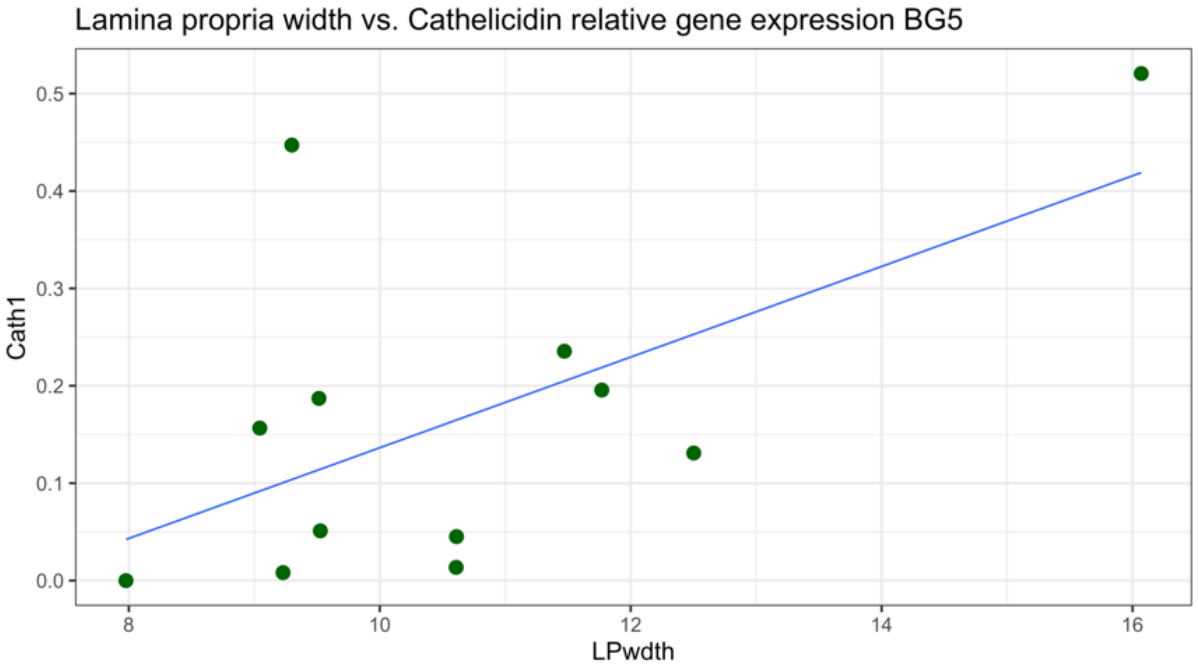
On gene level correlations were found between LPwdth & CATH1.

Lamina propria width in relation to cathelicidin gene expression

CATH1 showed no significant difference between treatment groups but did show approaching significant difference between BG1 & BG3 (P<.066) and BG1 & BG5 (P<.056) (Table 2: Appendix G1). Treatment group BG3 presented highest variation (Sørensen et al., 2020).

A linear relationship was found between LPwdth and CATH1 for treatment group BG5 (P.<043) (R^2: 0.35) (Fig. 16.). Meaning, for this treatment group, that LPwdth increases as CATH1 increases.

Fig. 16. A linear relationship was found between LPwdth (µm) and CATH1 for treatment group BG5 (P<.05) (R^2: 0.35). CATH1 showed no significant difference between treatment groups. Atlantic salmon (Salmo salar). n=12



Previously, treatment group BG1 showed an inverse relationship between LPr and CATH1 meaning as LPr increases CATH1 decreases. As mentioned before LPr and LPwdth have a linear relationship for several diets. Meaning CATH1 shows different responses between treatment groups BG1 and BG5.
Treatment groups BG1 and BG5 are significantly different from each other concerning mean CATH1 per treatment group with BG5 presenting higher CATH1 levels (Appendix G1). Treatment group BG1, as the positive control, was considered to not agitate the gastrointestinal tract which was reflected by low LPr values. Thus, a healthy gastrointestinal tract could be characterised by low levels of CATH1.

However, treatment group BG3 which performed best in relation to LPr, LPwdth and K did present with the highest CATH1 levels.

Condition factor

This section will focus on the correlations found between K and variables within the biological level of tissue, cell and genes.

Tissue level correlations

On tissue level correlations were found between K and LPr.

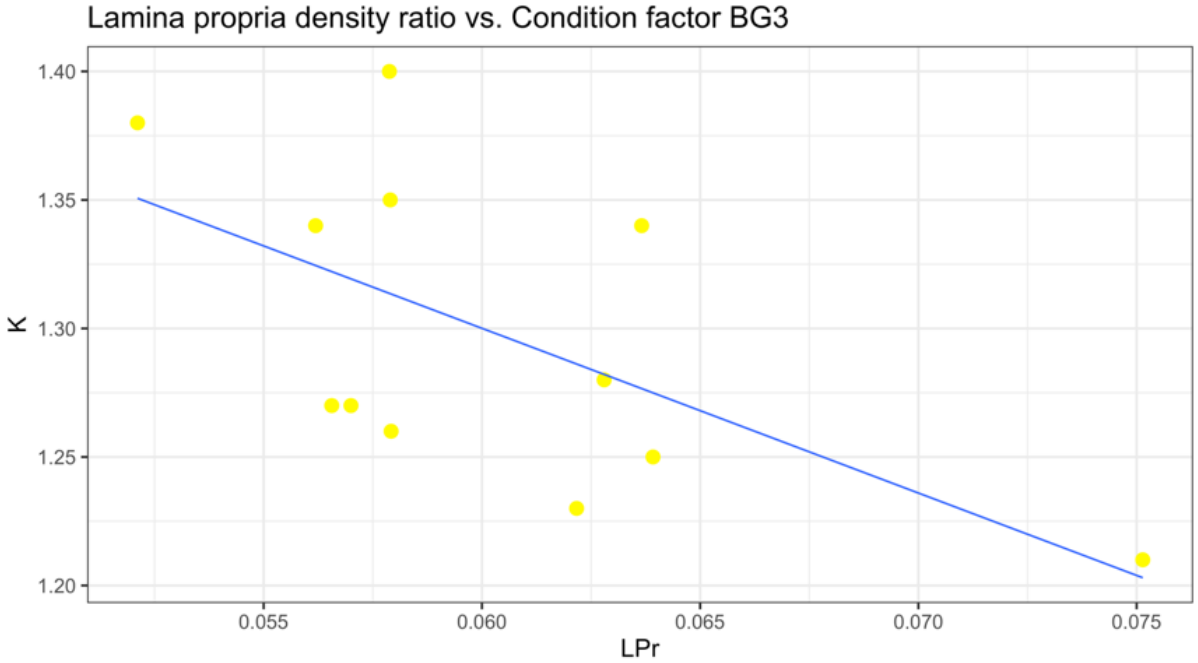
Condition factor in relation to lamina propria density ratio

Mean K for all treatment groups was 1.26 ± 0.08 with BG2 representing lowest mean value of 1.23 and treatment group BG3 representing highest K. Treatment groups did not display significant differences.

K in relation to LPr showed a significant inverse relationship for treatment group BG3 ($P<.05$) (R^2: 0.38) (Fig. 17.). So, for this treatment group, K decreases as the proportion of lamina propria tissue increases. Meaning that the proportion of lamina propria tissue decreases when physical health indication improves. This proportional decrease in lamina propria tissue is confirmed by thin LPwdth for treatment group BG3.

Linear relationships between LPr and LPwdth were found for several diets, meaning low LPr seem to coincide with thin LPwdth. It could be argued that healthy fish feature thin LPwdth in combination with low LPr.

Fig. 17. A significant inverse relationship was found between LPr and K for treatment group BG3 ($P<.05$) (R^2: 0.38). Treatment groups did not display significant differences. Experiment species: Atlantic salmon (Salmo salar) (n=12).



Cell level correlations

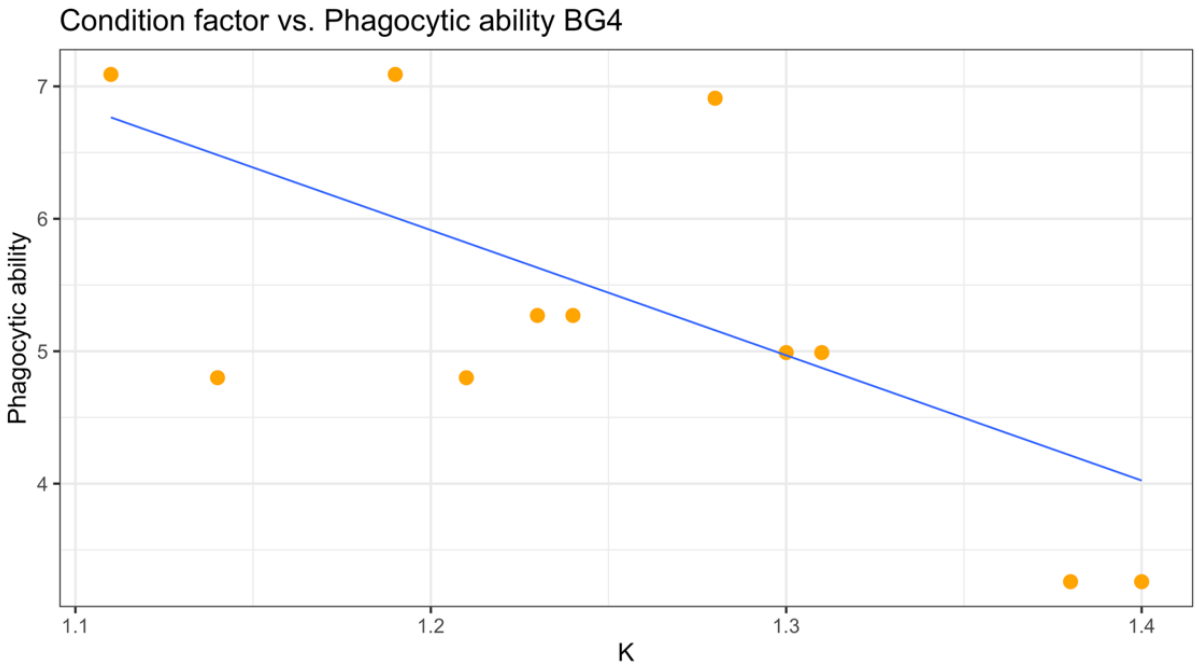
On cell level correlations were found between K & phagocytic ability, K & phagocytic capacity and K & lymphocytes.

Condition factor in relation to phagocytic ability

Mean phagocytic ability for all treatment groups was 7 ± 4. Phagocytic ability showed significant difference between treatment group BG1 and all other treatment groups (P<.05) (Table 2: Appendix E1) (Sørensen et al., 2020).

For phagocytic ability and K an inverse relationship was found as well for treatment group BG4 (P<.05) (R^2: 0.37) (Fig. 18.). Meaning as K increases phagocytic ability decreases.

Fig. 18. A significant inverse relationship was found between K and phagocytic ability for treatment group BG4 (P<.05) (R^2: 0.37). Phagocytic ability showed significant difference between treatment group BG1 and all other treatment groups (P<.05). Experiment species: Atlantic salmon (Salmo salar) (n=12).

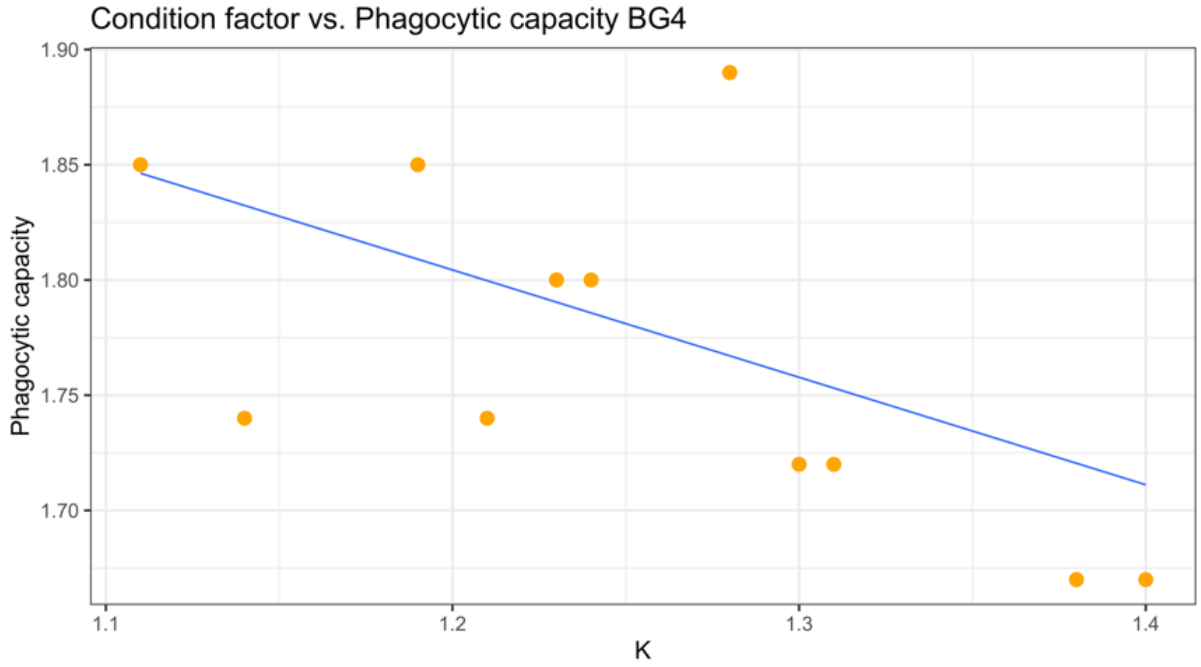


Condition factor in relation to phagocytic capacity

Mean phagocytic capacity for all treatment groups was 1.8 ± 0.1. Phagocytic capacity showed significant differences between treatment group BG1 and all other treatment groups (P<.05). In addition, significant difference was found between treatment groups BG4 & BG5 (P<.05) (Table 2: Appendix E4) (Sørensen et al., 2020).

As significance was found for K & phagocytic ability for treatment group BG4, a similar trend was seen for K & phagocytic capacity for the same treatment group however, with non-significant values (P<.087) (R^2: 0.26) (Fig. 19). Meaning as K increases phagocytic capacity decreases.

Fig. 19. A non-significant inverse relationship was found between K and phagocytic capacity for treatment group BG4 (P<.087) (R^2: 0.26). Phagocytic capacity showed significant differences between treatment group BG1 and all other treatment groups (P<.05). Experiment species: Atlantic salmon (Salmo salar) (n=12).



These two correlations found between K and phagocytic ability and capacity for treatment group BG4 show that both phagocytic ability and capacity decrease as K increases. A linear relationship was found between phagocytic ability and phagocytic capacity meaning they increase or decrease simultaneously, supporting the observation of both phagocytic ability and phagocytic capacity reacting in similar manners to condition factor for treatment group BG4 (Sørensen et al., 2020) (Appendix F1, F2, F3, F4). This could indicate both phagocytic ability and capacity increased when fish are in poorer physical condition.

However, previously an inverse relationship was found between LPr and phagocytic ability for treatment group BG5. Meaning, for this treatment group, that when LPr increases phagocytic ability decreases. It was also just shown that that LPr increases as K decreases for treatment group BG3. It could be argued

794 that phagocytic ability would then increase with K, which is opposite to what was
795 found for treatment group BG4.
796
797 Treatment groups BG4 and BG5 did have similar dietary compositions.
798 Treatment group BG4 contained higher levels of fish oil than treatment group BG5
799 but showed the inverse relationship for both phagocytic ability and capacity which
800 would contrarily have been expected for the other treatment group, if expected at
801 all. The trends and relationships found for this trial do not sufficiently explain the
802 occurrence of these discrepancies for treatment groups BG4 and BG5

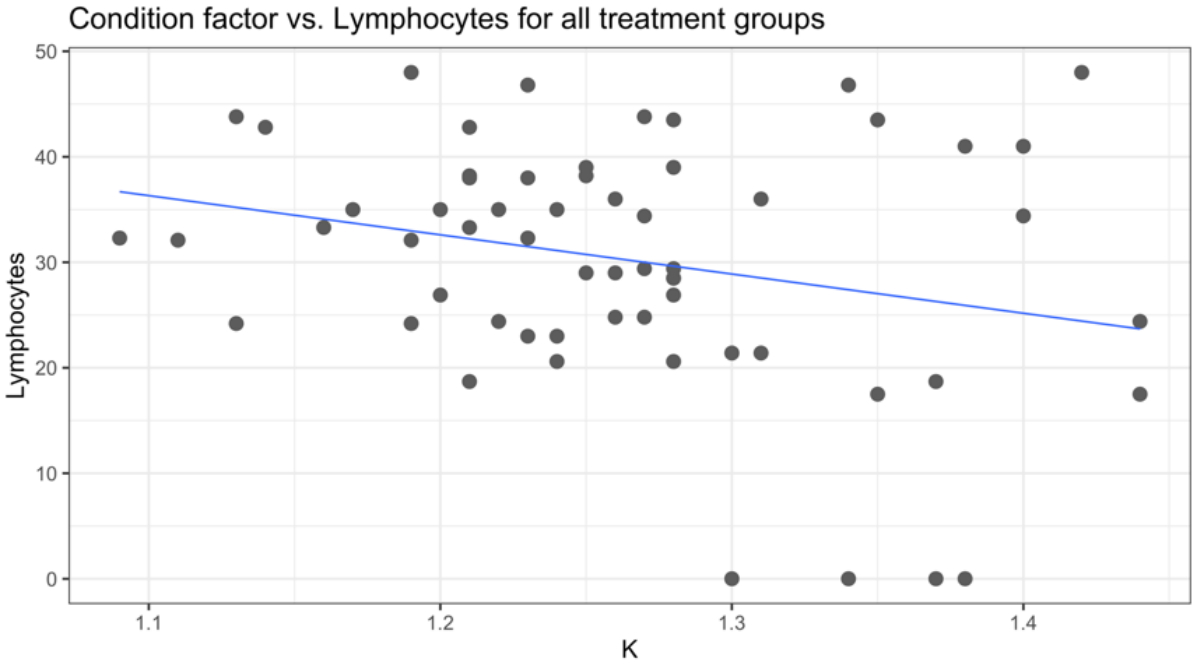
Condition factor in relation to lymphocytes

Mean lymphocytes for all treatment groups was 32.4 ± 8.6. Lymphocytes showed significant difference between treatment groups BG1 & BG5 and BG3 & BG5 (P<.05) (Table 2; Appendix E7) (Sørensen et al., 2020).

An inverse relationship was found between K & lymphocytes when applied to all treatment groups (P<.05) (R^2: 0.07) (Fig. 20.). Meaning as K increases lymphocytes decreases.

Even though this relationships is weak it does confirm previously found linear relationship between LPr and lymphocytes for treatment group BG3.

Fig. 20. A significant inverse relationship was found between K and lymphocytes when applied to all treatment groups (P<.05) (R^2: 0.07). Lymphocytes showed significant difference between treatment groups BG1 & BG5 and BG3 & BG5 (P<.05). Experiment species: Atlantic salmon (Salmo salar) (n=60).



Gene level correlations

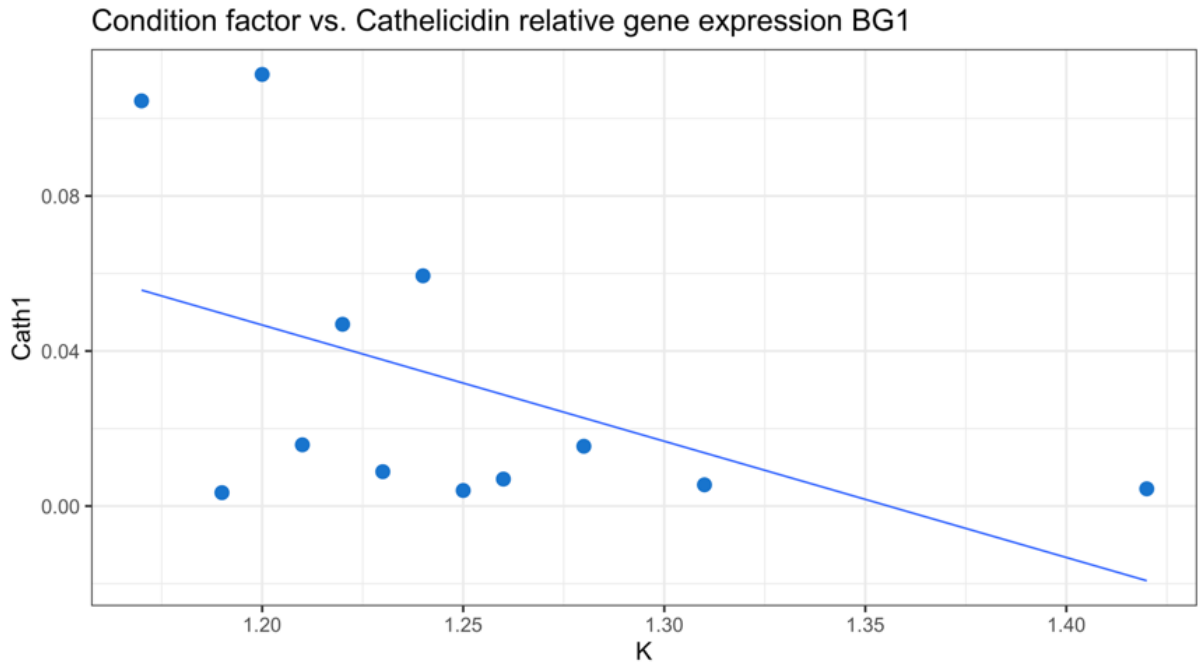
On gene level correlations were found between K & CATH1.

Condition factor in relation to cathelicidin relative gene expression

Mean CATH1 for all treatment groups was 0.1463 ± 0.2. CATH1 showed no significant difference between treatment groups but did show approaching significant difference between treatment groups BG1 & BG3 (P<.066) and BG1 & BG5 (P<.056) (Table 2: Appendix G1) (Sørensen et al., 2020).

An inverse relationship with approaching significance was found between K and CATH1 for treatment group BG1 (P.<094) (R^2: 0.25) (Fig. 21). Meaning as K increases CATH1 decreases.

Fig. 21. A non-significant inverse relationship was found between K and CATH1 for treatment group BG1 (P<.094) (R^2: 0.25). CATH1 showed no significant difference between treatment groups. Experiment species: Atlantic salmon (Salmo salar) (n=12).

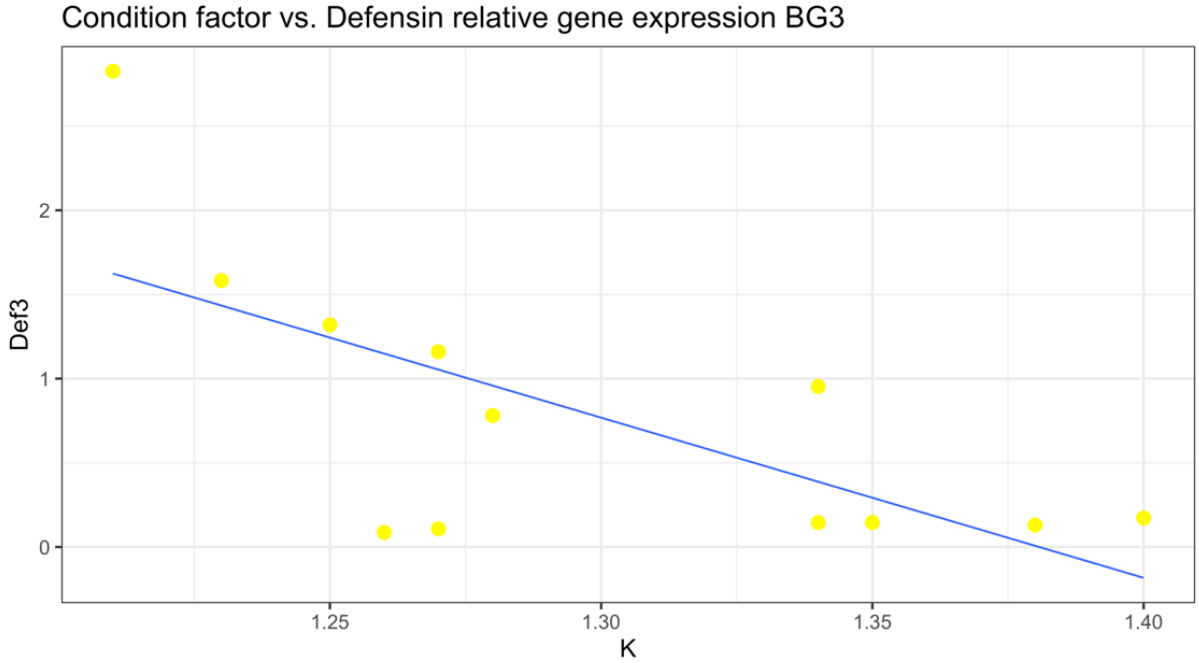


Condition factor in relation to defensin relative gene expression

Mean DEF3 for all treatment groups was 0.5 ± 0.6. DEF3 showed significant difference between treatment groups BG1 & BG2 and BG2 & BG5 (P<.05). In addition, close significance was found for BG1 & BG4 (P<.058) and BG2 & BG3 (P<.089) (Table 2; Appendix G7).

An inverse relationship was found between K & DEF3 for treatment group BG3 (P.<05) (R^2: 0.48) (Fig.22.). Meaning as K increases DEF3 decreases.

Fig. 22. A significant inverse relationship was found between K and DEF3 for treatment group BG3 (P<.05) (R^2: 0.48). DEF3 showed significant difference between treatment groups BG1 & BG2 and BG2 & BG5 (P<.05). Experiment species: Atlantic salmon (Salmo salar) (n=12).



This is confirmed by the previously found linear relationship between LPr and DEF3 for treatment group BG3 as well. Meaning as LPr increases the DEF3 increases. Indicating that when physical health decreases DEF3 increases.

It could be stated that CATH1 and DEF3 both increase when LPwdth and LPr increase as a result of a decreasing K. Peculiarly, treatment group BG3, that presented with healthy values for LPwdth, LPr and K presented the highest mean CATH1 value.

Condition factor in relation to mucin relative gene expression

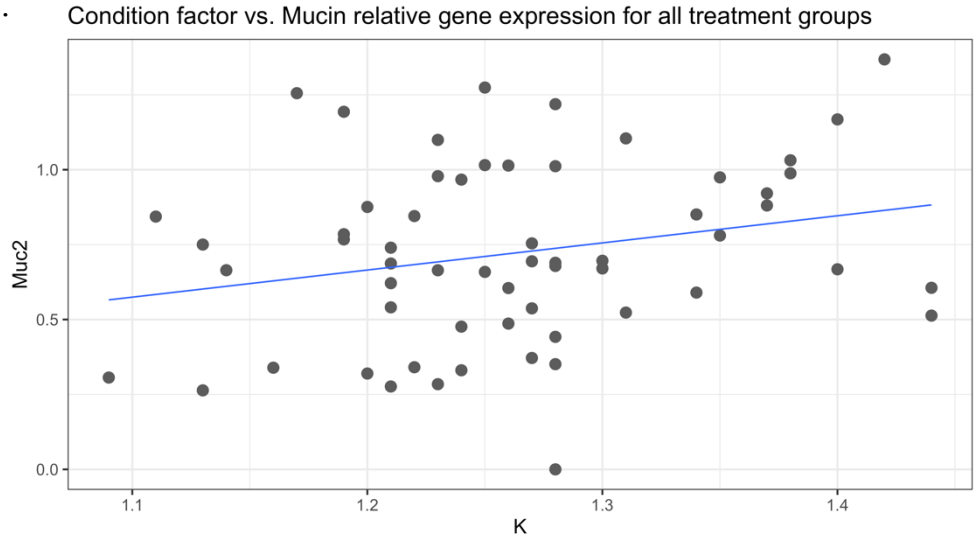
Mean MUC2 for all treatment groups was 0.7 ± 0.3. MUC2 showed significant difference between treatment group BG2 and all other treatment groups (P<.05) (Table 2: Appendix G4) (Sørensen et al., 2020).

A linear relationship was found between K & MUC2 for treatment group BG2 (P.<05) (R^2: 0.68) (Fig 23.a.), this relationship was also found when applied to all treatments however with non-significant but approaching values (P<.067) (R^2: 0.06) (Fig 23.b.). Meaning, for this treatment group, that K increases as MUC2 increases.

Fig. 23. A linear relationship was found between K and mucin relative gene expression (MUC2) for treatment group BG2 (P<.05) (R^2: 0.68) (n=12) and a non-significant relationship when applied to all treatment groups (P<.067) (R^2: 0.06) (n=60). MUC2 showed significant difference between treatment group BG2 and all other treatment groups (P<.05). Atlantic salmon (Salmo salar).

a.

b.



MUC2 is mainly involved in the maintenance of the mucous layer and aid in the defence against pathogens (Ellis., 2001; Olafsen, 2001). Treatment group BG2 presented significantly lower values for MUC2 than all other diets (Appendix G4) (Sørensen et al., 2020). Indicating that low levels of MUC2 indicate poor health.

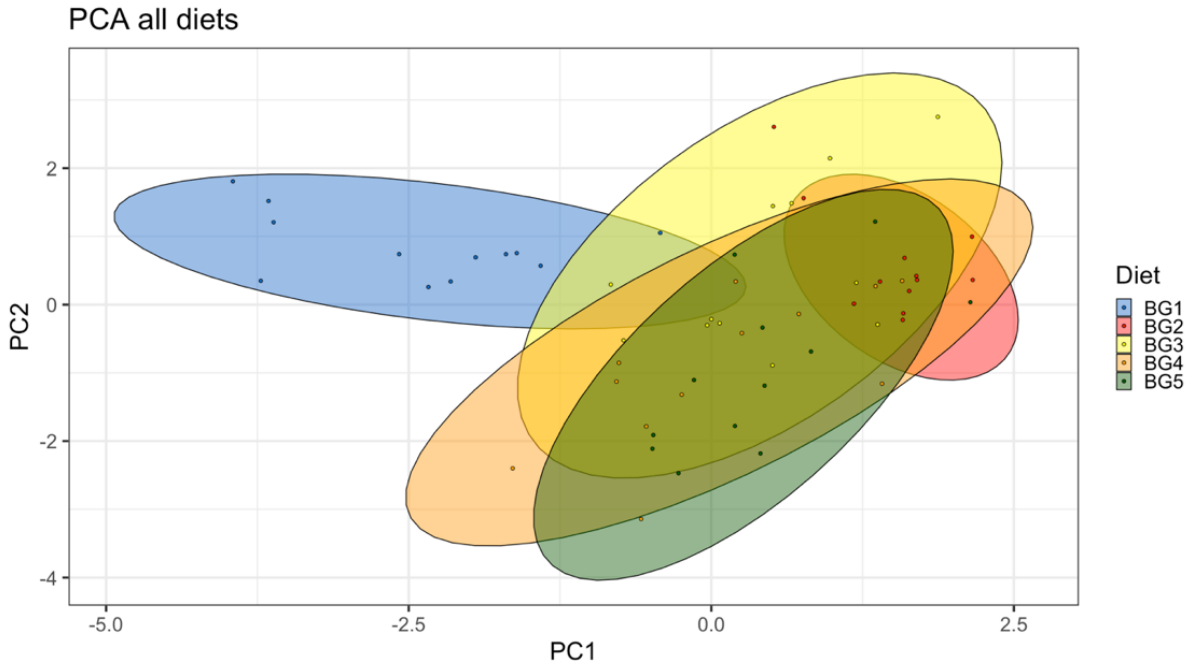
PCA

A PCA is deemed reliable in interpreting relationships between the different variables when proportion of variance exceeds 60%. Due to the novelty of the lamina propria trial it is used for exploratory measures and determination of future trial endeavours.

PCA for all treatment groups

Principal component analysis was performed for each diet in relation to all variables. Proportion of variance did not exceed 22%, meaning only 22% of proportion of variance is explained by PC1 (Appendix I6). The relationships indicated were not represented by the correlations found and explained in previous chapters. However, the PCA when converted to a polygon confirms previous indications by showing great overlap for treatment groups BG2, BG3, BG4 and BG5. However, BG1, positive control, showed the least overlap in relation to the other treatment groups and no overlap with BG2, the negative control (Fig. 24).

Fig. 24. Polygon representing PCA finding for all treatment groups. Original PCA presented a proportion of variance of 22% and indicated relationships did not present in correlations explained in previous chapters, thus was excluded form analyses. Atlantic salmon (Salmo salar). n=60



PCA for all treatment groups including diet composition ingredients

The principal component analysis including all treatment groups and diet composition ingredients showed a proportion of variance of 42% for PC1, meaning 42% of the variation is explained by PC1.

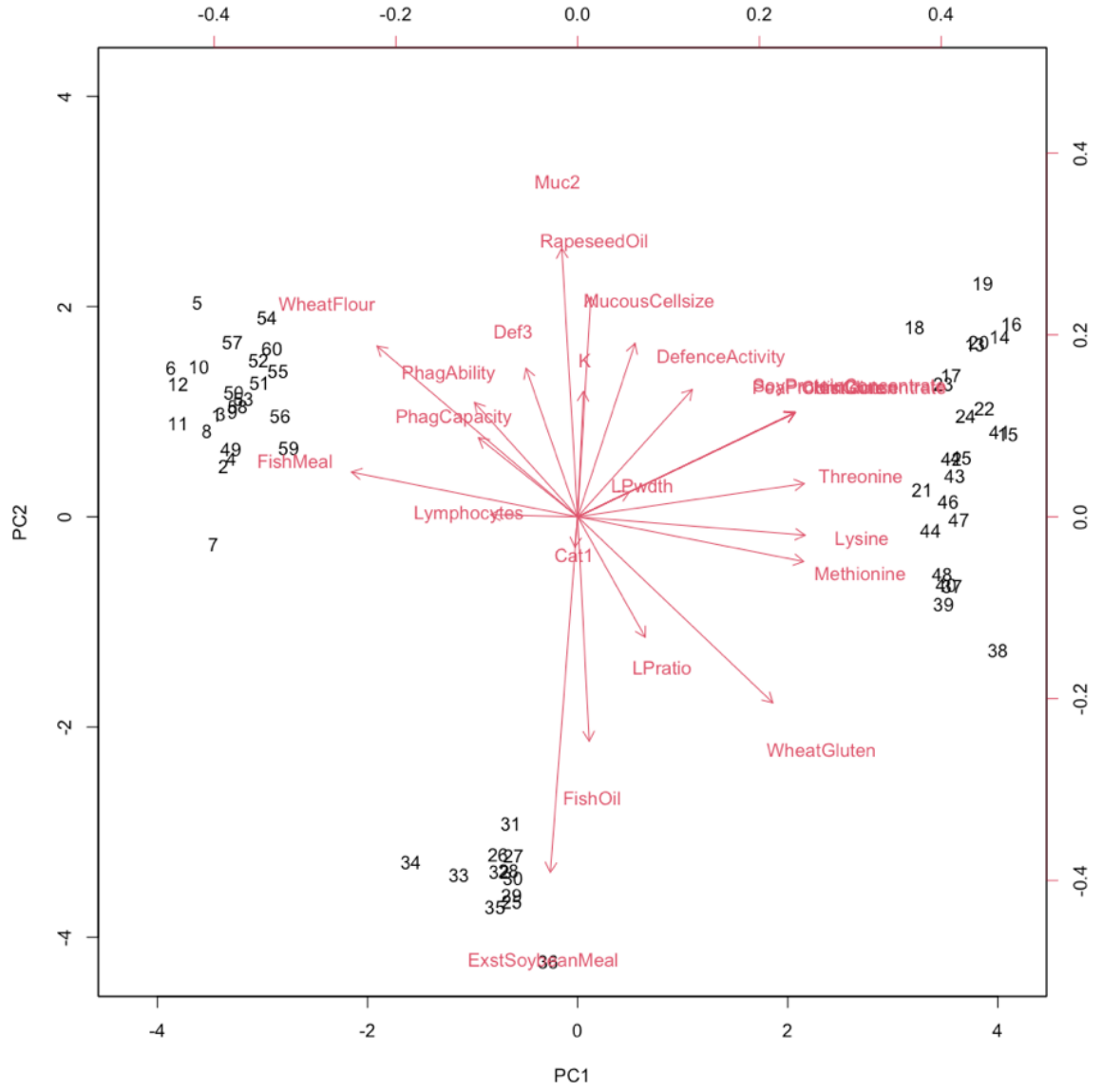
PCA showed grouping for MUC2, mucous cell size and condition factor including rapeseed oil with a possible inverse relationship to CATH1 including fish oil and extracted soybean meal.
MUC2 and K showed a linear relationships for BG2 confirming this grouping. Mucous cell size did not show relation to K but did show a linear relationship with

LPr for treatment group BG3. However, since LPr showed an inverse relationship with mucous cell size this grouping was not confirmed.
Phagocytic ability and phagocytic capacity grouped together including wheat flour with a possible inverse relationship to LPr including wheat gluten. Grouping for phagocytic ability and phagocytic capacity was confirmed by correlations found between the two variables (Sørensen et al., 2020).

Lymphocytes and fish meal grouped together with a possible inverse relationship to grouping threonine, lysine and methionine.

LPwdth and defence activity grouped together including soybean protein and pea protein but did not show a clear possible inverse relationship to any other groupings (Fig. 25.).

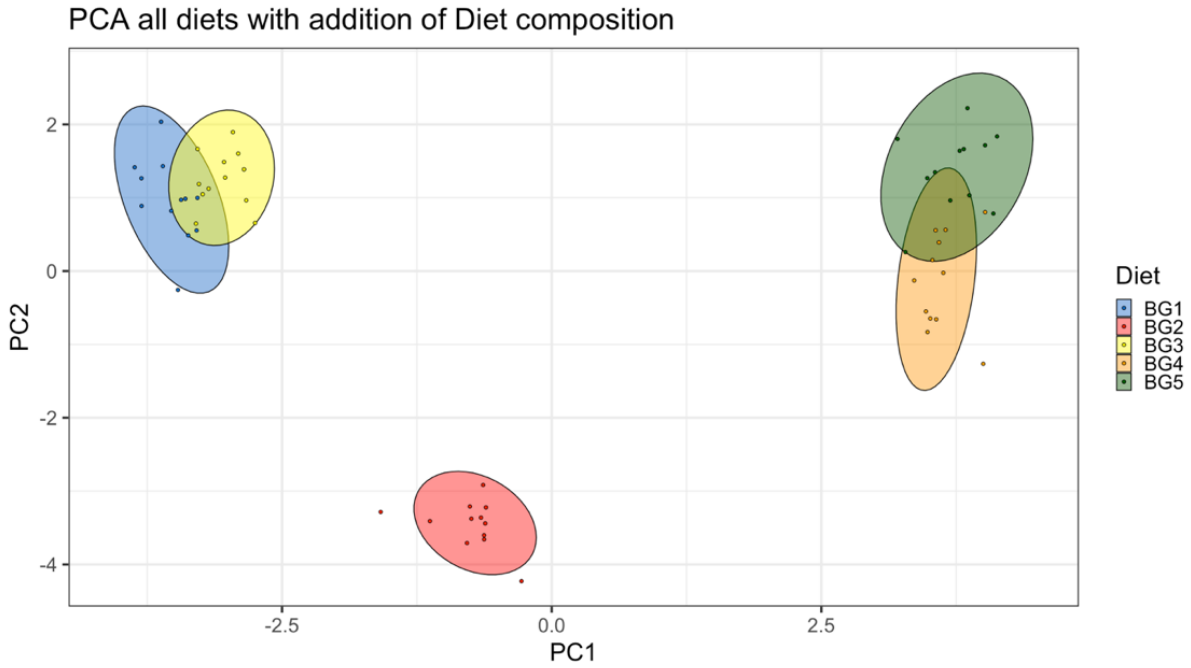
Fig. 25. PCA finding for all treatment groups including dietary ingredients. Presented a proportion of variance of 42%. Atlantic salmon (Salmo salar). n=60



The principal component analysis including all treatment groups and diet composition ingredients was transformed to a polygon. This showed distinct grouping for BG1 & BG3 and BG4 & BG5 with BG2 standing separate from all other treatment groups, which was supported by previous explained correlations between LPr and LPwdth and other variables (Fig.26).

Treatment groups BG1 & BG3 showed similar mean values for LPr, mean lymphocyte levels and mean defence activity values (Sørensen et al.,2020; Øyen, 2020). Treatment groups BG4 & BG5 showed similar results for mean LPr, K, mucous cell sizes and defence activity values (Øyen, 2020). Where treatment group BG2 underperformed for mean K, mucous cell sizes, defence activity, MUC2 levels and DEF3 levels, although treatment group BG4 showed very low values for DEF3 as well, where all other diets showed higher mean values with more variance (Sørensen et al.,2020; Øyen, 2020).

Fig. 26. Polygon representing PCA finding for all treatment groups including dietary ingredients. Original PCA presented a proportion of variance of 42%. Atlantic salmon (Salmo salar). n=60



Discussion

Pilot trial

The pilot trial showed a normal distribution for LPwdth and confidence intervals for the different measurement groups and showed that data stabilised for 50-groups, 100-groups and 200-groups. The methodology showed reproducibility within the main trial. The same methodology was implemented on hindgut samples, from the same fish used in this trial, by Øye, 2021. As a control the same samples that were used in this pilot trial, were analysed by them and produced similar results meaning the methodology is reproducible and unbiased, their application showed very stable and similar results to our own trial.

Main trail

Mean LPwdth for all treatment groups was 9.4 ± 1.7 µm. LPwdth showed no significant differences between treatment groups but approached significant difference between BG3 & BG5. Treatment group BG3 represented thinnest LPwdth and treatment group BG5 represented broadest LPwdth. Treatment group BG5 showed broadest range for LPwdth.

Treatment group BG4 and BG5 contained high levels of pea protein and corn gluten. Pea protein has been connected to decreased nutrient digestibility and had detrimental effects on growth and intestinal morphology presenting shortened mucosal folds and an increase in lamina propria widths (Penn et al., 2011). This confirms the observation of high values of LPwdth for treatment group BG5 and the elevated LPwdth values for treatment group BG4. It was found that corn gluten affects the appetite of the fish and results in reduced weight (Fauconneau, 1988; Cowey & Cho, 1992). However, this was not seen for these treatment groups since BG4 and BG5 presented higher K values than BG1 and BG3. Treatment group BG3, that presented thinnest LPwdth, had high levels of marine fish meal but had fish oil replaced by rapeseed oil. Rapeseed oil seemed to not have detrimental effect since treatment group BG3 presented best values concerning K, LPwdth and LPr. That was confirmed by Atlantic salmon fed high levels of rapeseed oil and actually increased in weight when rapeseed oil was proportionally increased (Bell et al., 2003). No qualitative methods have been developed before to measure LPwdth. Up till now LPwdth has been accessed through traditional histological observations (Penn et al., 2011). These observations always indicated that as inflammation would occur that LPwdth's would increase in relation to the other tissue structures (Penn et al., 2011; Ross & Pawlina, 2006; Baeverfjord & Krogdahl 1996).

Mean LPr for all treatment groups was 0.0655 ± 0.0127. LPr showed significant difference between treatment groups BG3 & BG4, treatment groups BG1 & BG4 showed approaching significant difference. Treatment group BG3 represented lowest LPr, meaning proportion of lamina propria tissue was smallest in relation to epithelia. Treatment group BG4 represented highest LPr meaning this treatment group represented the highest proportion of lamina propria tissue in relation to epithelia. Treatment group BG5 showed broadest range for LPr. LPr has never been assessed before so reactional patterns of LPR or interaction

between LPR and, for example, dietary ingredients is unknown. The fact that treatment group BG3 again presented best values and BG4, which was clustered with treatment group BG5, now showed highest levels for LPr shows that previously mentioned dietary compositions had similar effects on LPr as on LPwdth. This could have been expected since LPr and LPwdth showed to simultaneously increase or decrease in reaction to the same stressors.

The only treatment group that showed very different responses from the other treatment groups was treatment group BG2. For example, when BG3 showed that mucous cell size would increase with LPr, treatment group BG2 showed an opposite reactional pattern with mucous cell size decreasing with LPwdth. The reactional pattern for treatment group BG3 was confirmed by mucous cell size and density ratios that would increase when the gastrointestinal tract was agitated (Baeverfjord & Krogdahl 1996). Treatment BG2 was known to cause inflammation and poor health which was reflected in lowest K and elevated LPr and LPwdth values. But this opposite reaction suggested that when an immune system is depleted and meet a threshold that defence mechanisms are not able to respond accordingly anymore. This was confirmed by soybean meal being connected to a decrease in the immune system's ability to respond to dietary agitation or pathogens (Baeverfjord & Krogdahl, 1996; Torrecillas et al., 2015).

Discrepancies between BG4 and BG5 concerning relationships found for phagocytic ability and capacity could not be explained sufficiently by correlations available within this trial.

A linear relationship was found between mucous cell size and defence activity for treatment group BG5 and when applied to all treatment groups combined meaning as mucous cell size increases defence activity is elevated as well (Appendix D1, D2). This was supported through treatment group BG5 presenting the highest mean mucous cell size values and high defence activity values. This could indicate that when the gastrointestinal tract is agitated several defence mechanisms are activated like increase in mucous cell sizes, defence activity, lymphocytes, phagocytic ability, phagocytic capacity, increase in CATH1, DEF3 and MUC2 levels (Sørensen et al., 2020; Baeverfjord & Krogdahl 1996; Abós et al., 2015; Featherstone & Elliss, 1995; Ellis., 2001; Olafsen, 2001; Chang et al., 2006).

Due to the novelty of the lamina propria variables, LPr and LPwdth, finding a concise reactional pattern could be challenging due to the number of diets tested. For a preliminary trial it could be of interest to minimize the number of treatments tested and use two or three extreme diets to elicit different reactional patterns for these variables.

One unforeseen problem affected treatment group BG1. This treatment group was supposed to represent the natural diet of salmon in the wild but showed digestibility (nutrient-uptake) issues (Sørensen et al., 2020). This diet was meant to function as a benchmark for other diets to be compared to but due to these digestibility issues it is possible that this benchmark function was compromised clouding or distorting possible relationships.

The relative short trial period could have affected these outcomes by prematurely terminating the experiment and by that "stunting" the results. The data shows many trends and emerging patterns that were crystallising towards definitive answers but many of these have not reached fruition. Needless to say, lamina propria is in need but also deserves further investigation. In addition, it is advised to include several sampling points to give a better understanding of the undergoing changes the tissue goes through during the trial.

The aim of this trial was to investigate how lamina propria, as a tissue, reacted to different stressors delivered through dietary ingredients.

Conclusion

Bases for lamina propria tissue being an aid as an indicator for determination of physical health were found. LPr showed higher correlations with other measures than LPwdth. The relationships shown through LPr were more conclusive and explanatory where LPwdth acted as a supportive measure. In addition, the LPr seems better suited for integration with the mucosal mapping technique than LPwdth, since LPwdth seems more sensitive to sectional direction. For that reason, LPr will create a more well balanced digitalised and automated system than LPwdth would produce.

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