NUTRITIONALLY-MEDIATED EFFECTS ON BACTERIA AND PARASITE INFECTIONS IN GILTHEAD SEA BREAM

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CSIC Consejo Superior de Investigaciones Científicas

BioMar

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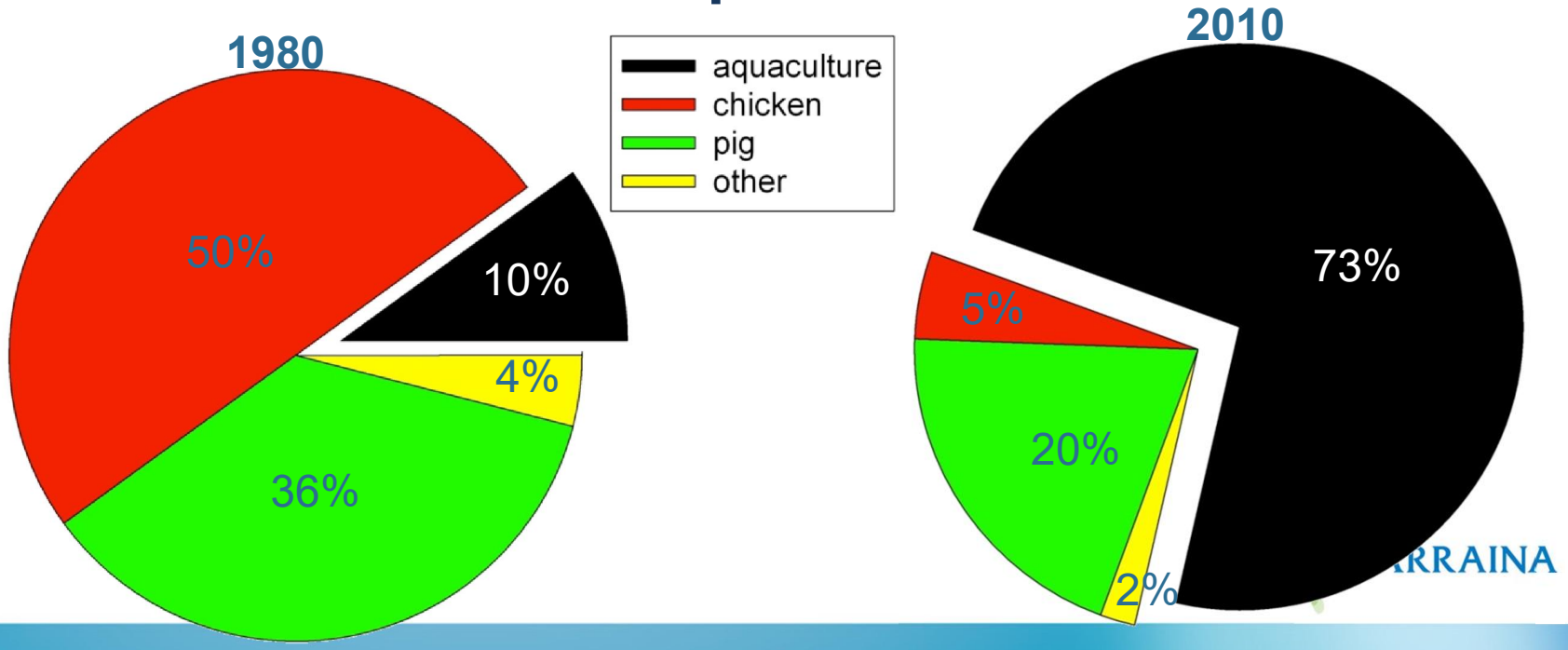
Seventh Framework Programme

Aquafeeds: first cost of production in fish farming

- Search for alternative protein and oil sources: microbial, animal, vegetal
- In gilthead seabream: no histopathological damage, good growth performance and no apparent immunodepression, with a combined replacement of FM and FO up to 65-75%

World consumption of fish meal



After Shepherd & Jackson 2013 J Fish Biol 83:1046-1066

Model species for parasitic enteritis: Gilthead sea bream and *Enteromyxum leei*

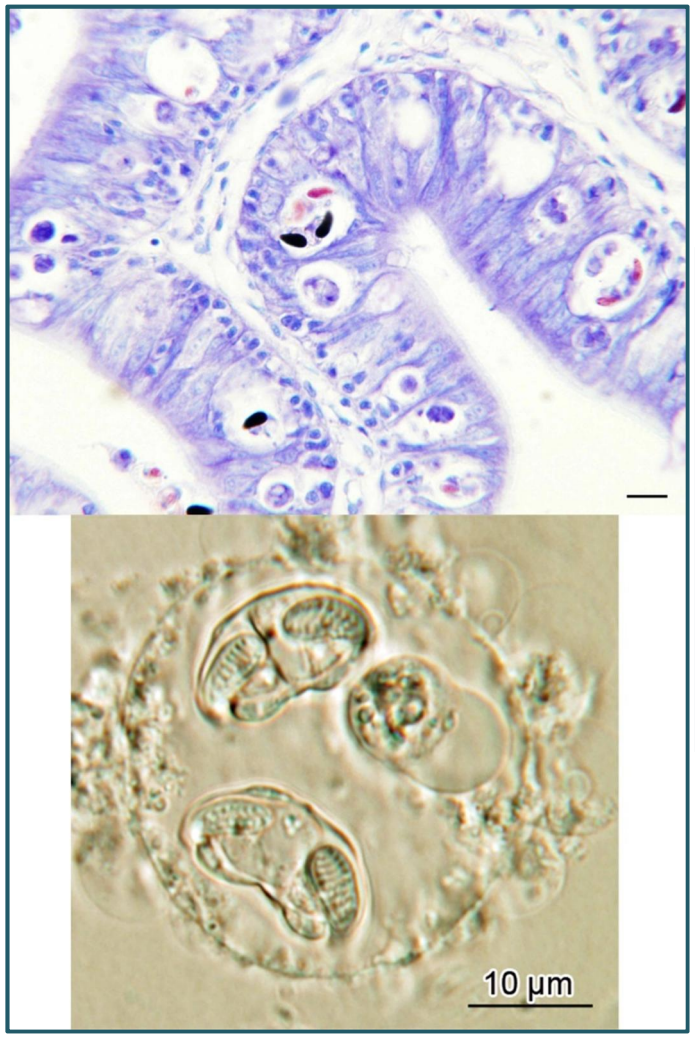
***Enteromyxum leei*:**

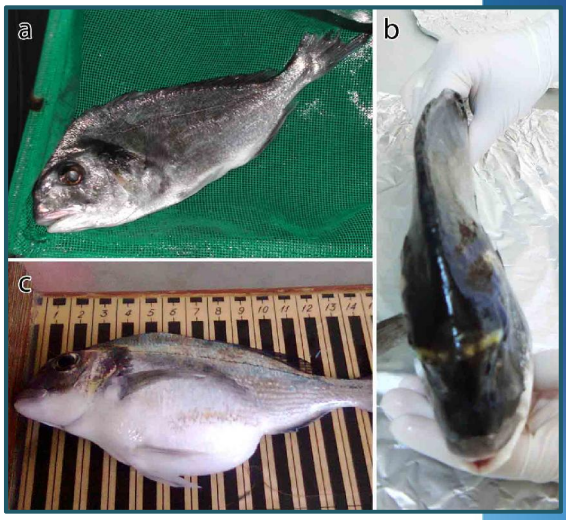
- Pathogenic myxosporean parasite with high economic impact in sea farming
- Disease signs: anorexia, weight loss, anaemia, emaciation
- Produces severe chronic enteritis with epithelial destruction

→Intraepithelial, extracellular parasite

→Inflammatory infiltrates

- Direct fish-to-fish transmission
- No efficacious treatments are available





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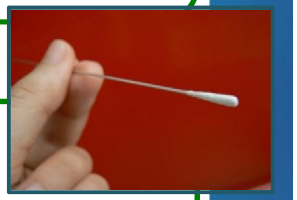
Nutritional background and disease outcome

Fish, diet & parasite challenge

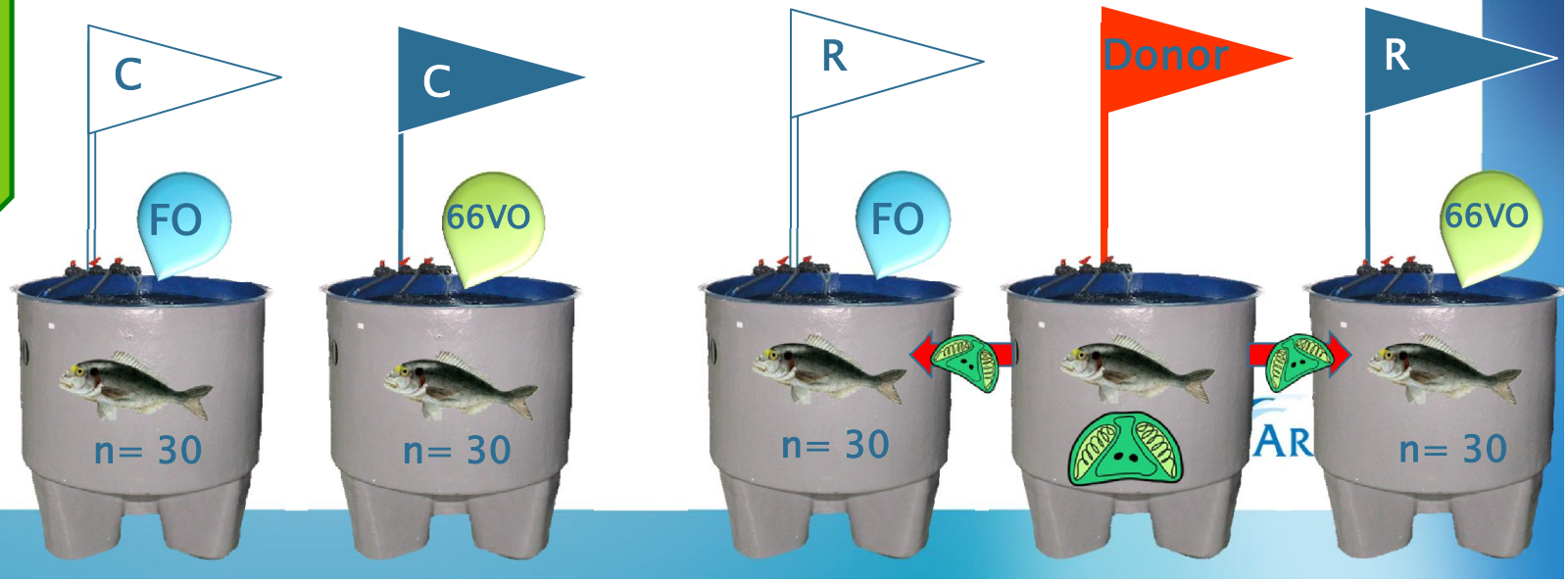
- Two different plant protein-based diets : **66VO** (major lipid source vegetable oils: 66% replacement) and **FO** (fish oil commercial diet) → for 9 months
- Effluent experimental transmission: 102 days of exposure → samplings at 0, 30, 53, 88, 102 days post exposure



Disease outcome

- Infective status: non-lethal PCR & histology
- Growth performance: weight, length, condition factor (CF), SGR
- Food intake
- Blood: haematocrit

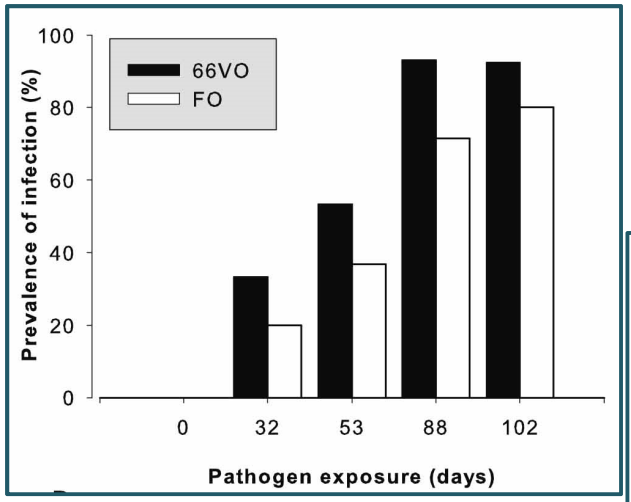
Innate immune factors

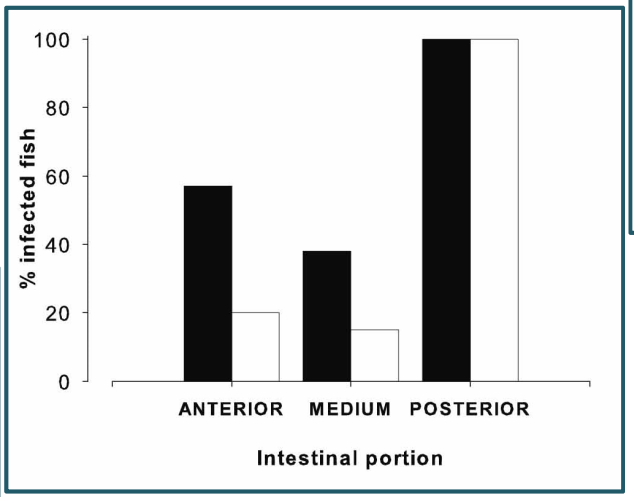


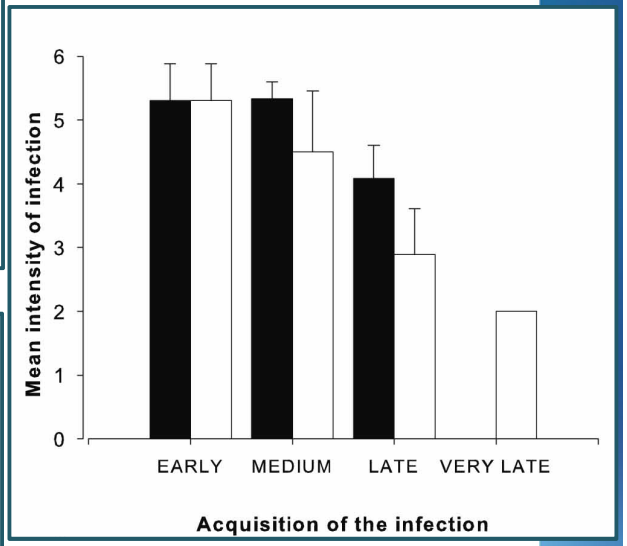
Nutritional background and disease outcome

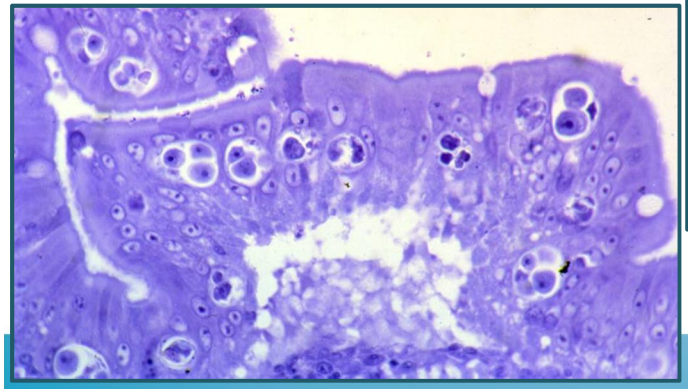
66VO-R:

- Higher prevalence and intensity of infection
- Faster parasite establishment
- More fish with infection in the entire intestinal tract









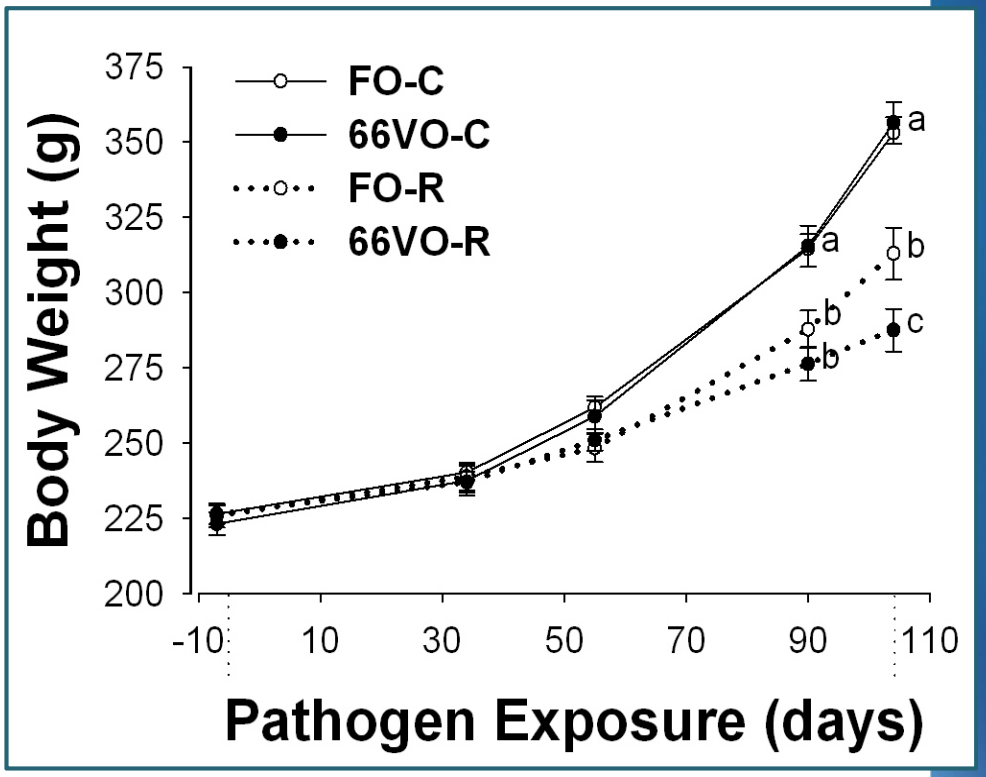
Early disease acquisition → higher intensity of infection at 102 days

Nutritional background and disease outcome

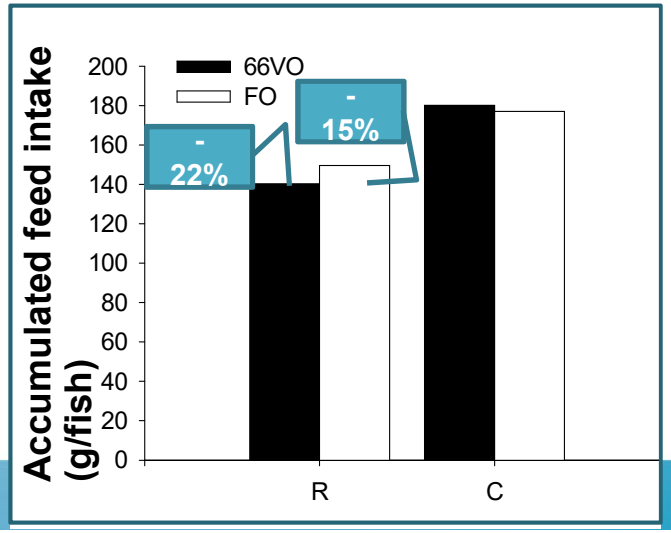
66VO:

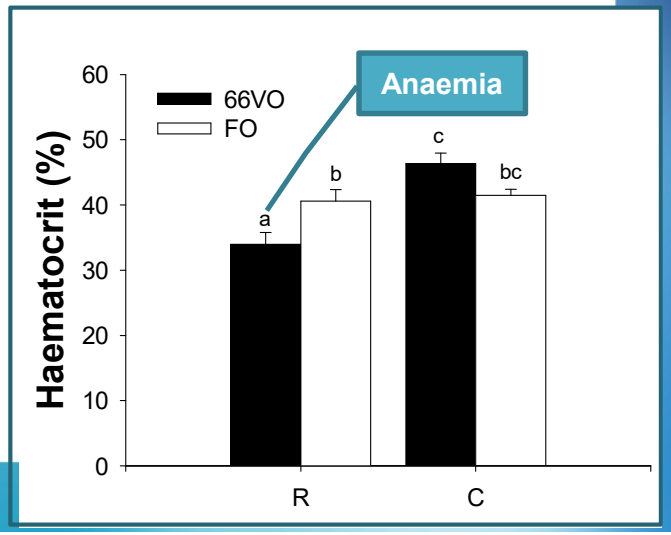
- More pronounced disease signs:

  lower growth, CF and SGR
- Higher anorexia (decrease of voluntary feed intake)
- Lower haematocrit

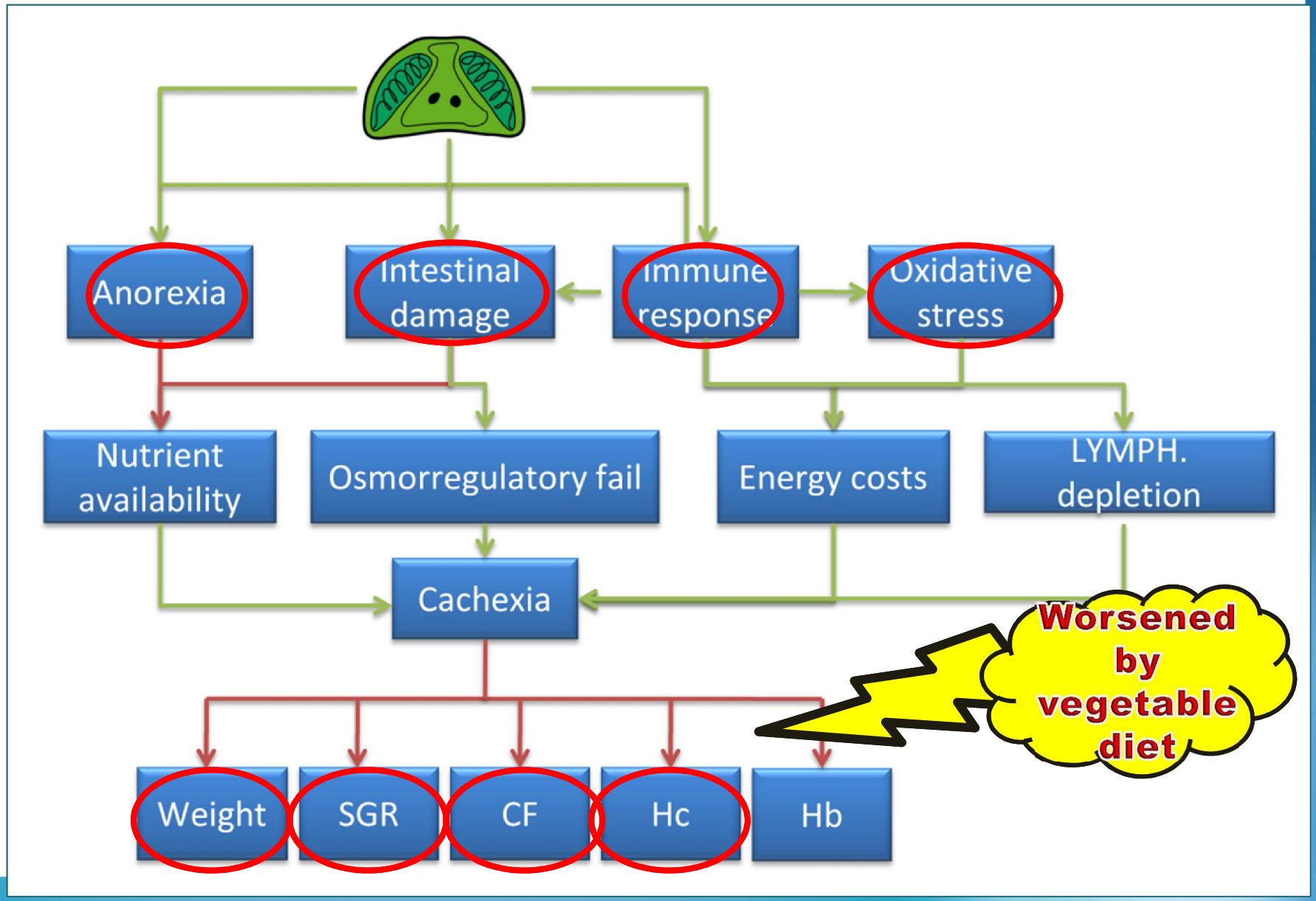


Increased progression of the infection and severity of the disease signs

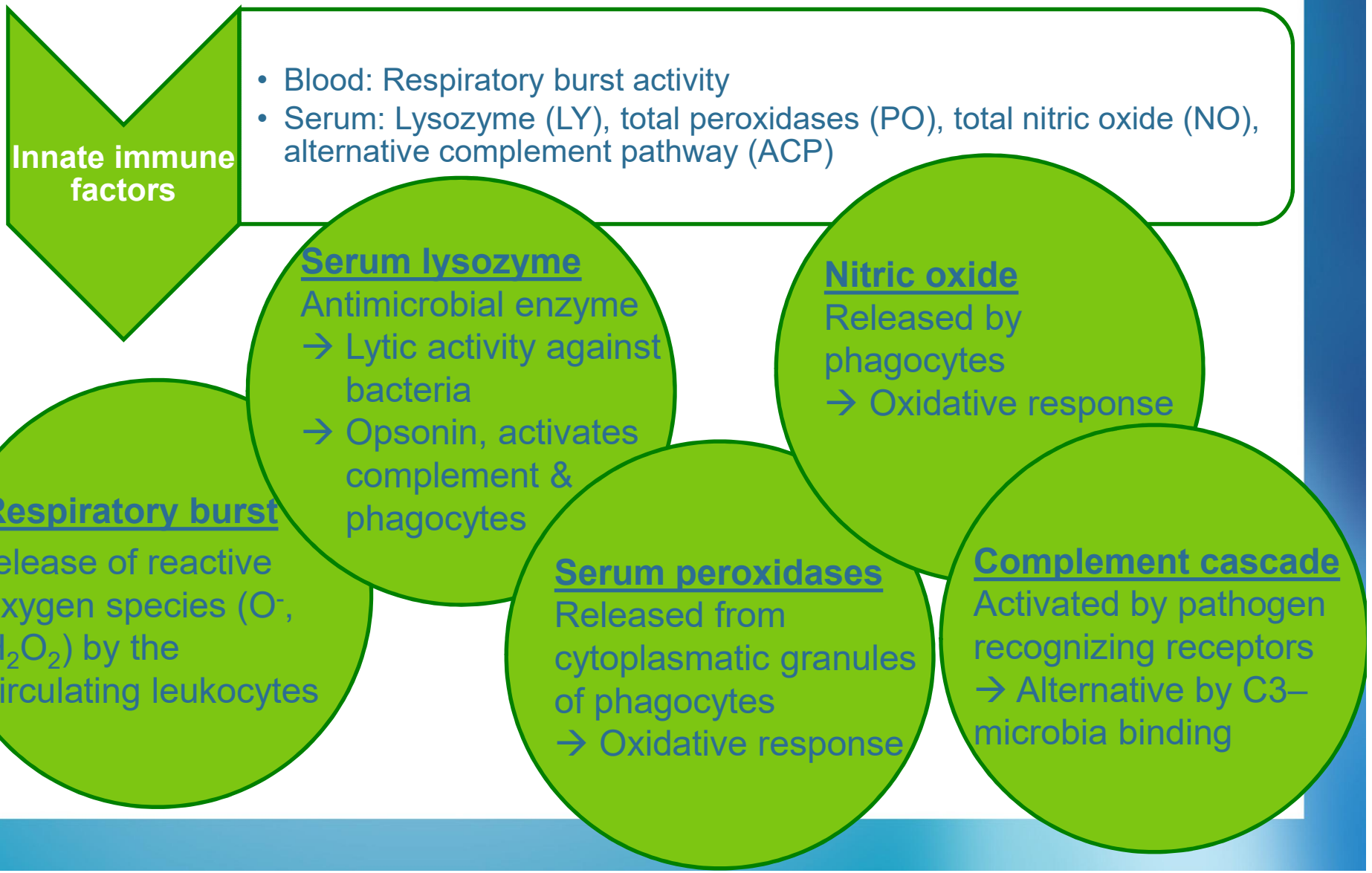




Nutritional background and disease outcome



Nutritional background and disease outcome: Innate immune factors



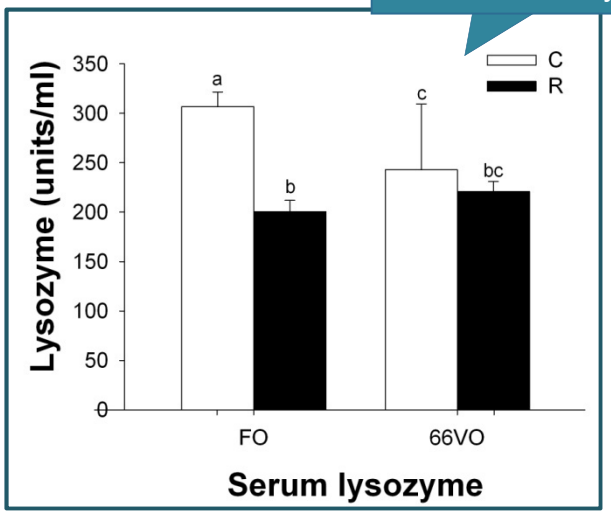
VO diet is a predisposing factor that accelerates the severity and progression of the disease

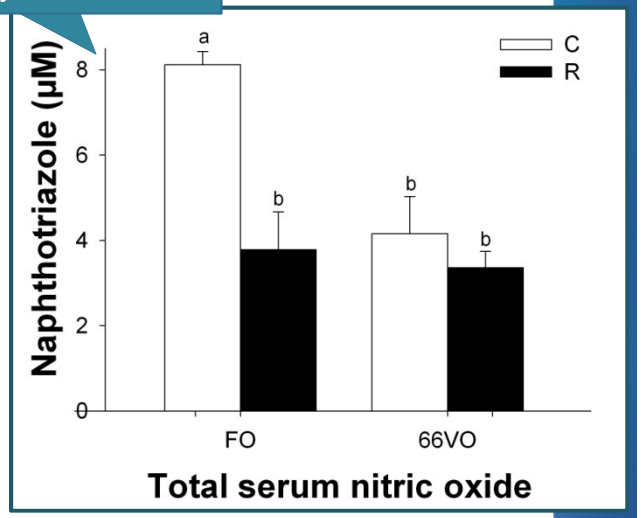
- Higher respiratory burst in both R groups

66VO:

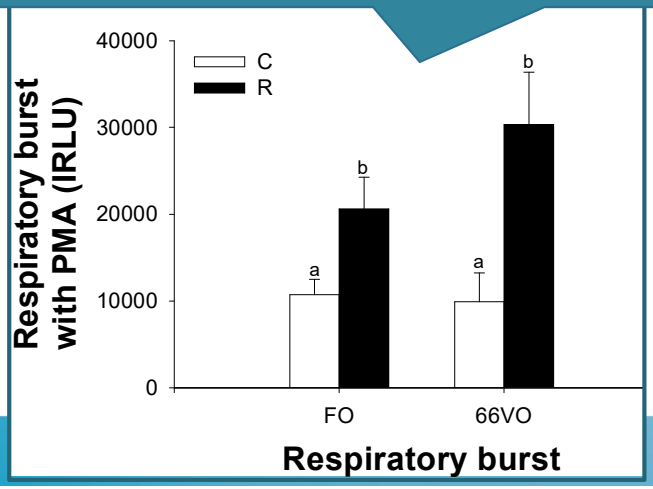
- Lower lysozyme and NO in C
- Lower peroxidase in R
- Higher ACH50 in C

→Decreased by the infection
→Decreased by 66VO

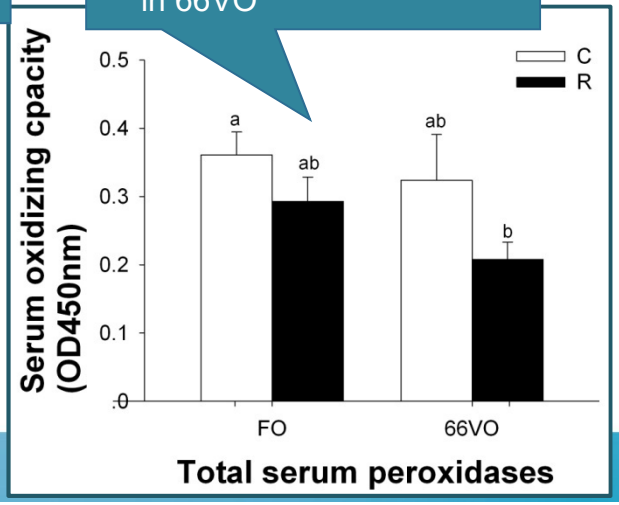




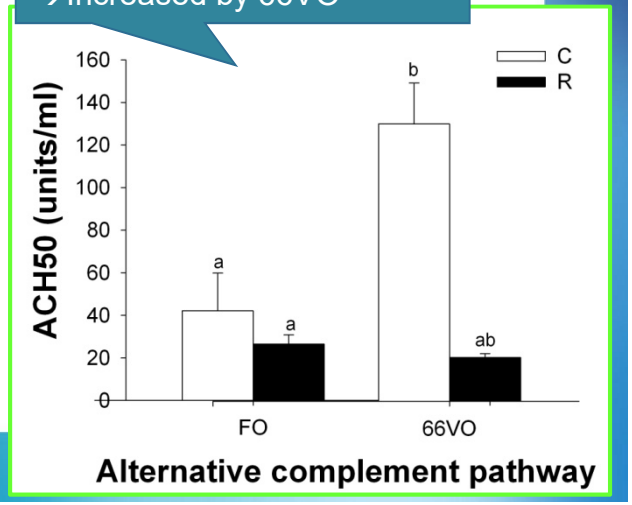
→Increased by the infection
→Positively correlated with infection intensity



→Decreased by the infection in 66VO



→Decreased by the infection
→Increased by 66VO



The intestinal mucus barrier: Effect of nutrition and enteromyxosis on mucins

Tissue samples

- Anterior, middle, posterior intestine sections
- 2 diets: FO and 66VO, 102 days effluent exposed fish
  → 2 categories: early infected (E) - late infected (L)

Histo-chemistry

- Periodic acid Schiff (PAS): neutral mucins →magenta
- Alcian blue (AB): acidic mucins →blue
- Aldehyde fuchsin-AB (AF-AB): acidic-sulphated mucins →purple
  acidic-carboxylic mucins →blue
- *Sambucus nigra* Agglutinin (SNA): sialic acid

} goblet cell (GC) quantification

Molecular approach

- BlastX searches on the updated GSB transcriptomic database
- Sequence and phylogenetic analyses
- Screening of the mucin tissue expression pattern
- Disease outcome and gut transcriptome analysis

C-66VO (vs C-FO):

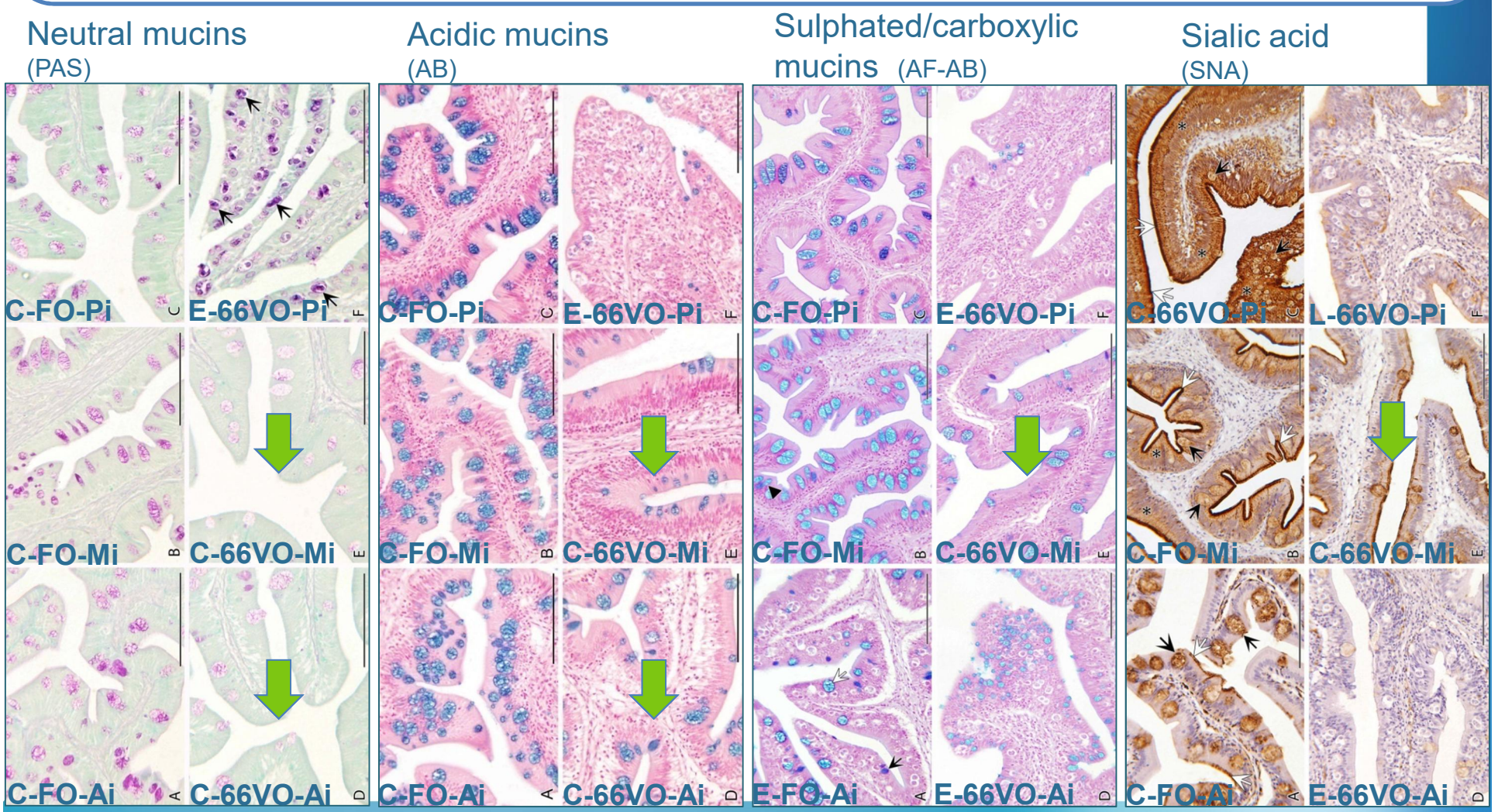
- Ai & Mi: lower neutral and acidic GCs
- Mi: lower carboxylic and sialic acid GCs

→ Less protection ?

R fish:

- Lower GCs at Pi (specially in E)
- Negative correlation between infection intensity and no. of GCs

Main factor for GC depletion: infection intensity



Six mucins characterized with tissue-specific expression pattern in skin, gills and gut epithelia

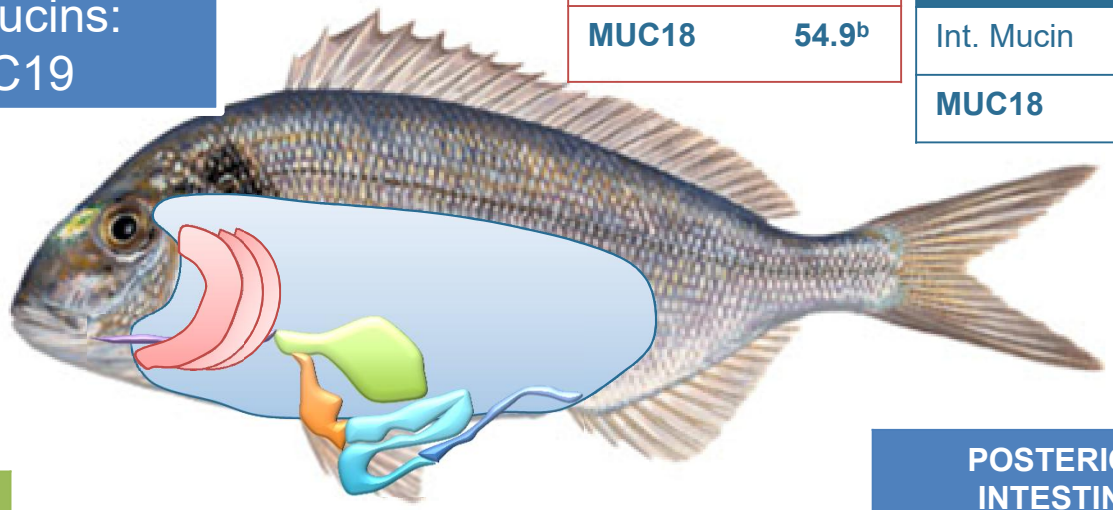
Membrane-associated mucins: MUC13, MUC18, Int. mucin

Secreted gel-forming mucins: MUC2, MUC2-like, MUC19

ESOPHAGOUS	
Int. Mucin	195.6[a]
MUC18	113.5[b]
MUC19	**3600.6[c]**

GILLS	
Int. Mucin	0.71[a]
MUC18	**54.9[b]**

SKIN	
Int. Mucin	4.1[a]
MUC18	**180.5[b]**



STOMACH	
MUC2	1.0[a]
MUC2-like	0.9[a]
Inst. Mucin	42.9[c]
MUC13	0.9[a]
MUC18	**59.1[c]**
MUC19	1.7[b]

ANTERIOR INTESTINE	
MUC2	1449.3[b]
MUC2-like	1481.4[b]
MUC13	**2407.7[c]**
MUC18	50.5[a]

MIDDLE INTESTINE	
MUC2	1111.8[b]
MUC2-like	1142.1[b]
MUC13	**2566.9[c]**
MUC18	55.8[a]

POSTERIOR INTESTINE	
MUC2	820.7[b]
MUC2-like	961.6[b]
Int. Mucin	1806.7[c]
MUC13	**2671.5[a]**
MUC18	82.2[a]

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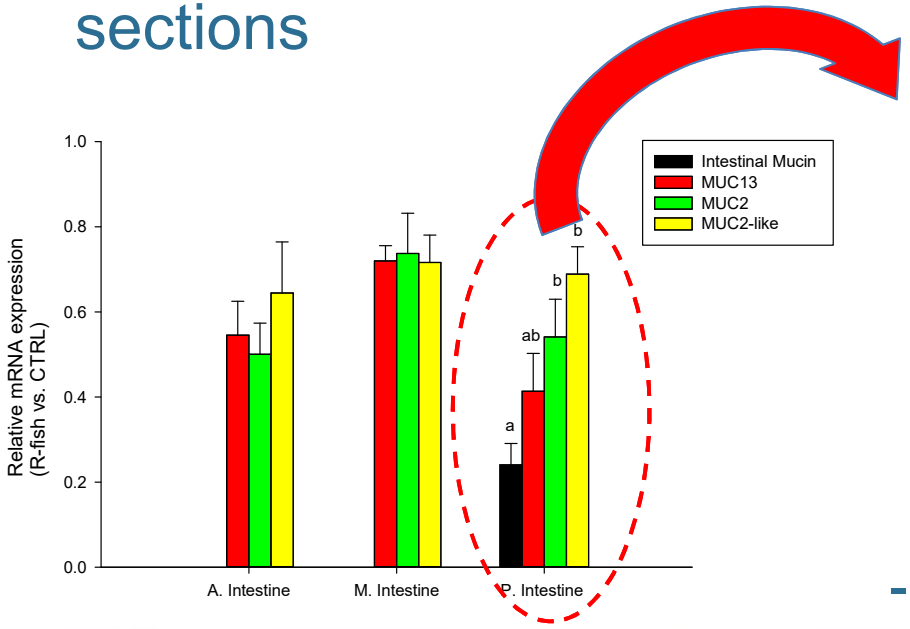
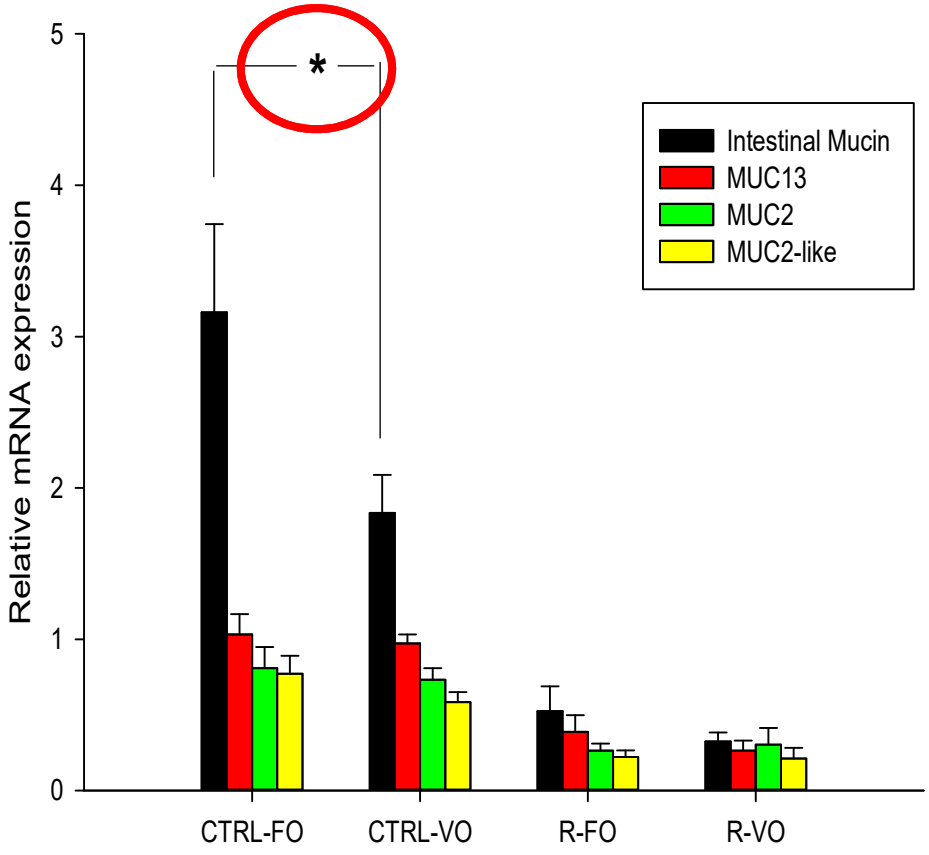
The intestinal mucin is a health biomarker of prognostic and diagnostic value

- Mucin mRNA expression is down-regulated by infection (fold change < 1)
- Mucin expression profile is similar in the three intestine sections

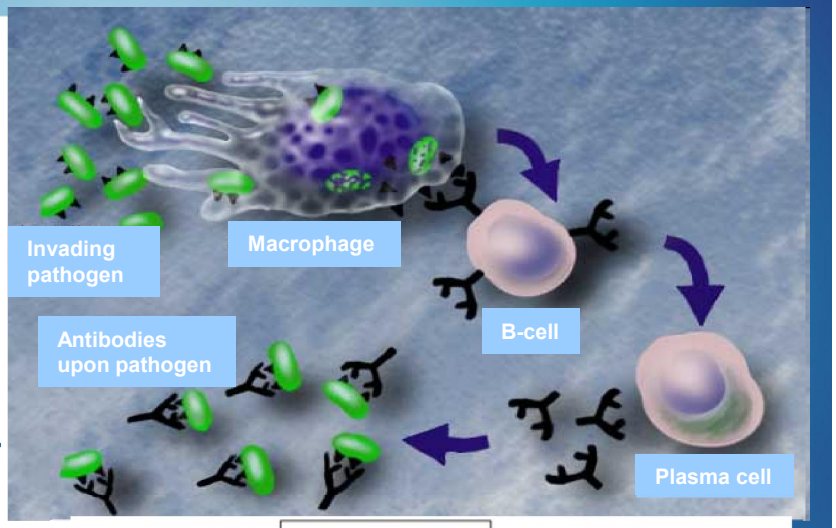


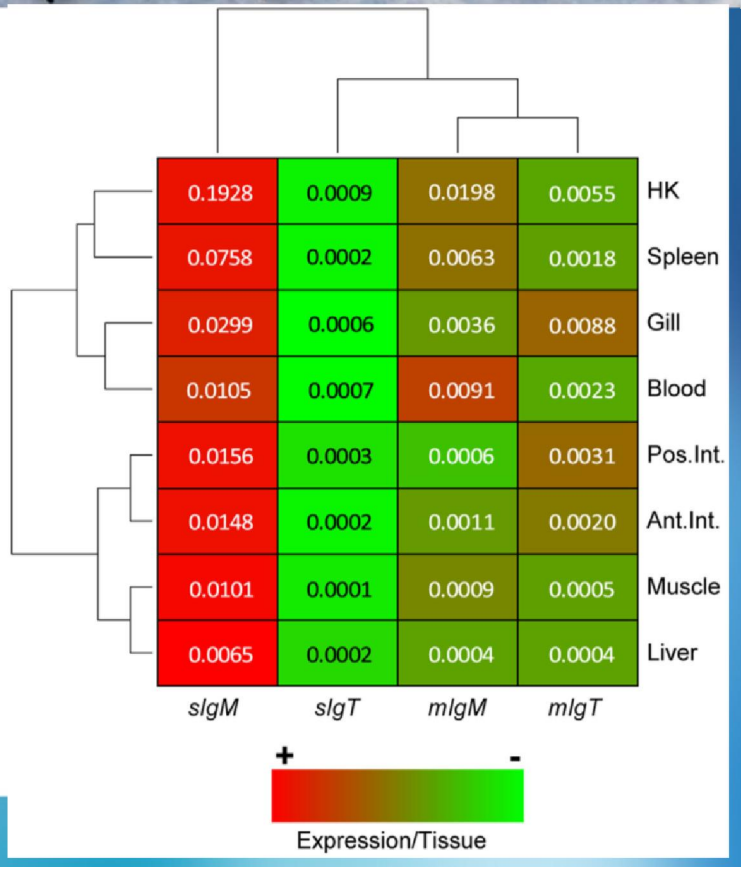
- "Intestinal mucin" is specific of posterior intestine and down-regulated by VOs



Adaptive immune response: Immunoglobulins

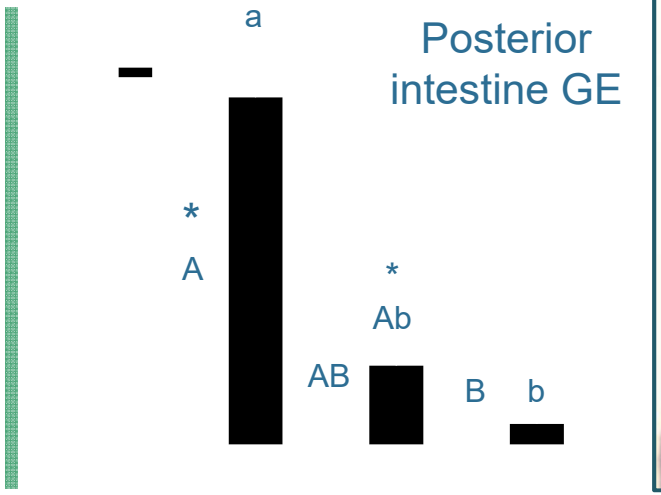
- Ig's produced by B cells: membrane bound or secreted (blood & mucus & tissues)
- Soluble IgM predominant isotype in gilthead sea bream
  - → Systemic activity
  - → ⇧membrane IgM in blood, head kidney and spleen
- IgT mucosal role: ⇧membrane IgT in intestine and gills

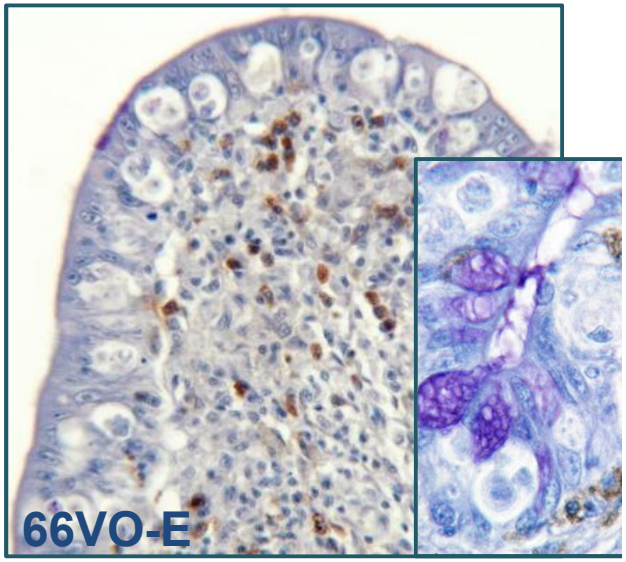


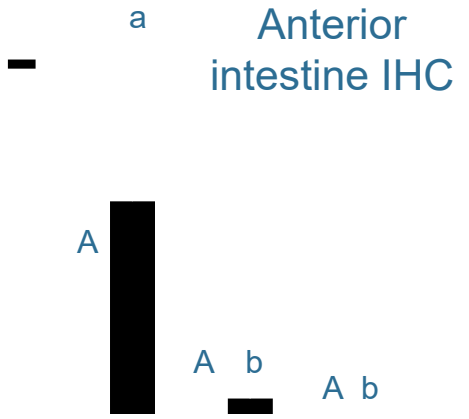


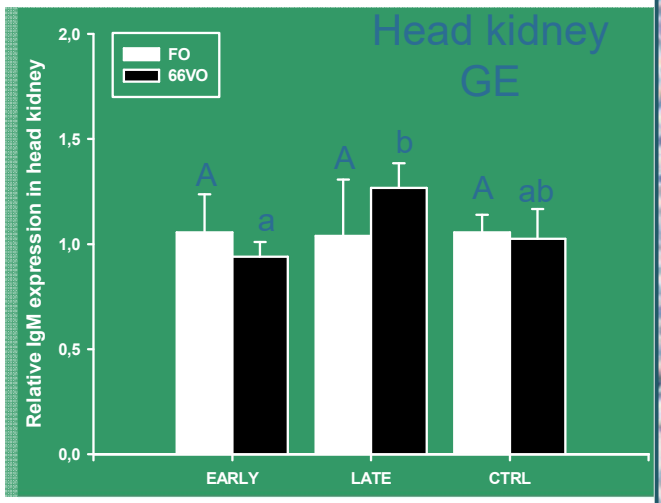
IgM levels are related to intensity of infection:

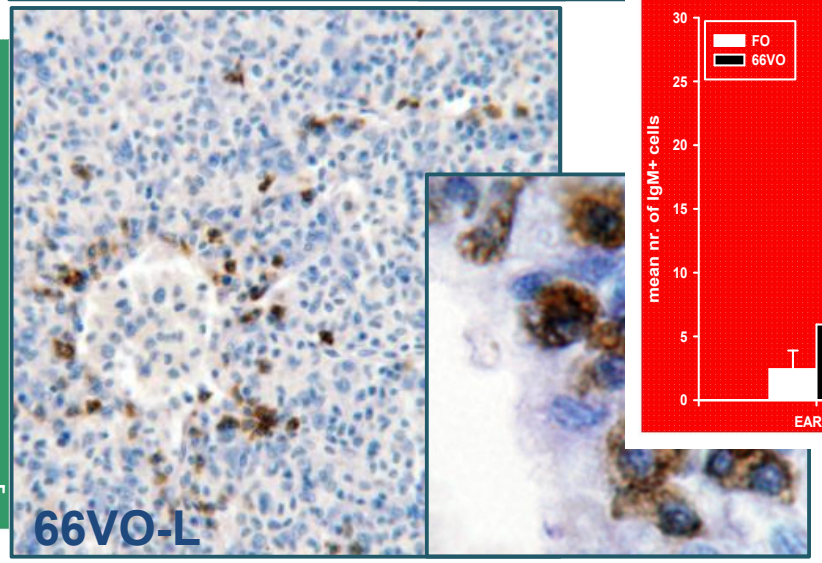
EARLY infected > LATE infected > CTRL

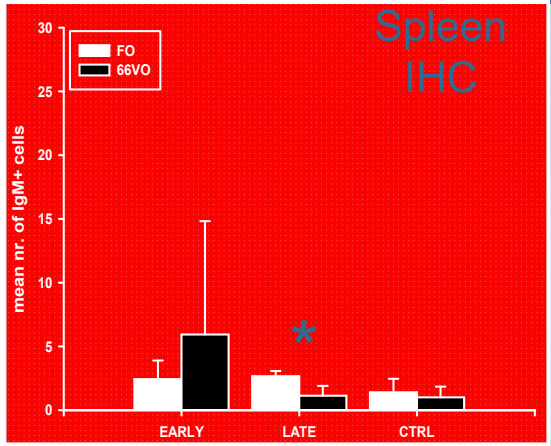












- No diet-effect in CTRL
- 66VO: stronger immune response & time effect (intensity of infection)

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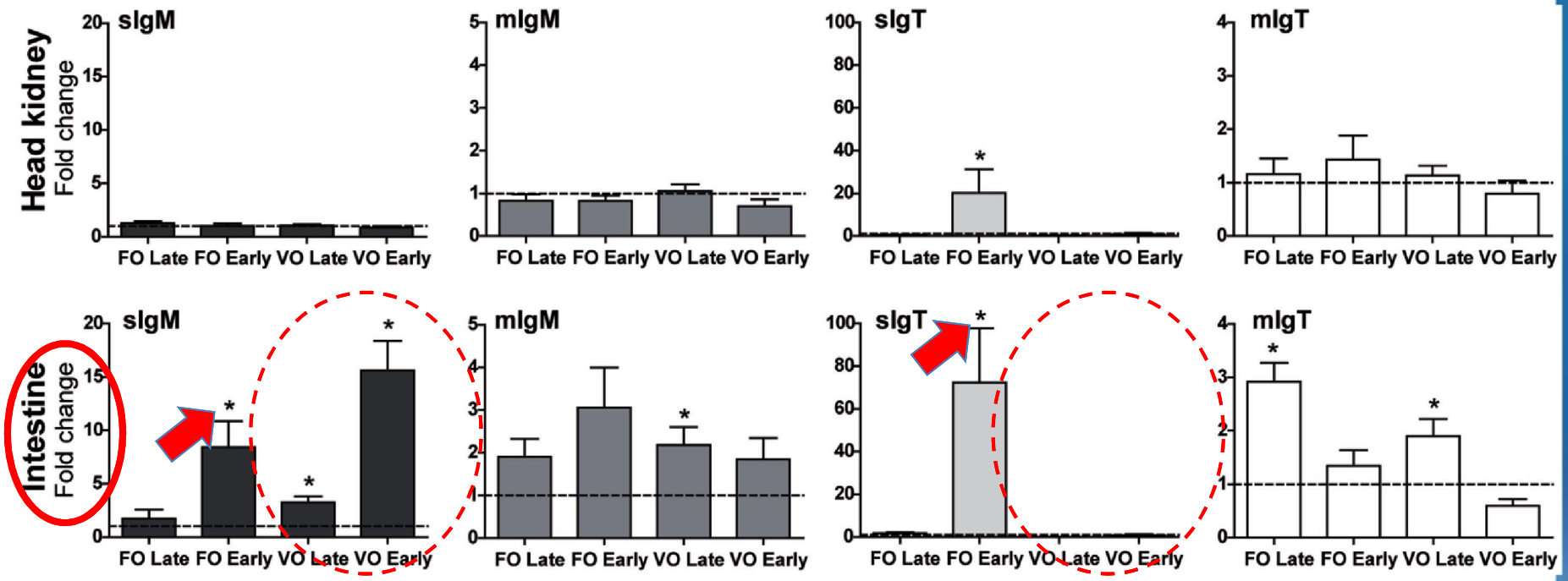
66VO inhibits secreted IgT up-regulation



→ No diet-effect in CTRL

→ FO: secreted Ig up-regulation in long term infection

→ 66VO: no IgT up-regulation but stronger IgM up-regulation

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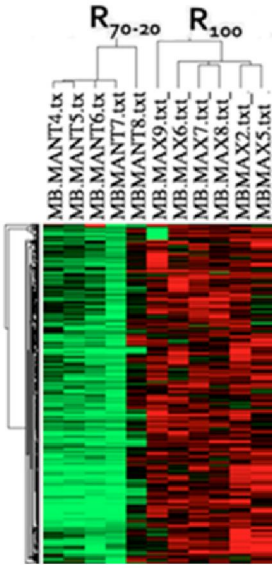
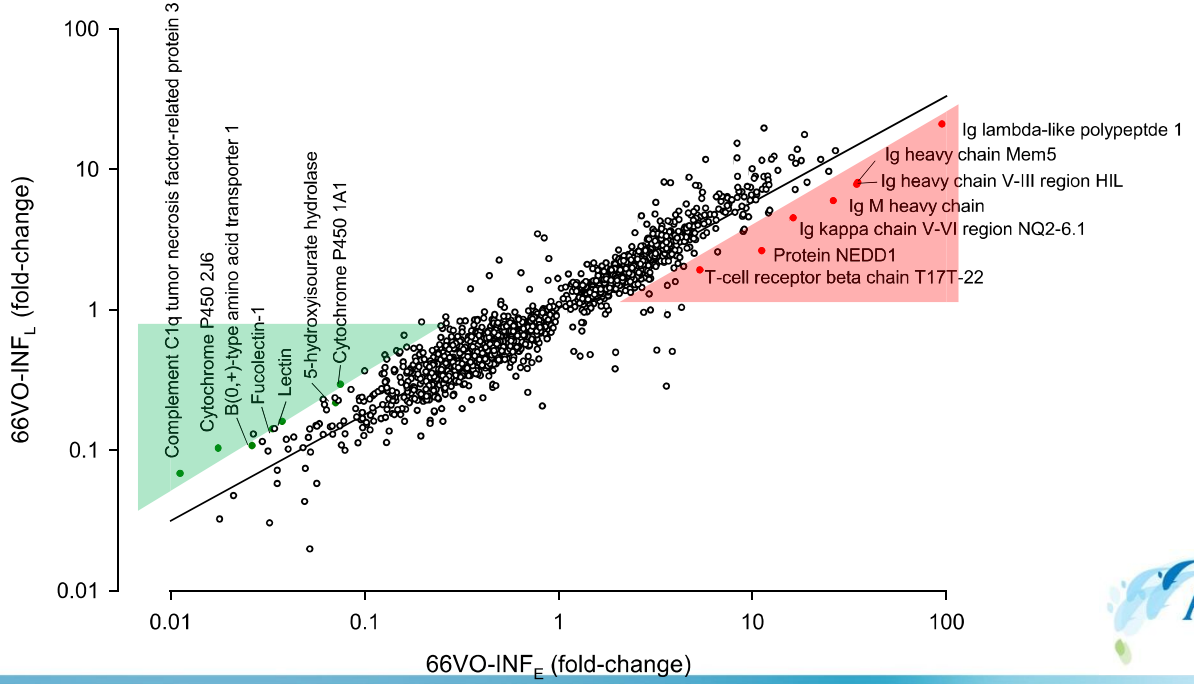
Which factors are involved in the differential susceptibility towards pathogen challenge?

- Study of the underlying mechanisms

  →wide gene expression analysis: intestinal trancriptome
- Oligo-microarray of 7500 genes





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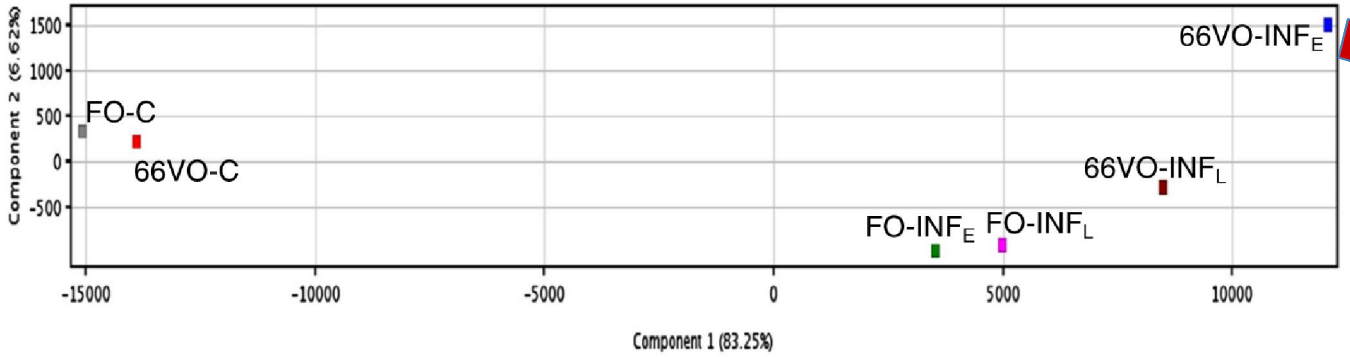
Dietary VOs do not alter the intestinal transcriptome of gilthead sea bream



Group Name	FO-C	FO-INF$_L$	FO-INF$_E$	66VO-C	66VO-INF$_L$	66VO-INF$_E$
FO-C	---	455	359	1	908	1392
FO-INF$_L$		---	2	338	5	117
FO-INF$_E$			---	253	7	131
66VO-C				---	787	1292
66VO-INF$_L$					---	36
66VO-INF$_E$						---

- No transcriptomic differences in CTRL fish
- Number of differential expressed genes and magnitude of change:

  → highest in EARLY infected 66VO

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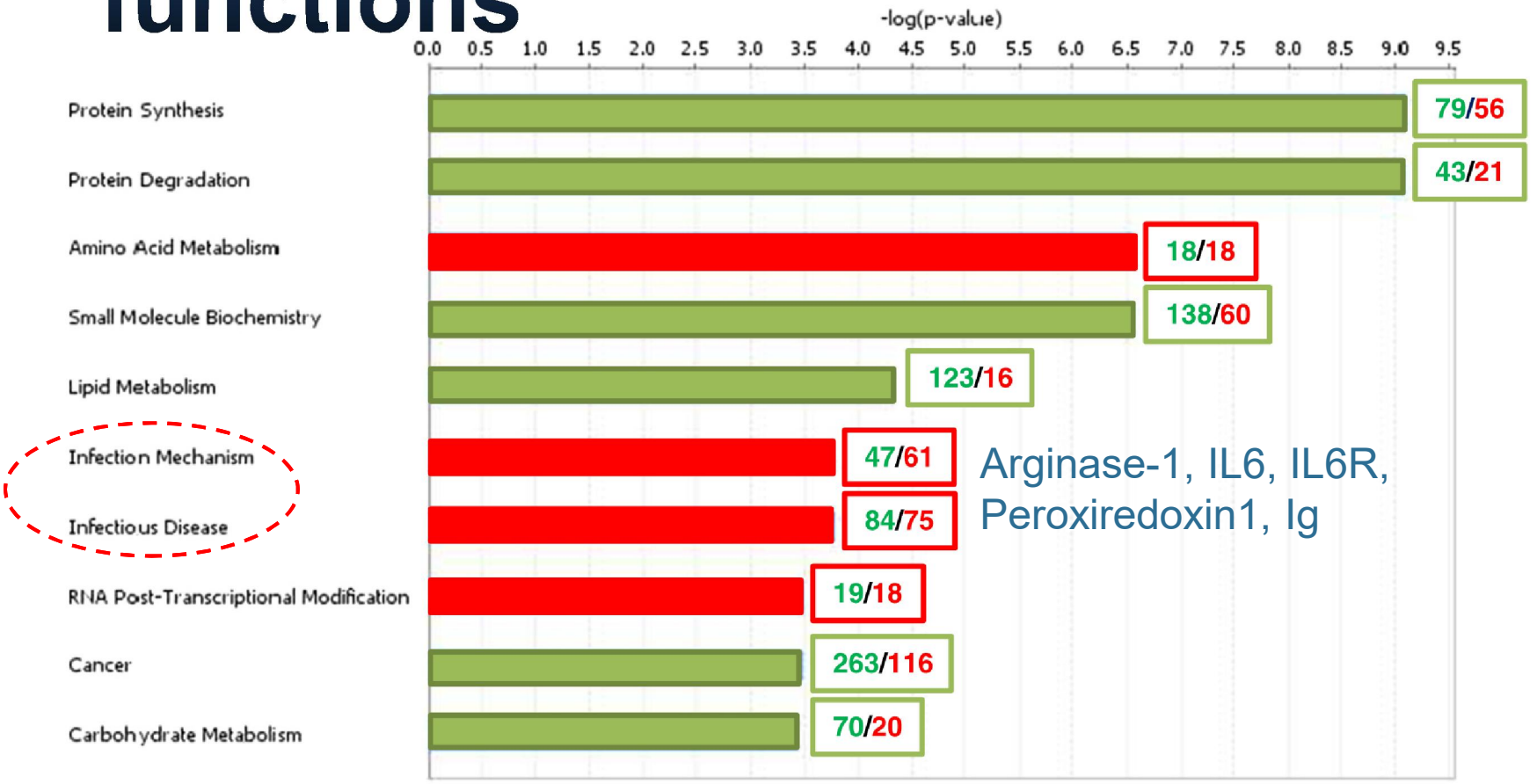
Top 10 regulated biological functions



Up-regulation: infection, immune response, stress-response

Down-regulation: xenobiotic metabolism & detoxification, lipid metabolism, somatotropic axis, complement pathway

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→ **66VO predisposing factor that worsens parasitic enteritis**

→ **⬇ innate immune factors, ⬇Goblet cells, ⬇mucin expression, ⬇secreted IgT expression**

→ **Alternatives needed to optimize plant replacement without detrimental effects on gut health**

→ **Supplementation with dietary immunostimulants**

References:

Estensoro *et al*., Veterinary Parasitology, 2011.
Estensoro *et al*., Diseases of Aquatic Organisms, 2012.
Pérez-Sánchez *et al*., PLoS One, 2013.
Estensoro *et al*., Fish and Shellfish Immunology, 2012.
Piazzon *et al.,* Frontiers in Immunology, 2016.
Calduch-Giner *et al.,* BMC Genomics, 2012.

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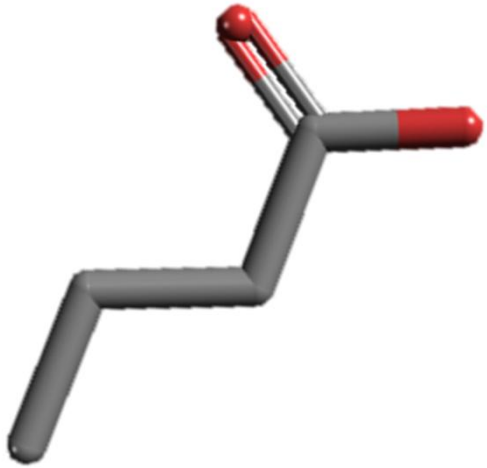
Is butyrate supplementation a good strategy to compensate the drawbacks of vegetable diets in fish?

Short chain fatty acid
Produced by bacterial fermentation of undigested carbohydrates

Metabolite produced by members of the intestinal microbiota



Sensed by the host as a signal:

- **Strengthen the epithelial barrier**
- **Reduce inflammation**
- **Increase production of mucins**
- **Increase production of antimicrobial peptides**

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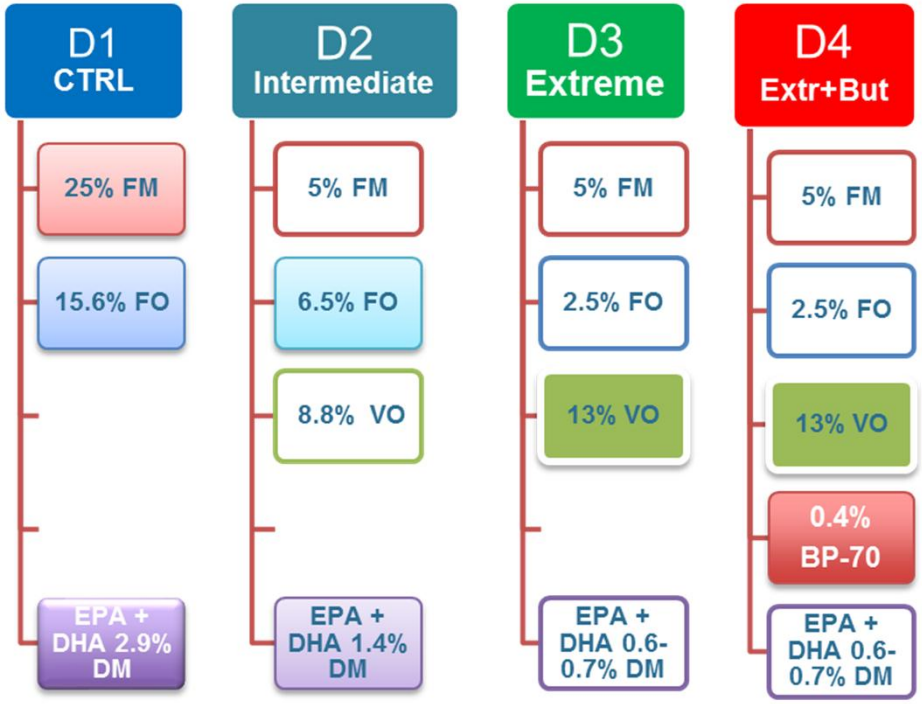
Evaluation of diets on intestinal health

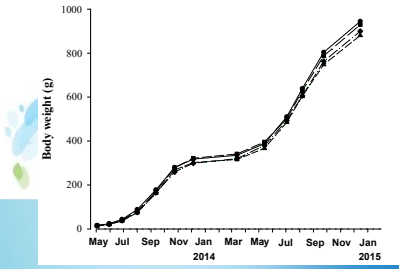


Gilthead sea bream: BWi 15g

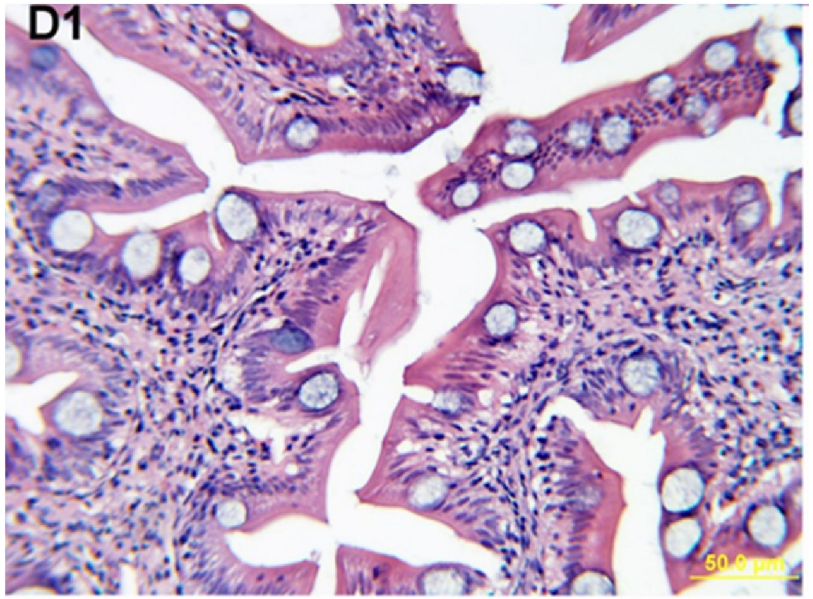
- Replicate tanks (3000L) 150 fish
- Natural photoperiod and Temp.
- Fed *ad libitum* up to **20 months**

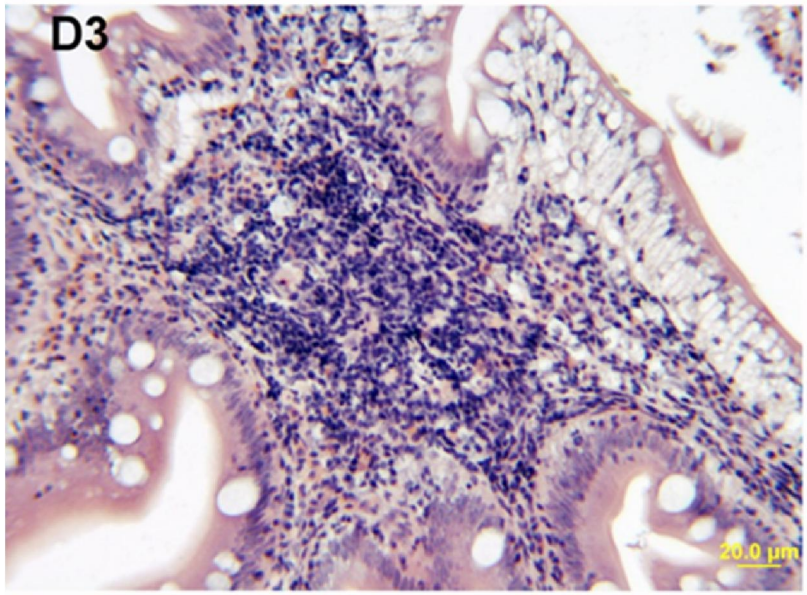
Outputs:

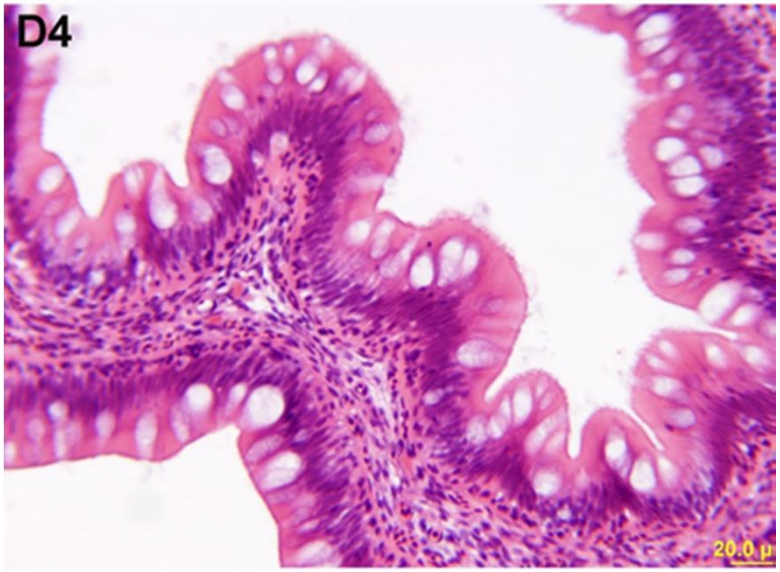
- Histological evaluation
- Transepithelial electrical resistance
- Transcriptomic profile
- Proteomic profile
- Microbiomic profile
- Disease outcome:
  parasite & bacteria



Histological observations







Butyrate supplementation (D4):

- Reversion of the proliferative profile
- Increased number of goblet cells

Extreme VO inclusion (D3):

- Pro-inflammatory profile
- Fat accumulation in the epithelium
- Infiltration of lymphocytes and granulocytes

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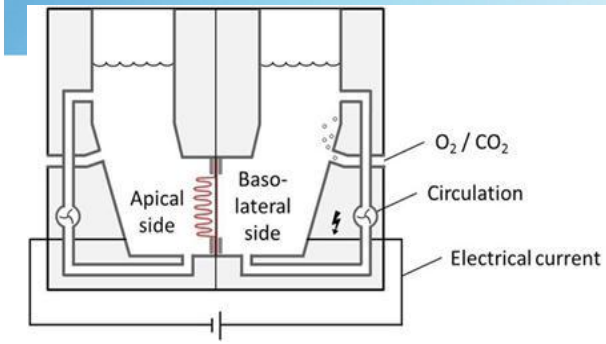
Estensoro *et al.*, PLoS ONE, 2016



Transepithelial electrical resistance

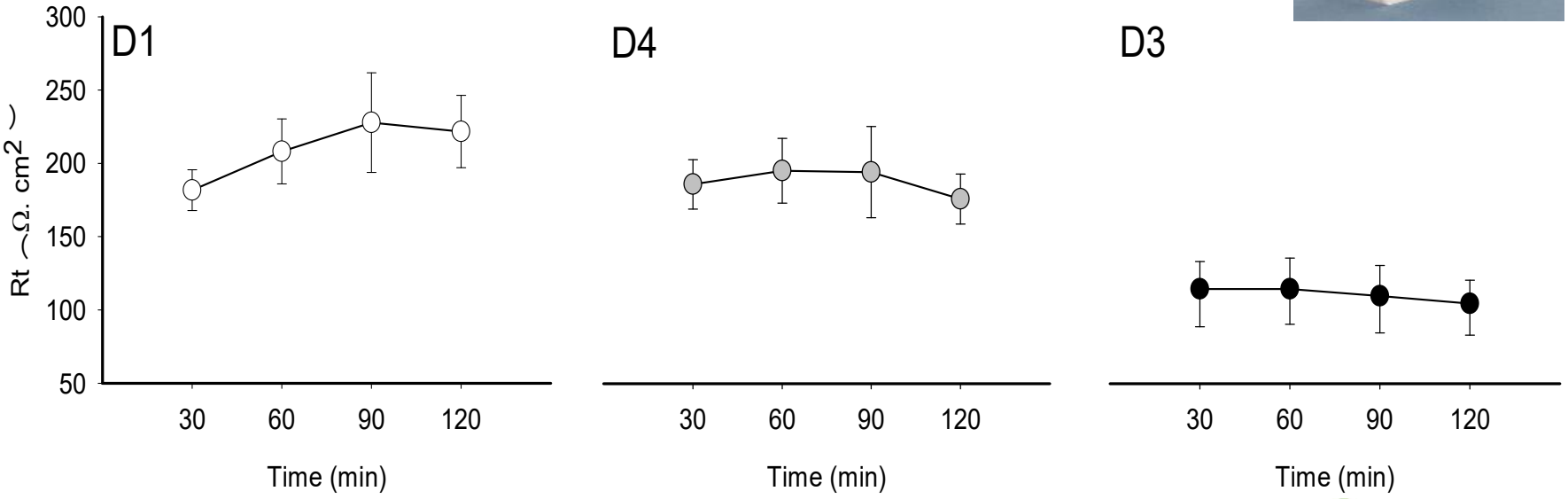
Measure of tissue integrity

Extreme diets (D3)

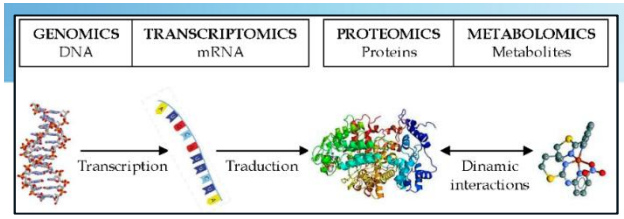
→ **lower transepithelial electrical resistance**

Butyrate supplementation (D4)

→ **restoration of the electrical resistance**



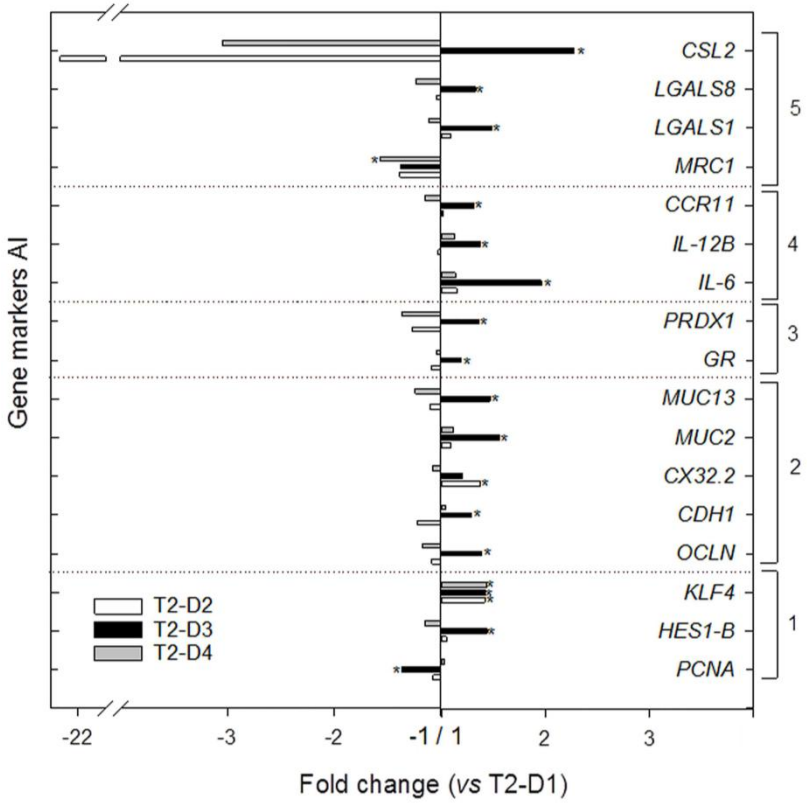
Estensoro *et al.*, PLoS ONE, 2016



Transcriptomic profile

AI	T2-D1	T2-D2	T2-D3	T2-D4
T2-D1		2	12	2
T2-D2			9	1
T2-D3				10

Differentially expressed genes: 17



PCR array for simultaneous profiling of 86 genes:

- Immune system
- Enterocyte mass and epithelial damage
- Intestinal architecture and permeability
- Cell differentiation and proliferation
- Mitochondria function and biogenesis

Effects more prominent in anterior intestine:

D3 ↑ **Proinflammatory genes**
↑ **Epithelial permeability**
↑ **Mucus production**

Butyrate reverted most of these changes

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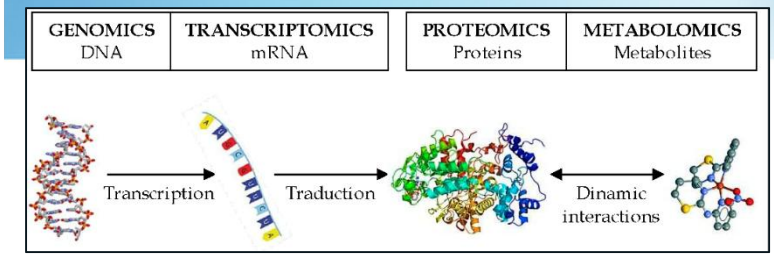
Estensoro *et al.*, PLoS ONE, 2016

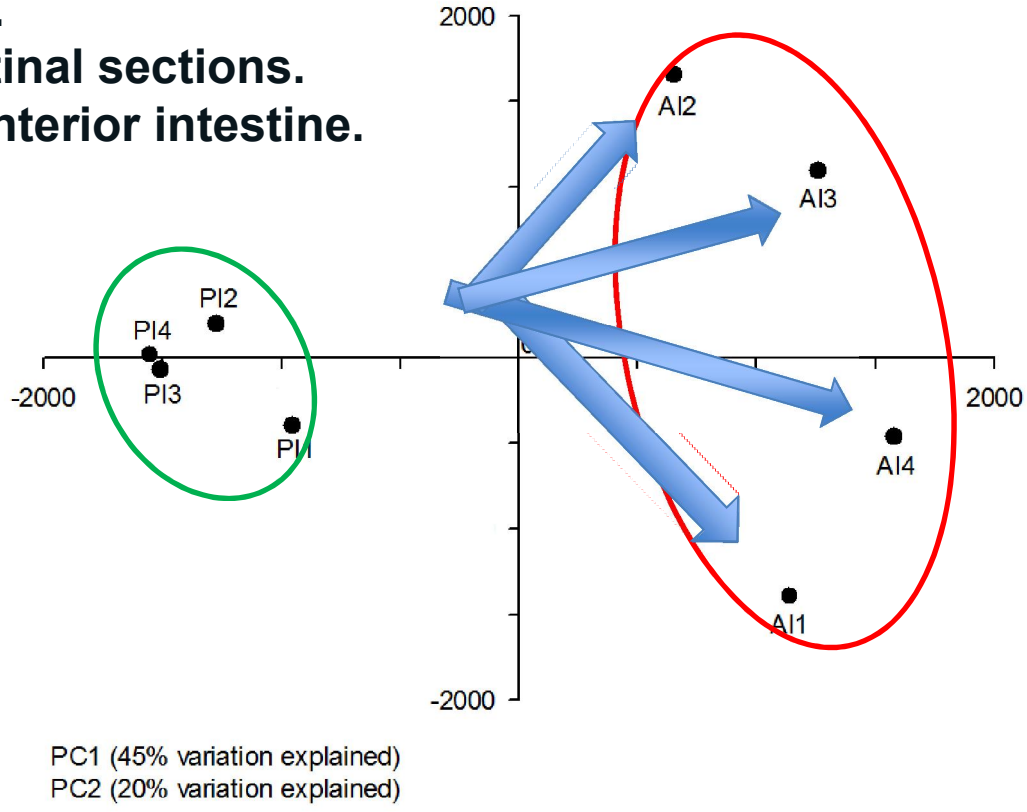


Proteomic profile (mucus)

iTRAQ method: High sensitivity, accurate quantification

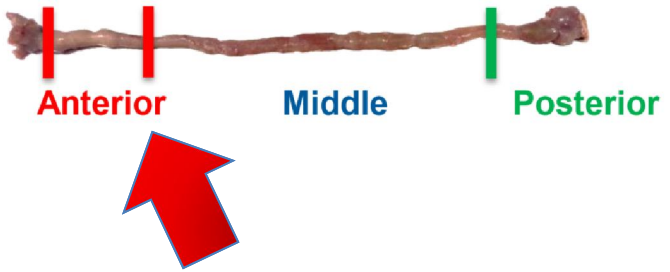
- **Comparing anterior and posterior profiles**

→ More than 2000 proteins identified.
→ Clear differences between intestinal sections.
→ Diet effect more prominent on anterior intestine.



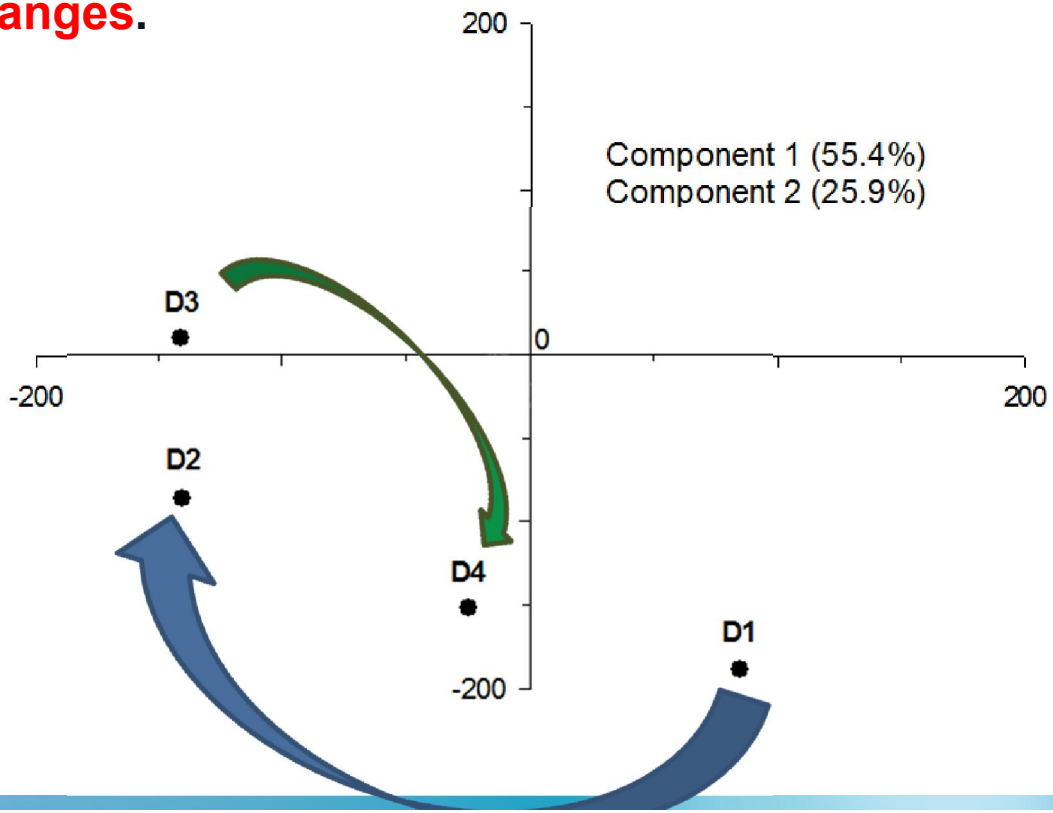
Piazzon *et al.*, In press, 2017

Proteomic profile (mucus)



Proteome: Mucus from anterior intestine

- **Comparing anterior profiles among diets:**
  - → **More than 1000 proteins identified.**
  - → **122 differentially expressed.**
  - → **Butyrate reverts changes.**



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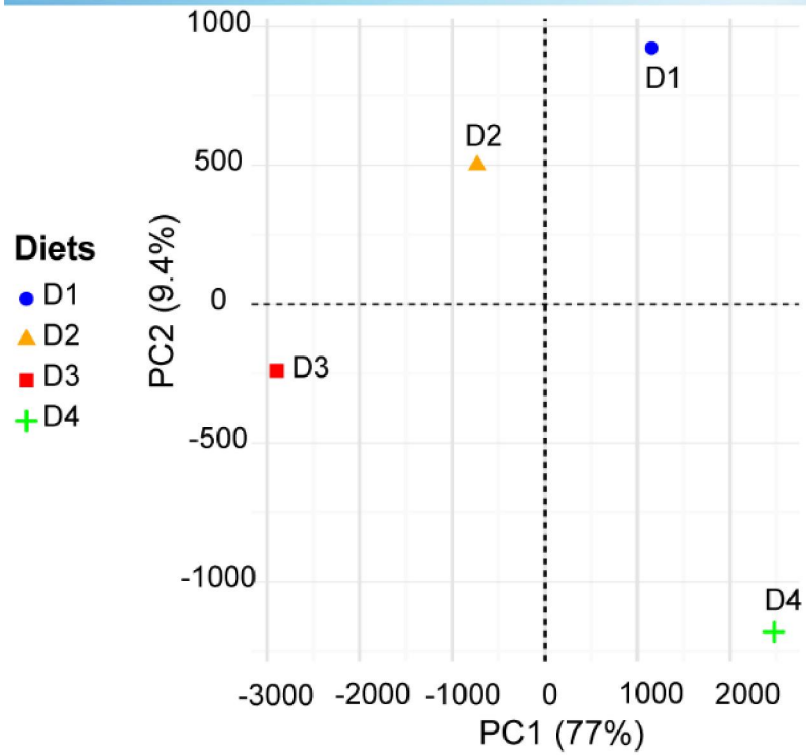
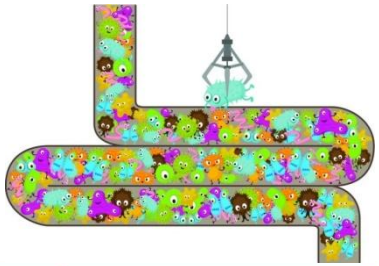
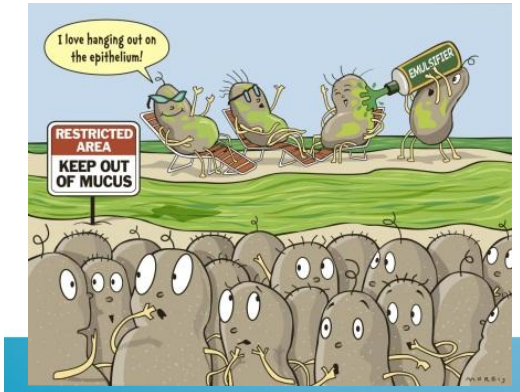
Microbiomic profile (mucus)



Gut microbiota was highly regulated by diet with a clear dominance of *Photobacterium* and lower diversity in plant-based diets

Butyrate supplementation increased bacteria biodiversity with a partial reversion to control diet phenotype





DIET	DOMINAT GENERA (%)		SHANNON DIVERSITY INDEX *
	Photobacterium	*Vibrio*	
D1	71	**19**	1.53
D2	69	19	1.36
D3	**82**	2	1.05
D4	44	7	**2.27**

Piazzon *et al.*, In press, 2017

D1 CTRL | D2 Intermediate | D3 Extreme | D4 Extr+But

Overall effects on intestinal health

Plant based diets:

- **Proinflammatory profile with infiltration of leukocytes**
  - Butyrate: Reversion & increased goblet cells
- **Decreased transepithelial electrical resistance**
  - Butyrate: Recovery
- **Changes in proinflammatory, epithelial integrity and mucus genes**
- **Shift in proteomic profile in agreement with gene expression changes**
  - Butyrate: Reverted most changes
- **Dominance of *Photobacterium* and lower bacterial diversity**
  - Butyrate: Reversion & higher diversity

Plant based diets → Worse disease outcome

Butyrate?

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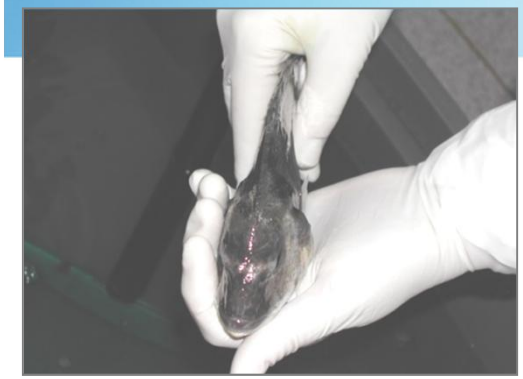


Parasite challenge (*E. leei*)

D1	D3	D4
CTRL	Extreme	Extreme+But

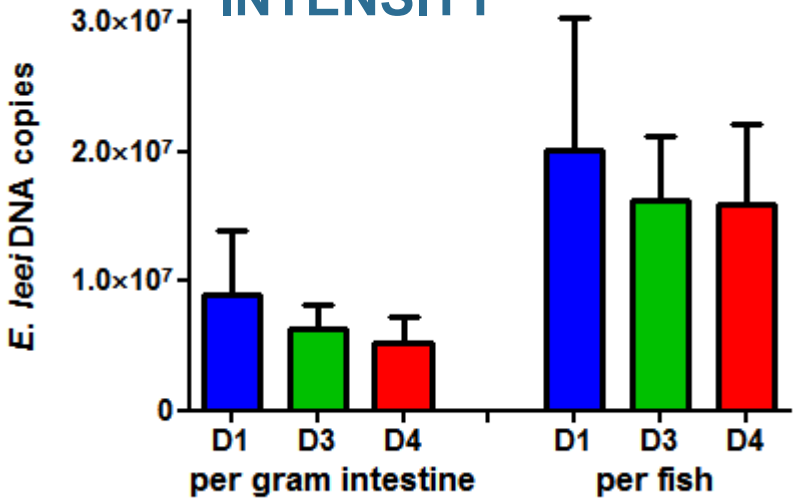
Parasite infection by anal intubation:

- **10 weeks p.i. sampling and diagnostics**

PREVALENCE

D1	D3	D4
79.2%	87.5%	91.7%

INTENSITY



Diagnosis by PCR:

- High prevalence and intensity of infection in ALL groups

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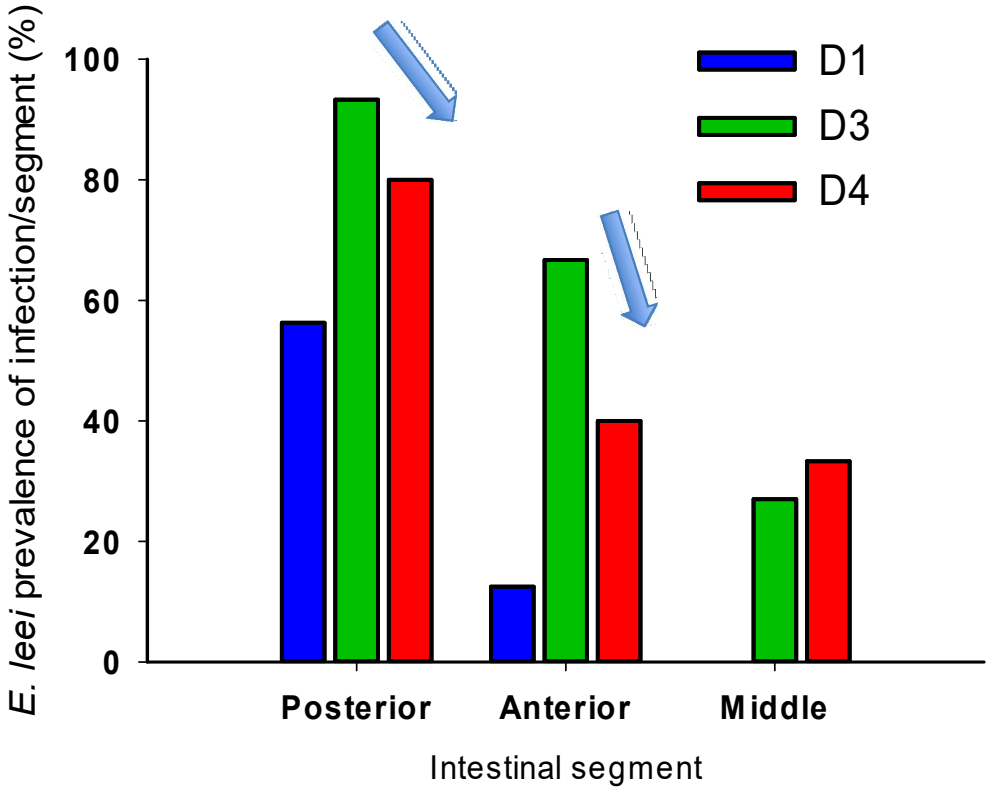
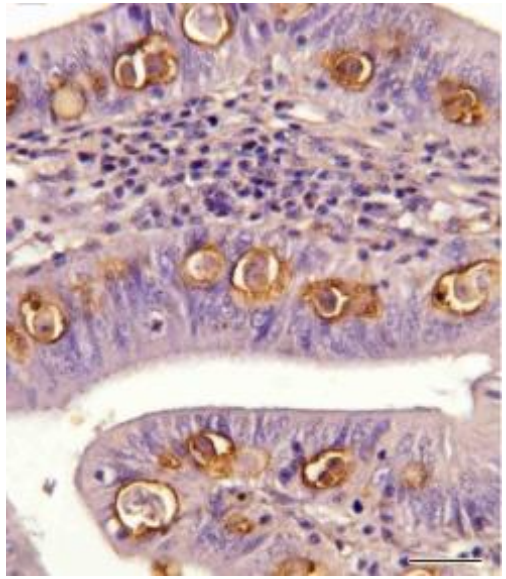
Parasite challenge (*E. leei*)

Diagnosis by HISTOLOGY:

- **Prevalence was highest in plant based diets**

- **Butyrate supplementation:**
  → decreased prevalence in anterior and posterior intestine





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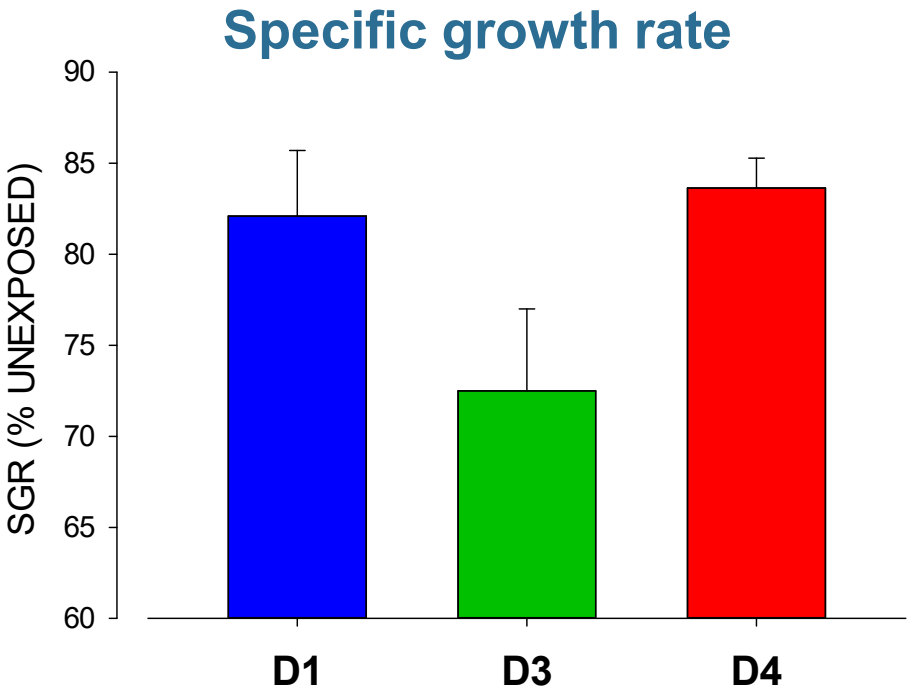
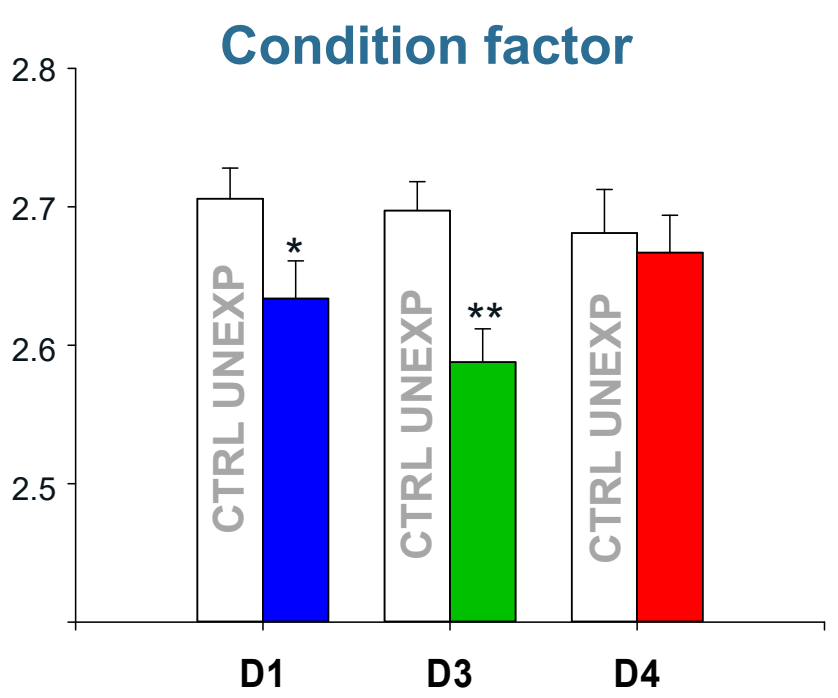
Parasite challenge (*E. leei*)

Disease signs:

- **Plant based diets** enhanced impaired growth and condition factor
- **Butyrate supplementation** improved disease signs even more than control diets





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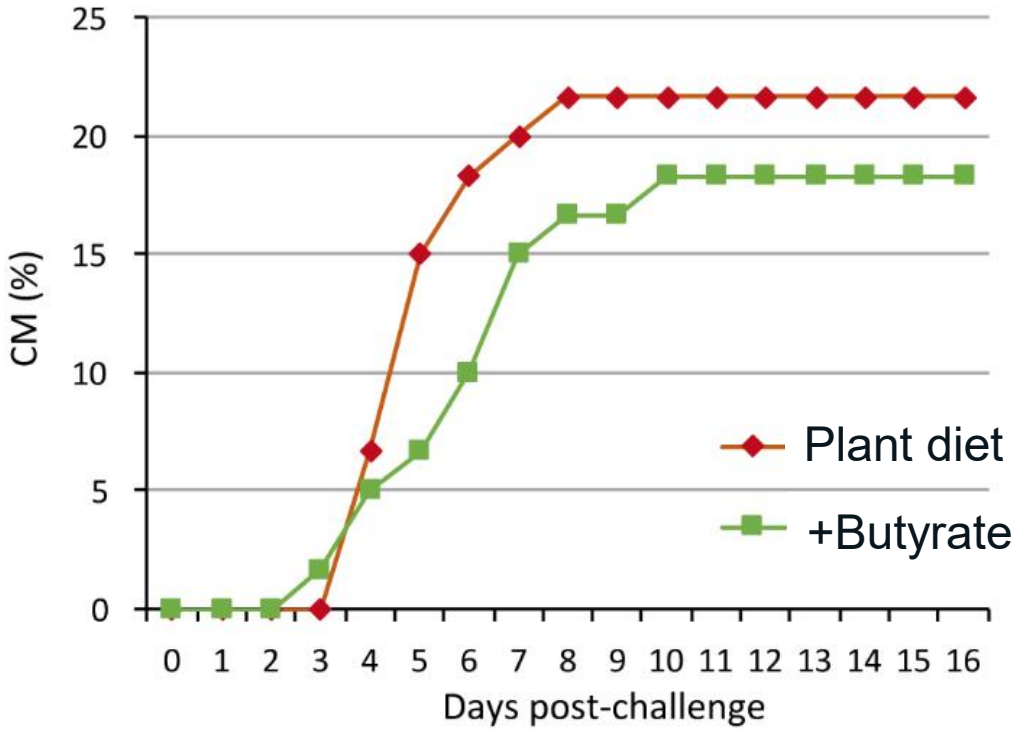
Bacterial challenge (*Photobacterium damsela* subsp. *piscicida*)

Bacterial infection by I.C. injection:

- **Mortality count**



Butyrate improved survival upon bacterial infection

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Piazzon *et al*., In press, 2017

Conclusions

Plant based diets:

- Show promising results in terms of growth
- Under pathogenic pressure can induce higher susceptibility
- Induce changes in the general intestinal health

Butyrate supplementation in plant based diets:

- Is able to revert most of the changes
- Showed promising results alleviating disease outcome

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Thank you for your attention

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