Synergistic benefits of marine derived bioactives to maintain the gut barrier in an ex vivo model

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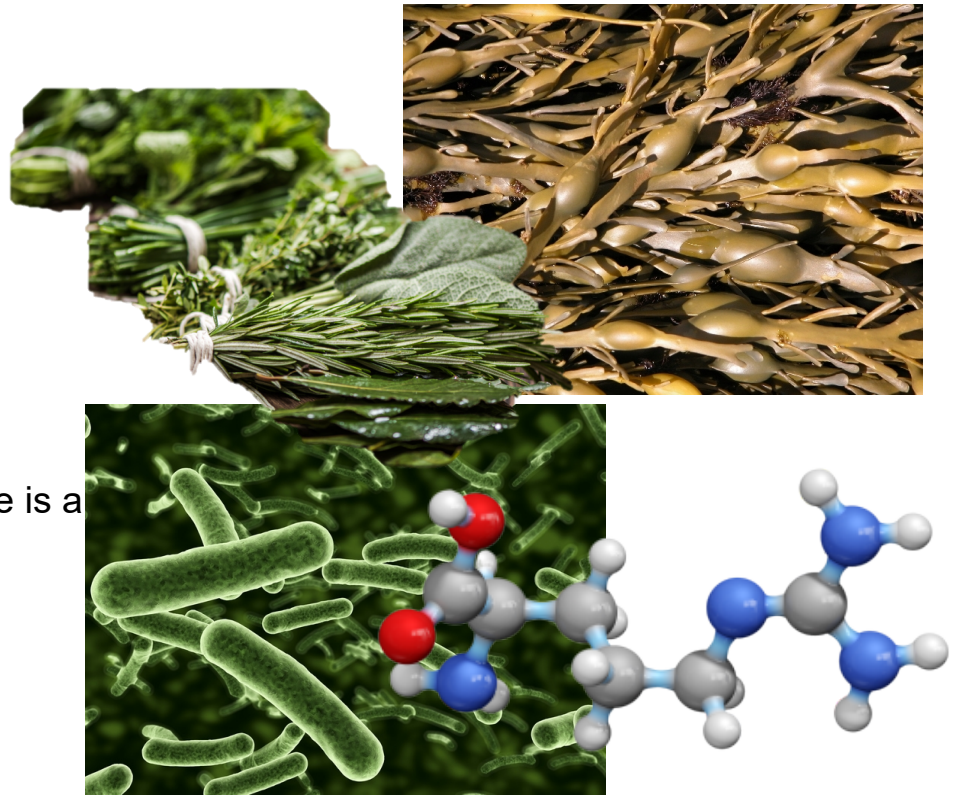
PIONEERING MARINE MINERALS SINCE 1993

Introduction

Need for new technologies and solutions in animal husbandry

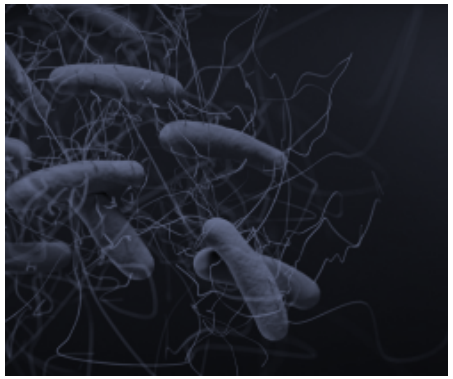
- The search for technologies to replace high levels of zinc oxide and in-feed AGP is still on-going (Canibe et al., 2022; O'Doherty et al., 2021; Bonetti et al., 2021; Costa et al., 2021).
- Natural solutions have a lot of undiscovered potential.
- Generally, there are multiple bioactives present in natural sources such as plants or marine products (O'Doherty et al., 2021; Costa et al., 2021).
  - To better understand the potential of these ingredients and their bioactives, there is a high demand for screening ingredients.
  - *In vitro* methodologies can be used to quickly screen for bioactives and help to develop these into potential applications.



Introduction

Application of new technologies from natural bioactives

- Successful technologies solve problems that are experienced by the end-users. Different challenges require different solutions. One of the challenges in pig production is the presence of *Salmonella* spp. in pig herds.
- In pigs, *Salmonella* spp. is often a subclinical disease and gets sometimes forgotten about in initial searches for challenges that are seen in practice.
- High *Salmonella* spp. levels in herds have effects on food safety:
  - Prevalence of *Salmonella* spp. is 30-55% in UK and Irish herds (Martelli et al., 2021; Deane et al., 2022).
  - Due to improved hygiene measures in slaughter houses, transfer to carcasses is limited (EFSA report 2021).
  - Pork is an important source of salmonellosis in humans: 60,000 reported cases in 2021 in the EU (EFSA report 2021).
- Screening methodologies to reduce *Salmonella* spp. prevalence in pigs will increase pig health and food safety.



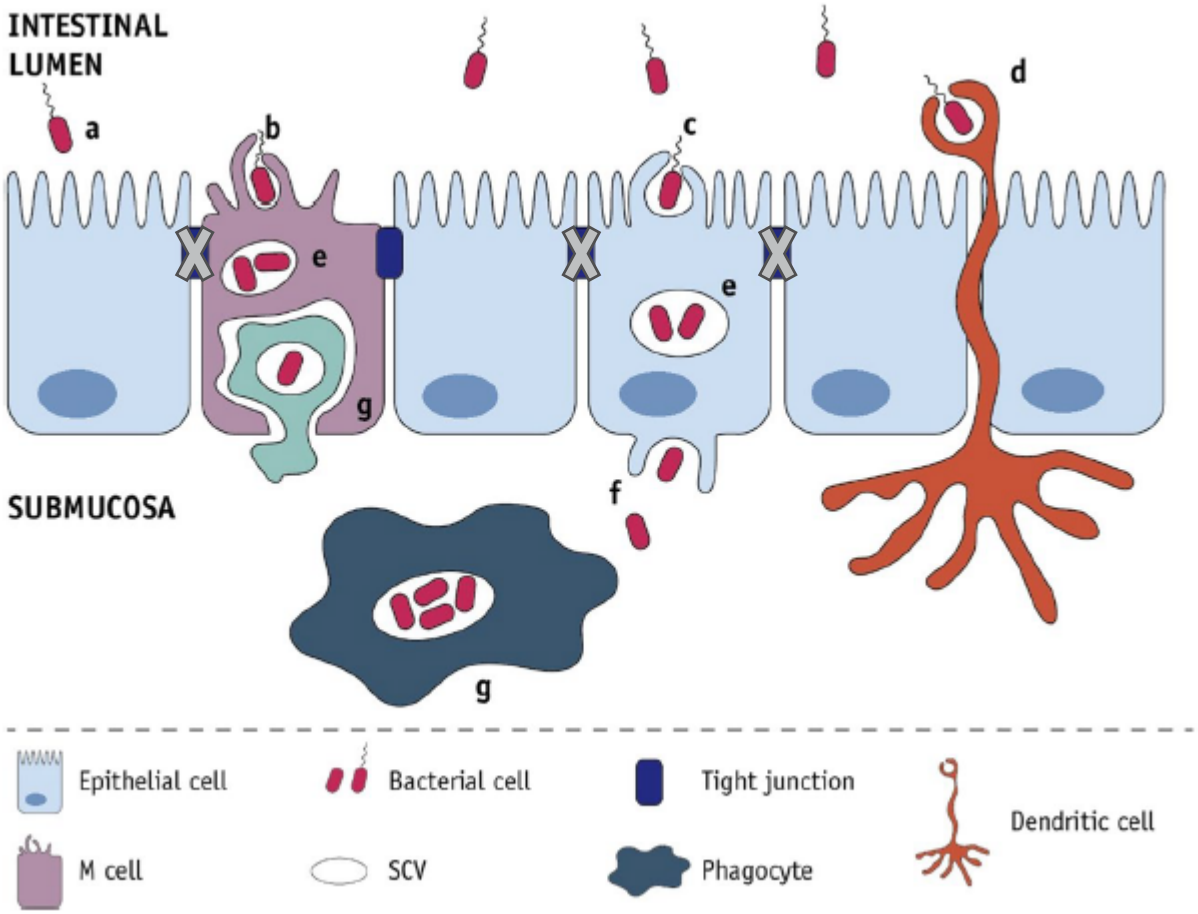
Introduction

A strong gut barrier can help to reduce colonization and subsequent (sub)clinical symptoms.

Salmonella spp. invades the cell through multiple manners:

- M-cell invasion
- Transepithelial invasion
- Dendritic cells

Salmonella spp. pathogenesis



- During invasion, *Salmonella* spp. produce proteases that break down the tight junctions to increase invasion.
- Subsequent inflammation also disrupts gut barrier.

Dos Santos *et al.*, 2018

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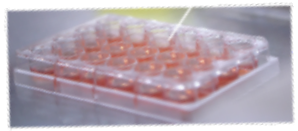
Introduction

What are the options to screen bioactives to work against *Salmonella* spp.?

In vitro

- Generally using Caco-2 or HT29 cell lines.
- Monocellular phenotype in 2D structure.
- Lacks dynamic, morphological, and physical features of intestinal epithelium.
- Permeability measured through transcellular transport (TEER): variable results, both between experiments and between labs.
- High throughput with low costs.



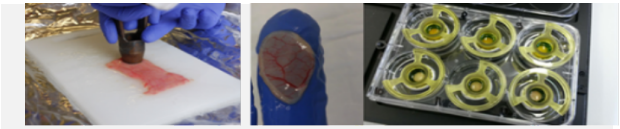
Awortwe et al., 2014

In vivo

- Using target animal or laboratory animals.
- Full animal model allows for assessment of bioactives in complete system.
- Interactions between microbes, history of the animal and inter-animal or species variability might dilute results.
- Ethical issues with usage of experimental animals.
- Low throughput with high costs.

Ex vivo

- Combines *in vitro* and *in vivo* model.
- Use of real animal tissue allows for a high number of bioactives to be screened.
- Only uses 1 animal so reduces inter-animal variability while using complete intestinal tissue with all cell types and layers.
- Measuring of both paracellular and transcellular transport.
- InTESTine™ model as developed by TNO



ex vivo intestinal tissue model (inTESTine™)

Hatton et al., 2015; Costa & Ahluwali, 2019; Kampfer et al., 2020; Amirabadi et al., 2022

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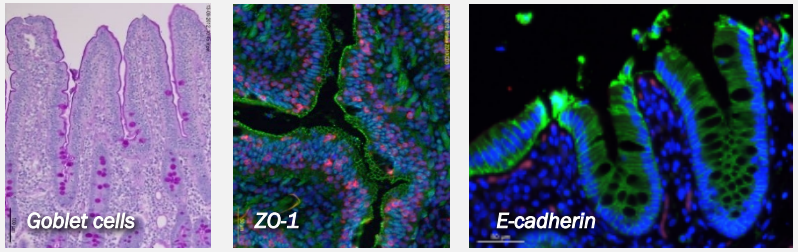
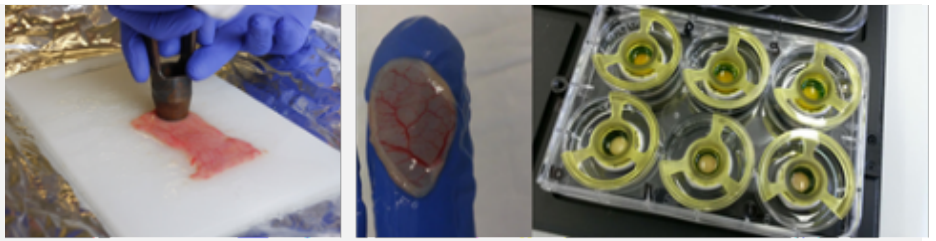
InTESTine ™:

A MEDIUM THROUGHPUT SYSTEM USING *EX VIVO* INTESTINAL TISSUE

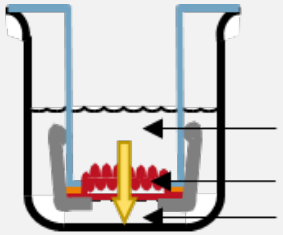
- Application of fresh intestinal tissue segments.
  - Upper to lower GI tract
- External muscle layer is removed.
- 6- or 24-well platform.
- Tissue from human, pigs, dogs, rat can be used.

- Tissue can be used afterwards for further processing





ex vivo intestinal tissue model (inTESTine™)



Apical compartment (i.e. lumen side)
Tissue
Basolateral compartment (i.e. blood side)

Publications

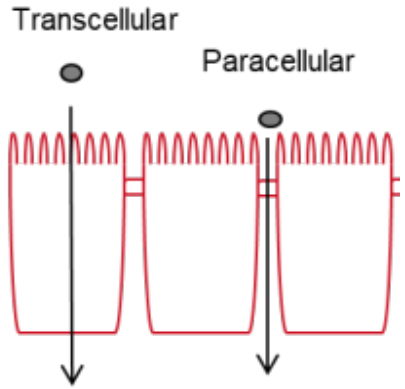
- Stevens et al., 2019 Eur J Pharm Sci
- Westerhout et al., 2017, Eur J Pharm Sci
- Westerhout et al., 2014, Eur J Pharm Sci
- Vaessen et al., 2017 Drug Met Disp
- Donkers et al., 2022, Microplastics and nanoplastics

FUNCTIONAL READ-OUTS

- **Read-outs**
  - Transport of small molecules
  - Effect on tissue functionality
  - Large molecule (FD4)
  - Tissue integrity

Functionality, integrity and viability markers are used in every study
- [^{3}H]-mannitol/atenolol (paracellular transport route)
- [^{14}C]-caffeine /antipyrine (transcellular transport route)
- FD4 , MW 4000 (tissue integrity marker)
- Lactate dehydrogenase secretion (viability)

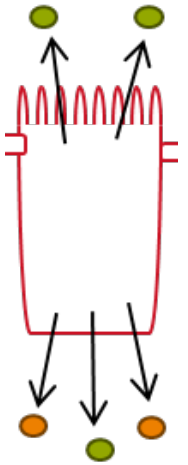
- Measurements are taken after 60 min, 120 min, 240 min, 300 minutes



Small molecules
Transcellular: Caffeine/antipyrine
Paracellular: Mannitol/atenolol
Ratio: C/M of A/A = tissue functionality

Large molecule
Paracellular: FITC-Dextran 4000 Da (FD4)
%/hr = tissue integrity

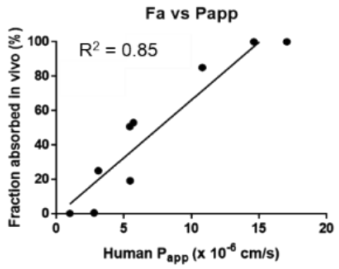
- Tissue viability/tox (LDH)

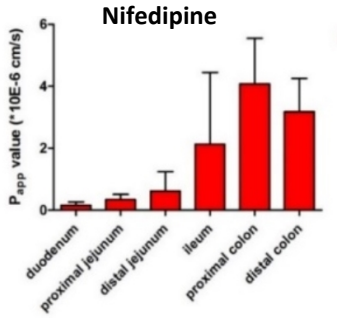


TNO *Ex vivo* intestinal tissue platforms

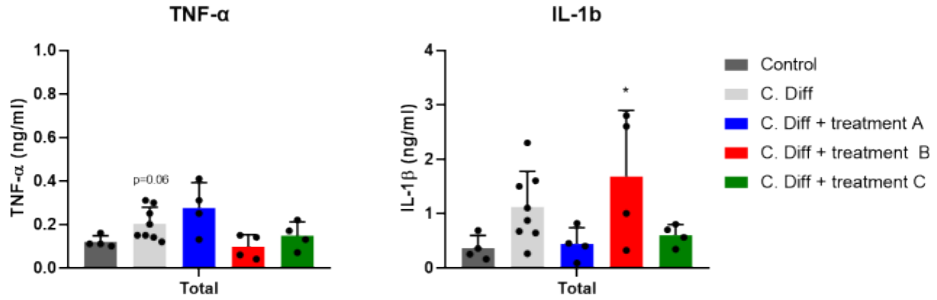
APPLICATIONS AT A GLANCE

- **Drug absorption:** measuring the oral bioavailability of drugs and nutrients.
- Including regional differences in absorption.

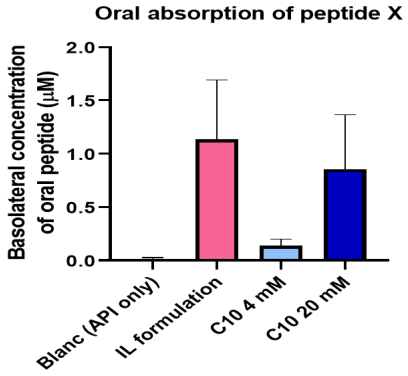




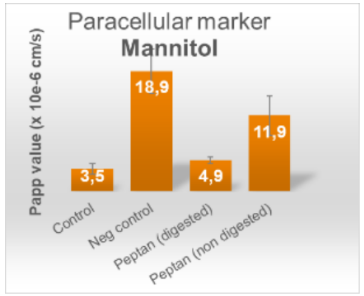
- **Inflammation:** Release of cytokines



- **Excipients:** effects on intestinal permeability

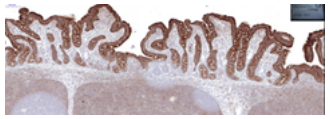


- **Barrier function:** measuring effects on the integrity of the epithelial layer.

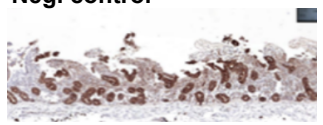


Cytokeratin 20 staining: Mucus layer

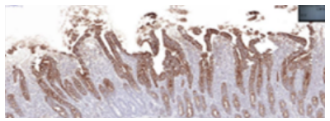
Control



Neg. control



Peptan (digestd)



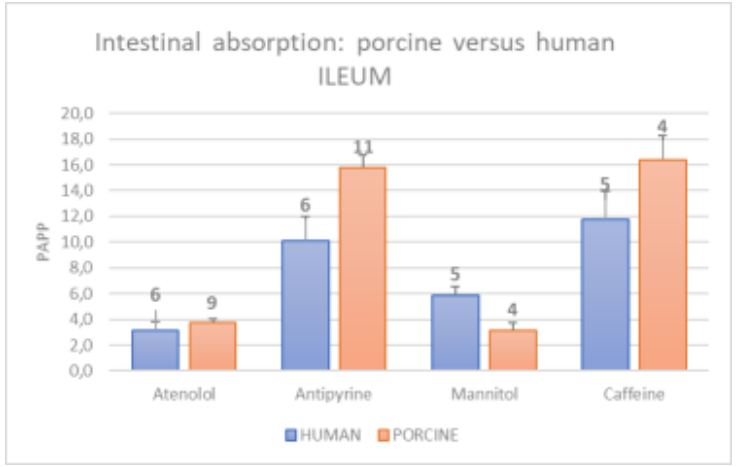
Porcine InTESTine is highly comparable to human InTESTine

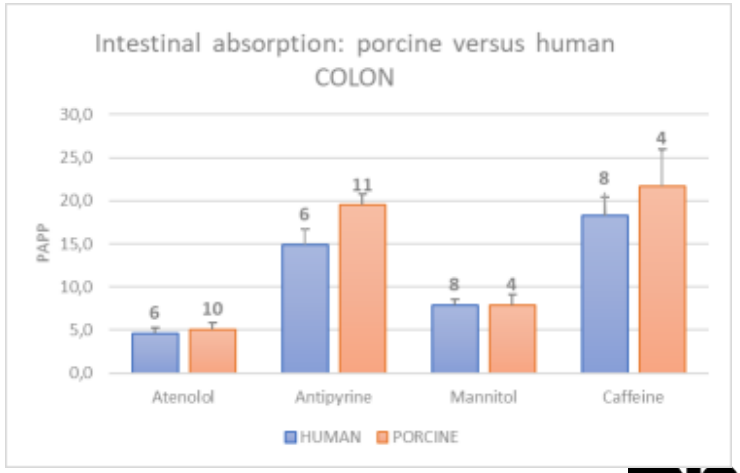
4 MODEL DRUGS FOR PARACELLULAR AND TRANSCELLULAR TRANSPORT

ex vivo intestinal tissue model (inTESTine™)

ILEUM	human			porcine		
	mean	*sem*	*n*	*mean*	*sem*	*n*
Atenolol	**3,1**	0,7	6	**3,7**	0,4	9
Antipyrine	**10,1**	1,9	6	**15,8**	1,0	11
Mannitol	**5,9**	0,6	5	**3,2**	0,6	4
Caffeine	**11,8**	2,2	5	**16,4**	1,9	4

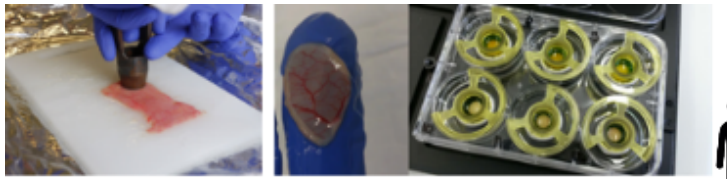
COLON	human			porcine		
	mean	*sem*	*n*	*mean*	*sem*	*n*
Atenolol	**4,6**	1,1	6	**5,0**	0,8	10
Antipyrine	**14,8**	3,0	6	**19,5**	1,3	11
Mannitol	**7,9**	1,7	8	**7,9**	1,2	4
Caffeine	**18,3**	2,7	8	**21,7**	4,3	4



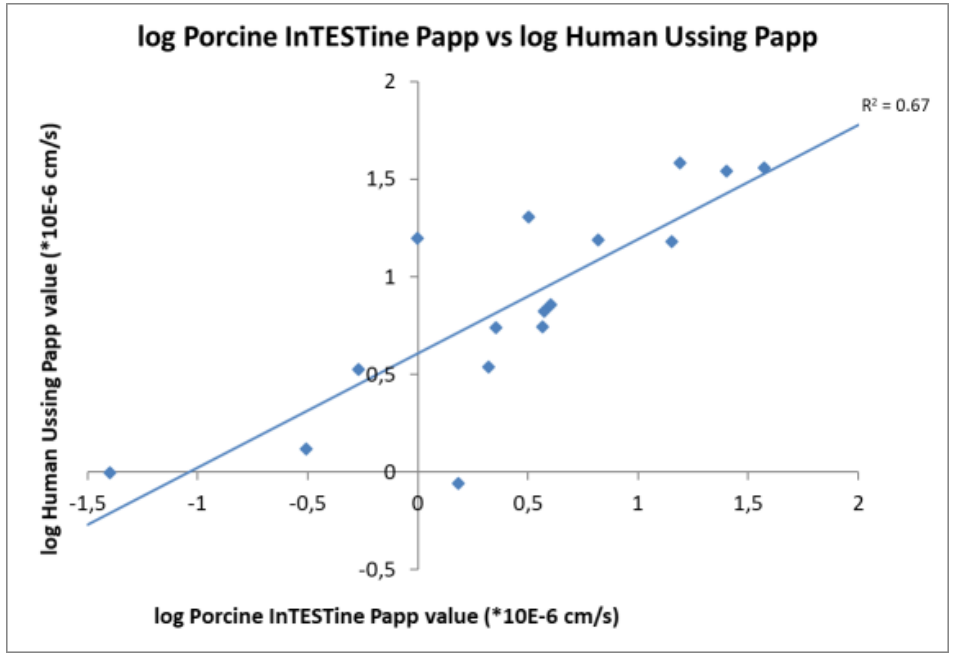


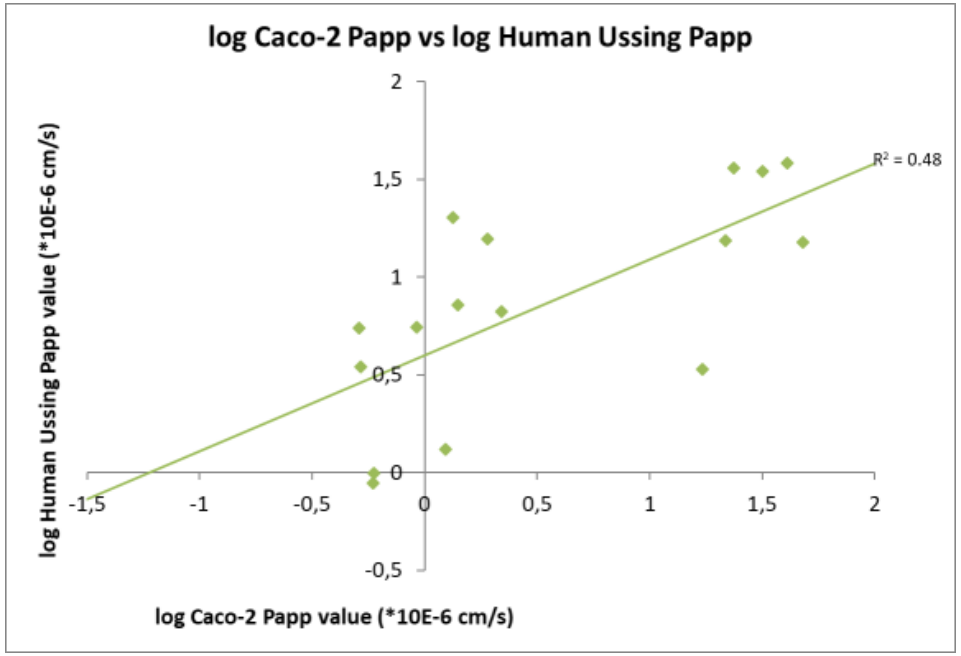
- Apparent permeability (P_{app}) of 4 model drugs is highly comparable between human and porcine ileum and colon tissue

Porcine InTESTine shows a good correlation with human Ussing



ex vivo intestinal tissue model (inTESTine™)





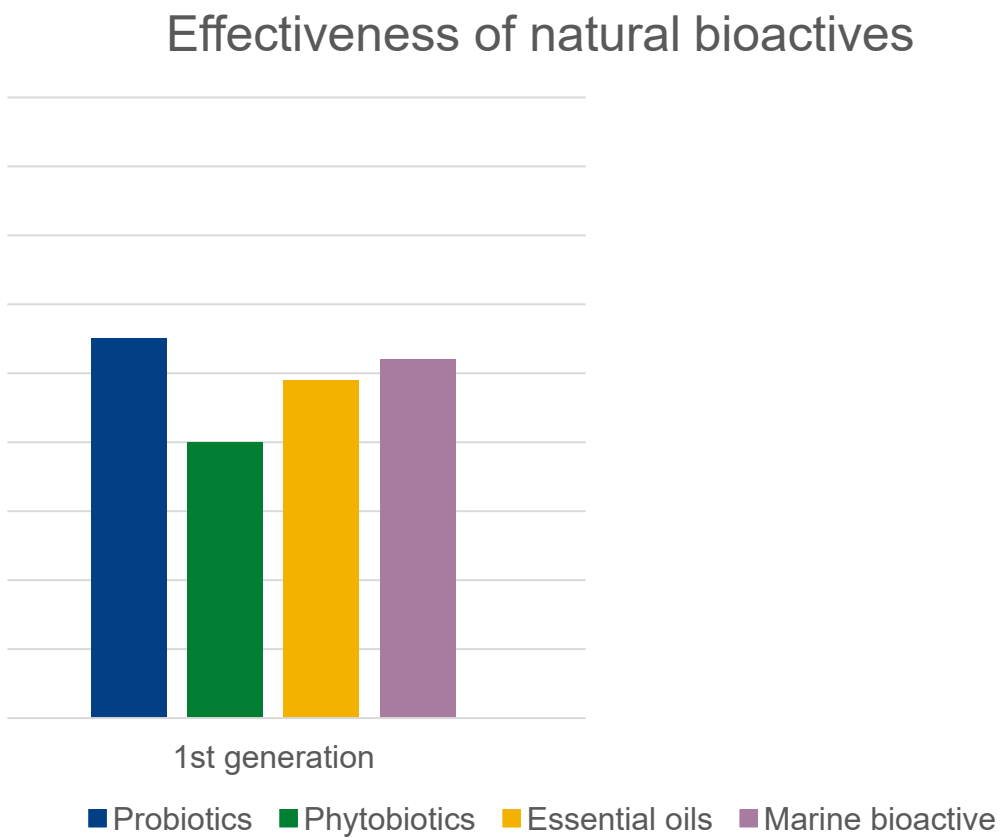
- Porcine InTESTine and Human Ussing have a positive correlation
- This correlation is higher than the correlation between Caco-2 cells and human Ussing
- Measured using 17 different medications

Introduction

Screening platform for next generation bioactives and technologies

Practice requires high performance animal health and welfare products to tackle issues.

- We need to move on to develop solutions for problems in practice that can't be solved anymore by old technologies like zinc oxide or antimicrobials.
- For many years, bioactives have been tested. The so-called first-generation products are effective but are giving variable results, depending on life stage of the animal, farm system, country, etc.



Canibe et al., 2022; Bonetti et al., 2021

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Introduction

Screening platform for next generation bioactives

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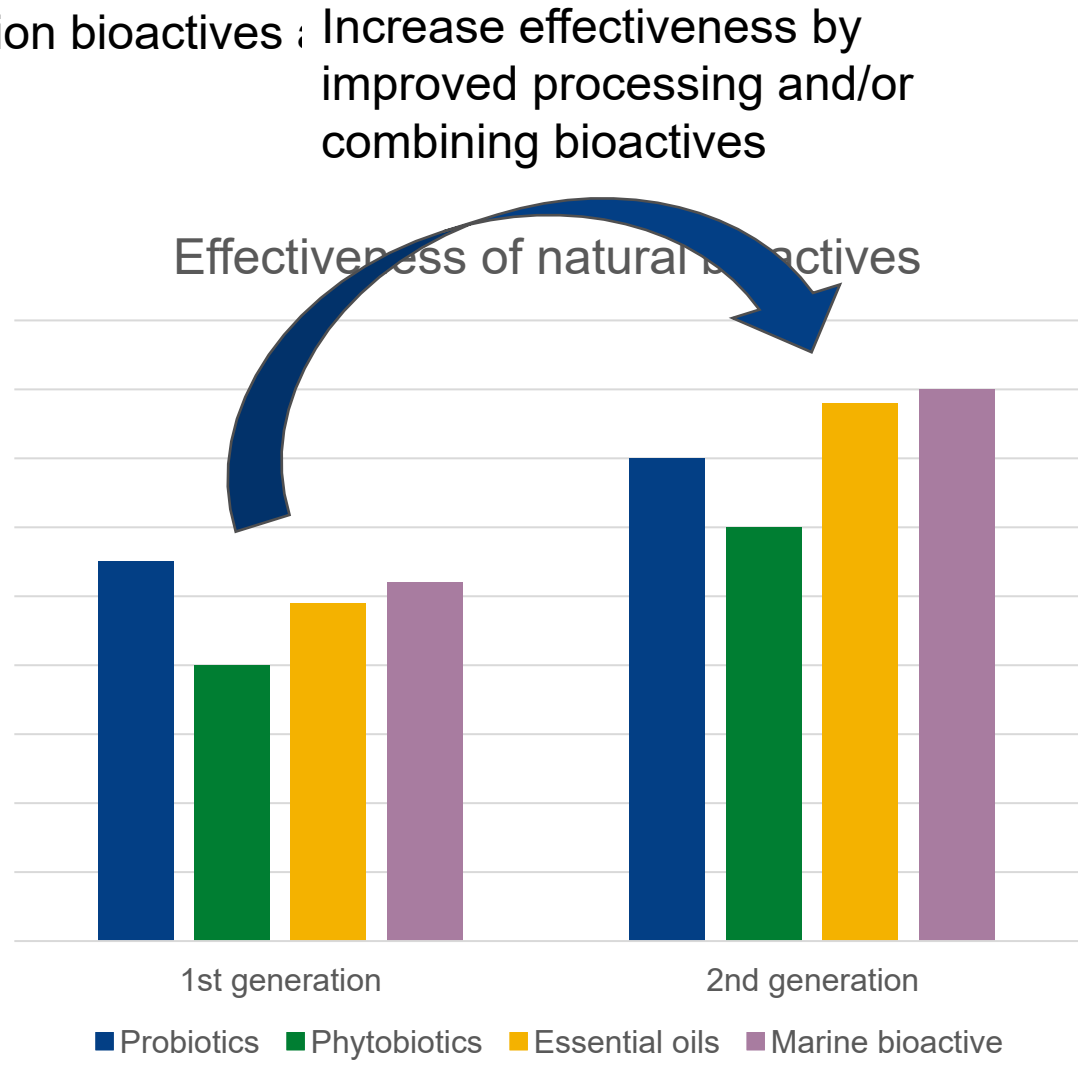
- For many years, bioactives have been tested. The so-called first-generation products are effective but are giving variable results, depending on life stage of the animal, farm system, country, etc.
- This requires 2nd generation technologies that need to achieve more. For example: combining of ingredients and/or better processing of technologies to increase performance in the field.
- *Ex vivo* screening method allows for quick screening of different dosages and combinations of natural solutions to achieve this higher performance.



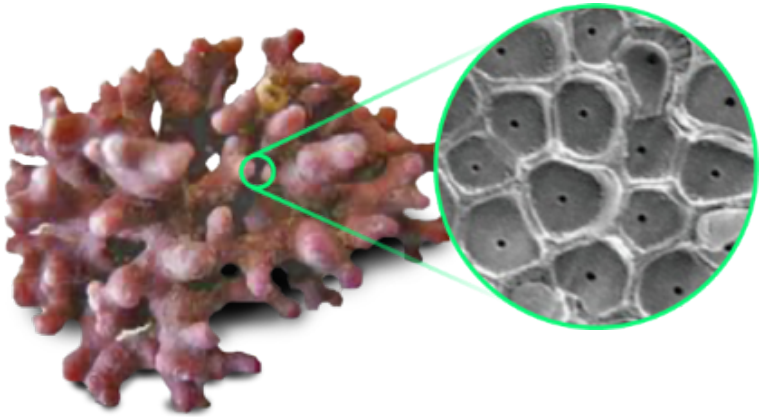
Adapted from Canibe et al., 2022; Bonetti et al., 2021

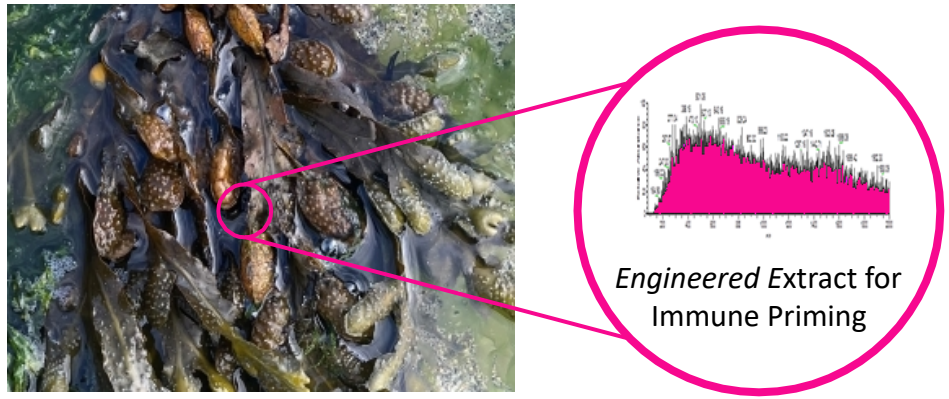
CELTIC SEA MINERALS

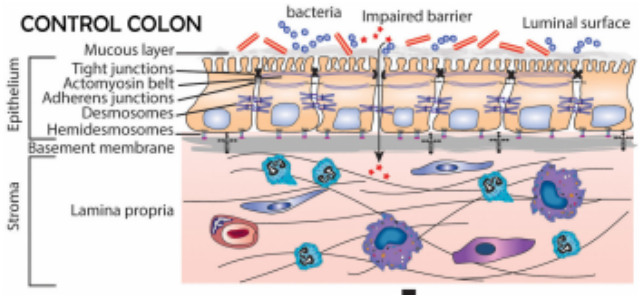
Introduction

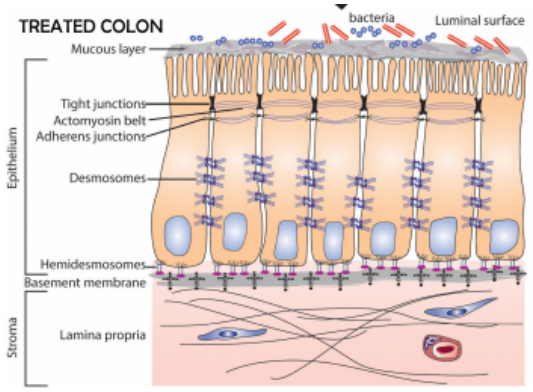
Proprietary processing of natural, marine ingredients to increase bioactives potential

- Expertise and understanding of individual raw materials is needed to understand their potential.
- This allows for the development of second-generation products and proprietary processing.
- This dictates the potential for synergies and combination of ingredients, reaching second generation quality products.
- Bioactives from *Lithothamnion glaciale* and *Ascophyllum nodosum* have individually been studied intensively.
- This has shown potential for a synergy to 1) enhance the gut barrier and 2) reduce the colonization of *Salmonella* spp.









Aslam et al., 2020

Bouwhuis et al., 2016; Bouwhuis et all., 2017; Aslam et al., 2019; Aslam et al., 2020; Aslam et al., 2021; Varani et al., 2023 Yuo et al., 2023

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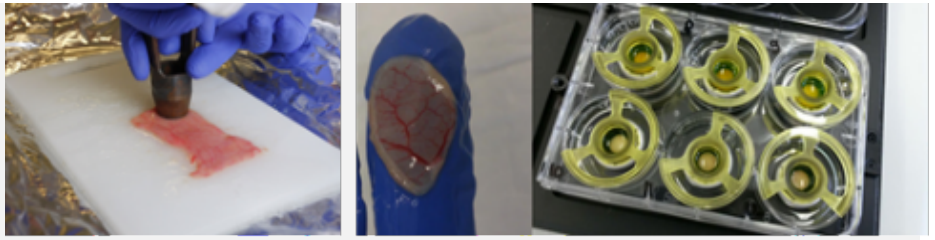
Materials and Methods

Trial set-up



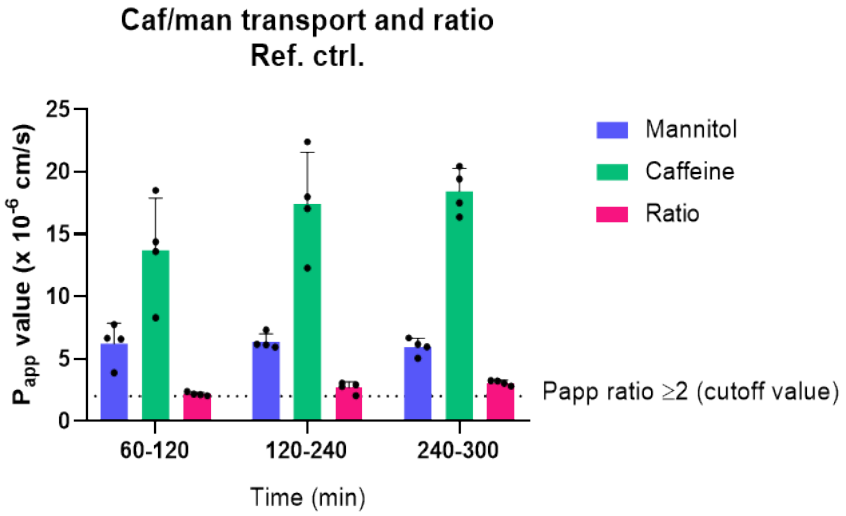
ex vivo intestinal tissue model (inTESTine™)

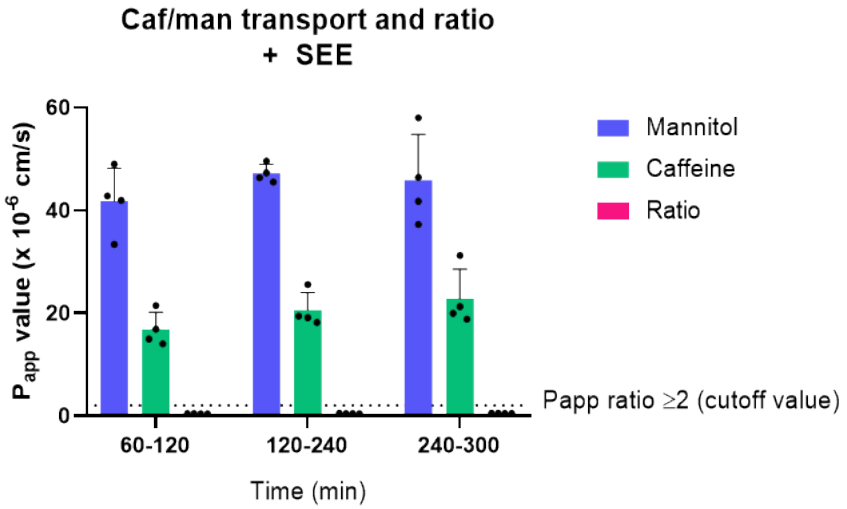
- Porcine ileal tissue was used in the InTESTine™ system.
- Tissue was incubated for 5 hours to ensure RNA quality was sufficient for gene expression analysis.
- All treatments were replicated 4 times (N=4 per treatment).
- Half the tissue was incubated with *Salmonella enterica enteriditis* (SEE) to induce inflammation and disruption of the gut barrier.
- 3 x 3 factorial design between 3 doses of *Lithothamnion glaciale* (LG) and 3 doses of *Ascophyllum Nodosum* extract (ANE), as well as the individual components.
- Concentrations of the bioactives were based on in-feed experience.
- *Lithothamnion glaciale* was pre-digested with HCl to mimic stomach digestion.
- Data was analysed using ExcelStat (16.7, 2002.4.1) using Tukey's adjustment, using factors time, SEE challenge and test condition.

Treatments		LG			
		None 0 µg/well	Dose 1 37.8 µg/well	Dose 2 63.2 µg/well	Dose 3 88.7 µg/well
ANE	None 0 µg/well	1		2	
	Dose 1 2.3 µg/well	3	4	5	6
	Dose 2 4.5 µg/well		7	8	9
	Dose 3 9.1 µg/well		10	11	12

Results

Validity of the experiment





- P_{app} for mannitol and caffeine
  - Determined for the different time points.
  - The ratio between mannitol and caffeine needs to be ≥ 2 in control conditions to ensure tissue quality.
  - SEE challenge reduced P_{app} ratio as expected
- FD4 permeability (tissue integrity)
  - Cut-off value of ≤1% FD4 permeability per hour for all test conditions at all stages.
  - Values were below cut-off value.
  - SEE challenge increased FD4 permeability but maintained within normal parameters.
- LDH leakage (cell viability)
  - Cut-off value of ≤3% LDH leakage per hour.
  - Values were below cut-off value.
  - SEE challenge did not affect LDH leakage.

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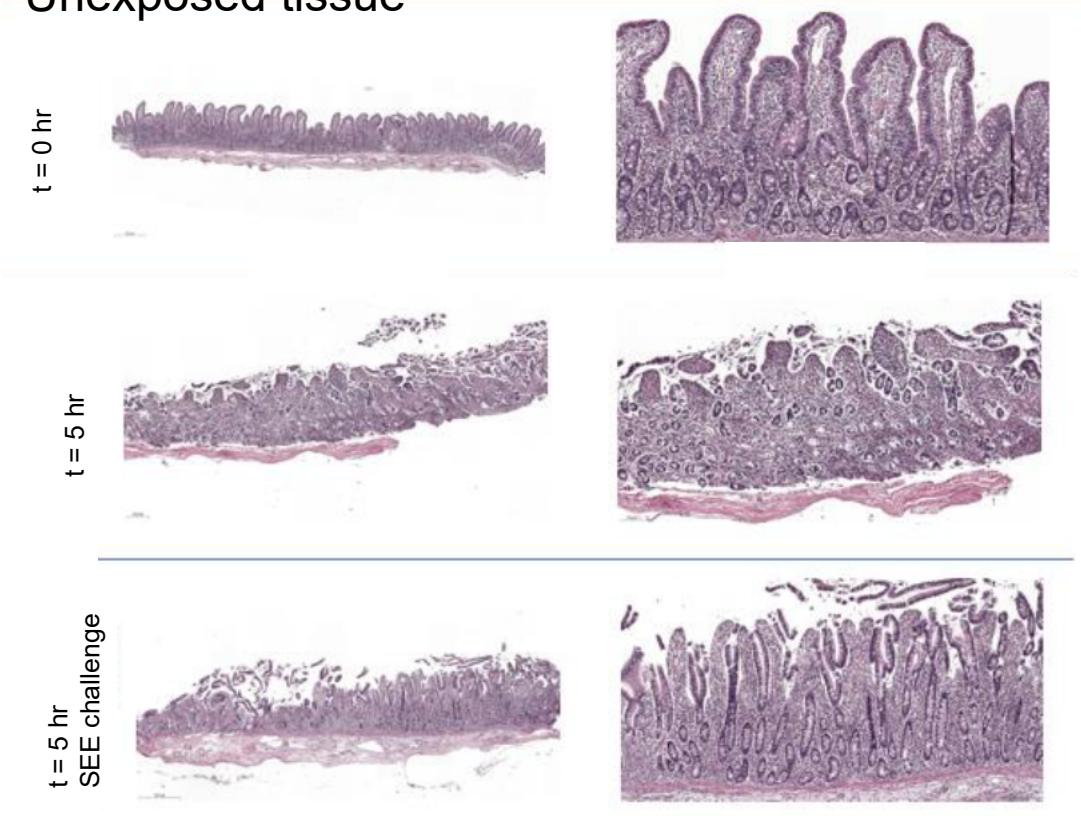
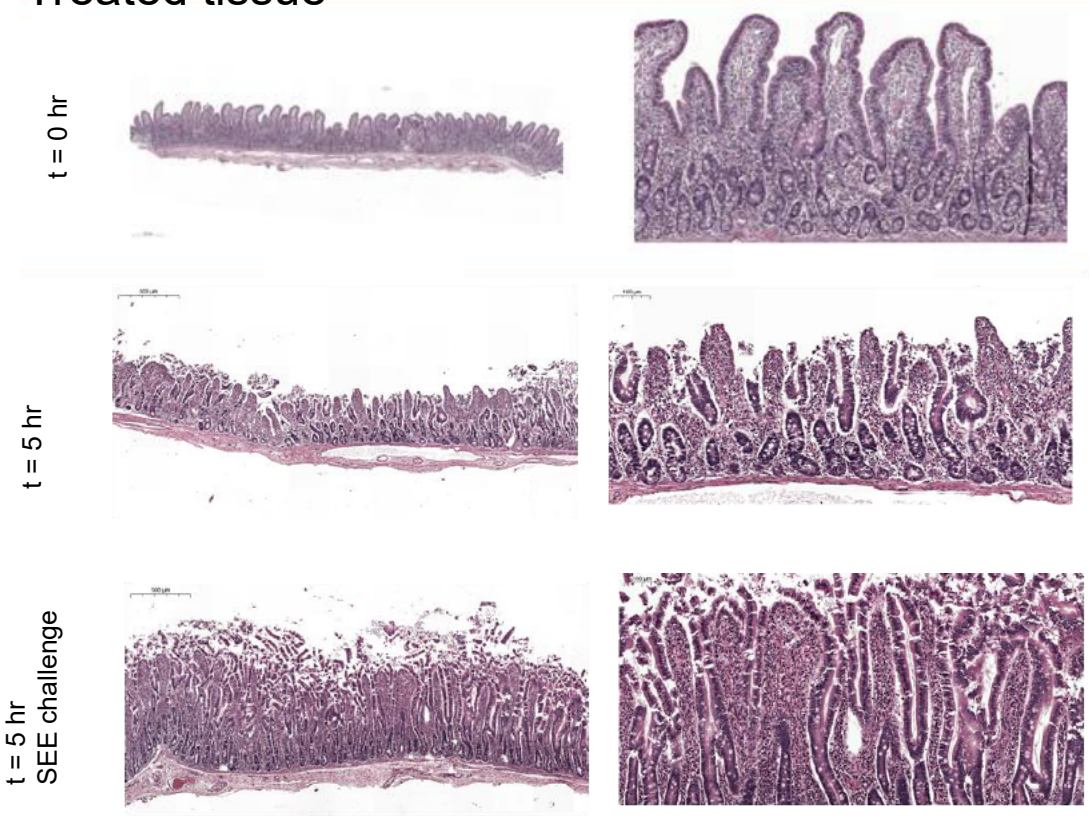
Results

Validity of the experiment: histological analysis

Unexposed tissue



Treated tissue



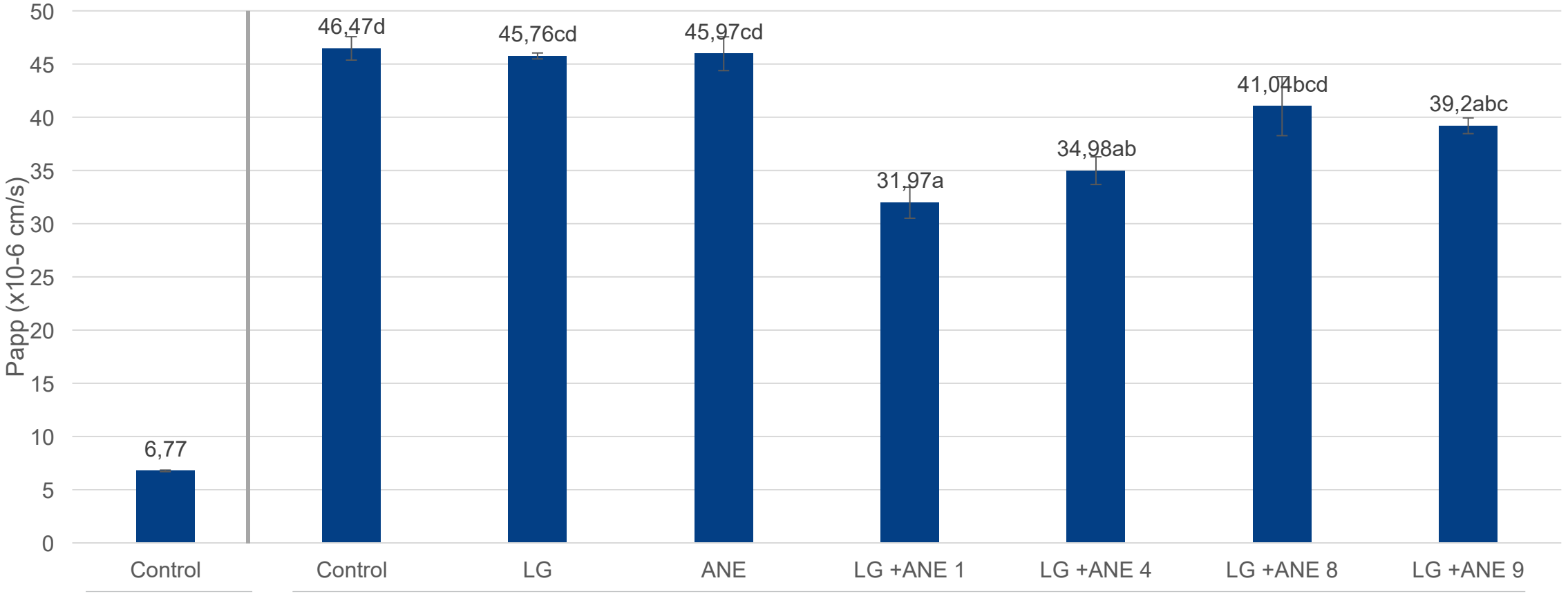
The 5 hr incubation affected morphology but still allowed for a clear structure.

The SEE challenge further damaged the gut morphology.

Results

P_{app} : Mannitol (paracellular) transport Time point 120 -240 minutes

The combination of LG and ANE at the lower concentrations reduced paracellular transport, indicating towards a stronger barrier.



Time P<0.001
Condition P<0.001
Challenge P<0.0001

Time x Condition x Challenge: P<0.0001

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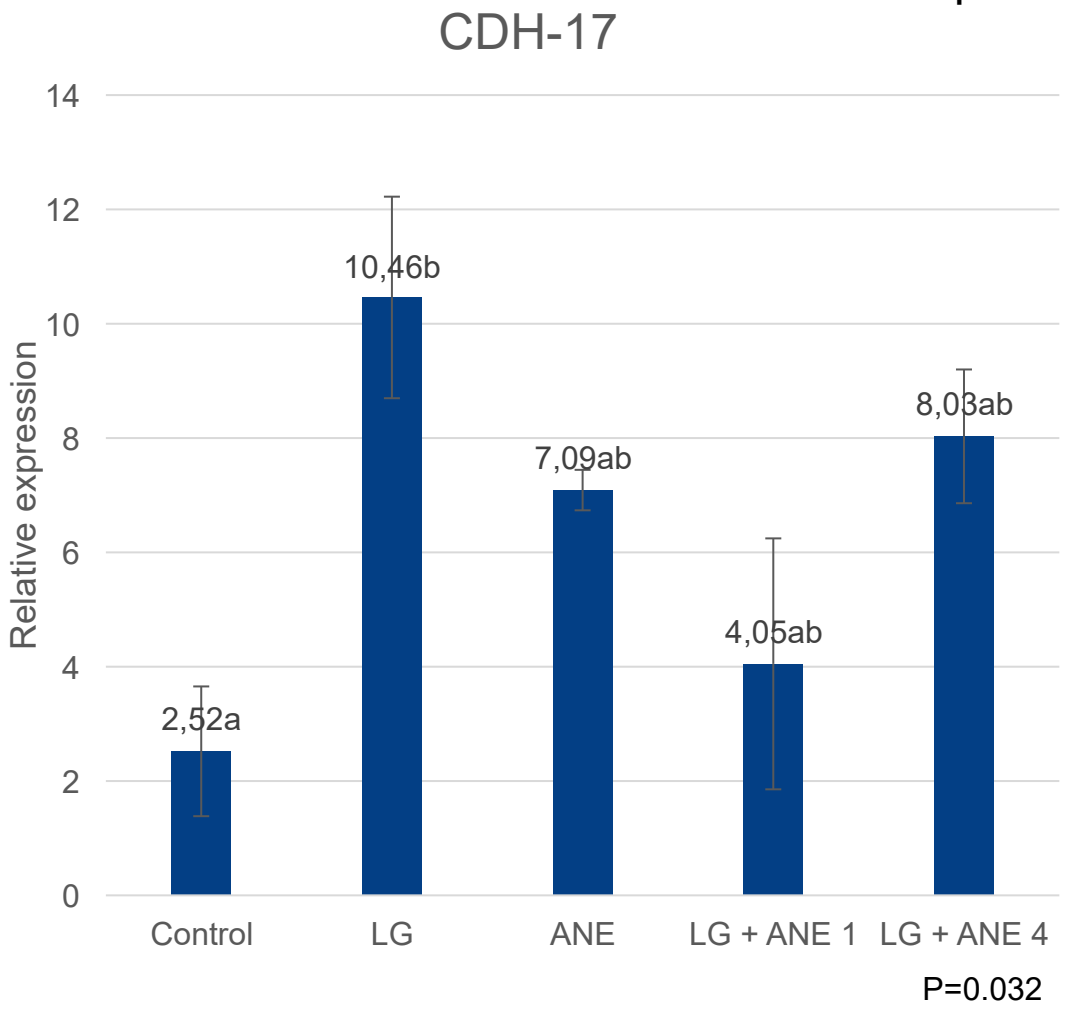
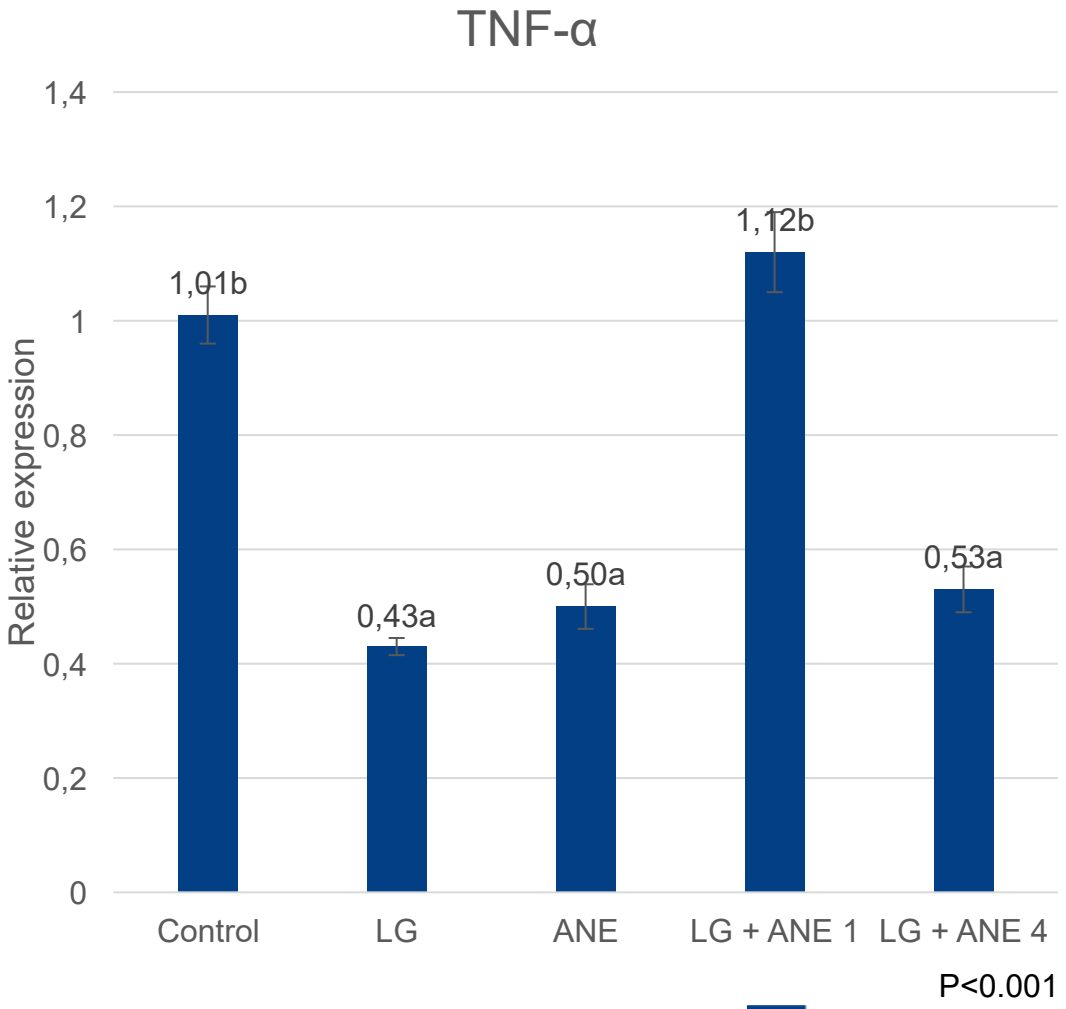
Results

Gene expression of cadherin-17 and TNF-α

The inclusion of LG increased the expression of CDH-17, while LG, ANE and LG+ANE4 showed a reduced expression of TNF-α.





CELTIC SEA MINERALS

Following from screening

On-farm validation of concept

- Based on literature research and the *ex vivo* data, the LG + ANE combination is being tested on a commercial pig farm in Ireland to reduce *Salmonella* spp. shedding.
- The study is set up as a 2x2 factorial design, with the factors being sow and piglet PW. The LG+ANE combination is fed to the sow and/or piglet PW through drinking water.
- Measurements include looking at *Salmonella* spp. shedding in both sow and piglets.
- Initial results indicate that piglets suckling sows fed the LG + ANE have reduced *Salmonella* spp. shedding during lactation.
- Trial is currently running, more data to come…

Conclusions

Using an *ex vivo* screening methodology will speed up the process to find bioactives that improve animal health and well-being.

New technologies

Required to replace the usage of high levels of ZnO and antimicrobials.

Salmonella spp. has potential to cause (sub)clinical disease in pigs and can cause salmonellosis in humans.

Damaging the gut barrier plays an important role in pathogenesis of *Salmonella* spp.

Quick screening methods for natural bioactives will help to identify the most potent solutions.

Ex Vivo model

An *ex vivo* approach allows for rapid screening while using the full intestinal morphology, combining the best of both *in vitro* and *in vivo* methodologies.

The InTESTine™ system allows for this *ex vivo* approach to measure gut barrier strength and other gut health parameters.

The InTESTine™ system is a fully validated methodology for human, porcine, rat or dog tissue.

This study

Lithothamnion Glaciale and *Ascophyllum Nodosum* extracts can enhance the gut barrier and act as anti-inflammatory agents.

To find second-generation technologies, these two were combined to investigate a synergy to reduce gut permeability during an SEE challenge in the InTESTine™ system.

The combination of LG and ANE strengthened the gut barrier during the SEE challenge.

A reduced gene expression of the pro-inflammatory cytokine TNF-α and the tight junction protein Cadherin17 was also observed.

We are a global leader in natural, marine phytonutrients with specialist expertise in seaweed.

Thank You

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