

Safety evaluation of an intra-nasally applied cocktail of *Lactococcus lactis* strains in pigs

Ruth Rattigan¹, Lukasz Wajda¹, Abel A. Vlasblom², Alan Wolfe³, Aldert L. Zomer², Birgitta Duim², Jaap A. Wagenaar², Peadar Lawlor^{1*}

¹Pig Development Department, Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark, Fermoy, Co. Cork, Ireland

²Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL, Utrecht, Netherlands

³School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland.

*Corresponding author: peadar.lawlor@teagasc.ie

Supplementary Table S1. Composition of the experimental diets (on an air-dry basis; kg/tonne).

Diet Number	1	2	3	4	5
Diet type	Dry sow	Lactation	Starter	Link	Weaner
Ingredients					
Barley	759.7	259.7	50.0	68.4	495.9
Wheat	0	455.2	0	100.0	216.8
Maize	0	0	231.0	300.0	0
Soybean meal	76.2	179.8	143.4	186.9	163.2
Full fat soybean meal	0	0	130.8	70.0	50.0
Lactoflo ¹	0	0	200.0	150.0	0
Skim milk powder	0	0	125.0	50.0	0
Soya hulls	125.3	0	0	0	0
Soya oil	14.0	66.0	85.0	38.2	40.0
Premix ²	1.5	1.5	3.0	3.0	3.0
L-Lysine HCl	2.3	5.0	6.2	6.7	5.9
DL-Methionine	0.4	1.5	3.6	3.2	2.2
L-Threonine	1.0	2.7	3.7	3.4	2.7
L-Tryptophan	0	0.8	1.4	1.3	0.6
L-Valine	0	2.7	1.3	1.3	0.6
Limestone flour	8.5	11.5	7.0	7.5	10.5
Mono dicalcium phosphate	7.0	8.5	5.5	7.0	5.5
Salt	4.0	5.0	3.0	3.0	3.0
Phytase ³	0.05	0.05	0.05	0.05	0.05
Chemical composition					
Dry matter ⁴	883.0	893.0	907.0	897.0	888.0
Crude protein ⁴	125.0	163.0	188.0	166.0	178.0
Ash ⁴	42.0	48.0	57.0	53.0	47.0
Ether extract ⁴	33.7	85.4	119.1	58.4	65.2
Crude fibre ⁴	87.0	26.0	16.0	33.0	32.0
Lysine ⁵	7.8	11.5	16.2	15.0	13.0
Methionine ⁵	2.4	3.9	7.0	6.1	4.7
Cystine ⁵	2.5	3.0	2.7	2.9	3.1
Threonine ⁵	5.6	8.3	10.9	10.1	8.8
Tryptophan ⁵	3.66	3.36	2.66	2.22	1.54
Digestible energy (MJ/Kg) ⁵	12.51	14.86	16.20	15.00	14.27
Net energy (MJ/Kg) ⁵	8.86	10.90	12.06	10.94	10.30
SID lysine ^{5,6}	6.6	10.7	15.3	14.1	12.0
Total calcium ⁵	7.2	8.3	8.2	7.5	7.4
Digestible phosphorus ⁵	3.5	3.8	4.6	4.2	3.3

¹Lactoflo, non-hygroscopic whey permeate powder (Volac, Royston, United Kingdom)

²Premix provided per kilogram of complete diet Diets 1 and 2): Premix provided per kg of complete diet: Cu from copper sulphate, 15 mg; Fe from ferrous sulphate monohydrate, 70 mg; Mn from manganese oxide, 62 mg; Zn from zinc oxide, 80 mg, I from calcium iodate, 0.6 mg; Se from sodium selenite, 0.2 mg; vitamin A as retinyl acetate, 3.44 mg; vitamin D₃ as cholecalciferol, 25 mg; vitamin E as DL-alpha-tocopheryl acetate, 100 mg; vitamin K, 2 mg; vitamin B₁₂, 15 µg; riboflavin, 5 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 500 mg; Biotin, 200µg; folic acid, 5mg; vitamin B₁,2 mg; and vitamin B₆, 3 mg.Premix provided per kilogram of complete diet (Diet 3, 4 and

5): Premix provided per kilogram of complete diet: Cu from copper sulphate, 85 mg; Fe from ferrous sulphate monohydrate, 90 mg; Mn from manganese oxide, 47 mg; Zn from zinc oxide, 120 mg; I from potassium iodate, 0.6 mg; Se from sodium selenite, 0.3 mg; vitamin A as retinyl acetate, 2.1 mg; vitamin D₃ as cholecalciferol, 25 µg; vitamin E as DL-alpha-tocopheryl acetate, 100 mg; vitamin K, 4 mg; vitamin B₁₂, 15 µg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 250 mg; vitamin B₁, 2 mg; and vitamin B₆, 3 mg.

³The diet contained 500 phytase units (FYT) per kg feed (RONOZYME HiPhos GT; DSM, Belfast, UK).

⁴Analysed nutrient composition

⁵Calculated nutrient composition

⁶SID lysine = Standardized ileal digestible lysine.

Supplementary Table S2. Health check scoring system.

Category	Score			
	0	1	2	3
Faecal score	NORMAL <ul style="list-style-type: none"> • Dry, pelleted faeces 	SOFT <ul style="list-style-type: none"> • Soft with shape 	MILD DIARRHOEA <ul style="list-style-type: none"> • Very soft or viscous liquid 	SEVERE DIARRHOEA <ul style="list-style-type: none"> • Watery or with blood
Behaviour	NORMAL <ul style="list-style-type: none"> • Active 	APATHETIC <ul style="list-style-type: none"> • Must be stimulated to get up 	LYING <ul style="list-style-type: none"> • Gets up with help 	DEEP PROSTRATION <ul style="list-style-type: none"> • Cannot get up
Dehydration	NORMAL <ul style="list-style-type: none"> • No signs/symptoms of dehydration 	MILD <ul style="list-style-type: none"> • Normal eyes • Slight reduction of skin elasticity • Normal capillary refill time • Normal mucous membranes 	MODERATE <ul style="list-style-type: none"> • Slightly sunken eyes • Loss of skin elasticity • Normal capillary refill time • Normal mucous membranes 	SEVERE <ul style="list-style-type: none"> • Sunken eyes • Loose, wrinkled skin very evident • Retarded capillary refill time • Dry mucous membranes
Clinical signs	NORMAL <ul style="list-style-type: none"> • Normal Temperature, Respiration/Cardiac rates 	MINOR <ul style="list-style-type: none"> • Minor changes in Temperature, Respiration/Cardiac rates 	MODERATE <ul style="list-style-type: none"> • Temperature ± 1°C, Respiration/Cardiac rates ±30% 	SEVERE <ul style="list-style-type: none"> • Temperature± 2°C, Respiration/Cardiac rates ±50%

Supplementary Table S3. qPCR primers

Gene	GenBank accession number	Primers sequences (5' – 3')
<i>β-actin</i>	U07786.1	F: CATCACCATCGGCAACGA R: GCGTAGAGGTCCTTCCTGATGT
<i>CXCL8</i>	NM213867	F: TAGGACCAGAGCCAGGAAGA R: GAACTGCAGCCTCACAGAGA
<i>IL6</i>	M86722.1	F: TGGATAAGCTGCAGTCACAG R: ATTATCCGAATGGCCCTCAG
<i>IL1β</i>	M86725.1	F: AAGTGATGGCTAACTACGGTGAC R: ATCTGCCTGATGCTCTTGTTC
<i>TNFα</i>	X57321.1	F: CACCACGCTCTTCTGCCTACTGC R: TCGGCTTTGACATTGGCTACAA
<i>pBD2</i>	AY506573.1	F: ACCTGCTTACGGGTCTTG R: CTCTGCTGTGGCTTCTGG
<i>TLR2</i>	AB072190	F: ACATGAAGATGATGTGGGCC R: TAGGAGTCCTGCTCACTGTA
<i>TLR9</i>	AB071394	F: GTGGAAGTGTGTTTGGCATC R: CACAGCACTCTGAGCTTTGT

β-actin, beta-actin; *CXCL8*, C-X-C motif chemokine ligand 8 (IL8); *IL6*, interleukin 6; *IL1 β*, interleukin 1-beta; *TNFα*, tumour necrosis factor alpha; *pBD2*, beta-defensin 2; *TLR2*, toll like receptor 2; *TLR9*, toll like receptor 9.

Supplementary Table S4. The effect of treatment on histopathology of the nasal conchae.

Treatment Group	Placebo	Bacterial Cocktail
No abnormalities		
24h after birth	3/6	6/6
96h after birth	6/6	6/6
14d after birth	6/6	6/6
Non-significant findings		
24h after birth	3/6	0/6
96h after birth	0/6	0/6
14d after birth	0/6	0/6

* Minor changes in epithelium and/or cellular debris in the lumen

Supplementary Table S5. The effect of treatment on the histopathology of the left and right tonsil paraepiglottal.

Treatment Group	Left		Right	
	Placebo	Bacterial Cocktail	Placebo	Bacterial Cocktail
No lymphoid tissue collected				
24h after birth	6/6	5/6	5/6	6/6
96h after birth	5/6	6/6	6/6	6/6
14d after birth	4/6	4/6	4/6	3/6
No abnormalities				
24h after birth	0/6	1/6	1/6	0/6
96h after birth	0/6	0/6	0/6	0/6
14d after birth	2/6	2/6	2/6	3/6
Non-significant findings				
24h after birth	0/6	0/6	0/6	0/6
96h after birth	1/6	0/6	0/6	0/6
14d after birth	0/6	0/6	0/6	0/6

*** Normal findings of neutrophils and eosinophils scattered throughout tissue.**

Supplementary Table S6. Effect of treatment on the histopathology of the tonsil palatine.

Treatment Group	Placebo	Bacterial Cocktail
No lymphoid tissue collected		
24h after birth	0/6	3/6
96h after birth	0/6	0/6
14d after birth	0/6	0/6
No abnormalities		
24h after birth	3/6	0/6
96h after birth	0/6	0/6
14d after birth	1/6	0/6
*Non-significant findings		
24h after birth	3/6	3/6
96h after birth	6/6	6/6
14d after birth	5/6	6/6

*** Normal findings of neutrophils and eosinophils scattered throughout tissue and/or cellular debris and degenerate inflammatory cells in the crypts.**

Supplementary Table S7. Effect of treatment on the histopathology of the left and right tonsil retropharyngeal.

	Left		Right	
Treatment Group	Placebo	Bacterial Cocktail	Placebo	Bacterial Cocktail
No lymphoid tissue collected				
24h after birth	4/6	1/6	2/6	4/6
96h after birth	3/6	3/6	1/6	4/6
14d after birth	3/6	2/6	2/6	1/6
No abnormalities				
24h after birth	1/6	5/6	4/6	2/6
96h after birth	1/6	1/6	1/6	1/6
14d after birth	2/6	4/6	0/6	2/6
*Non-significant findings				
24h after birth	1/6	0/6	0/6	0/6
96h after birth	2/6	2/6	4/6	1/6
14d after birth	1/6	0/6	4/6	3/6

***Normal findings of neutrophils and eosinophils scattered throughout tissue and/or cellular debris and degenerate inflammatory cells in the crypts.**