Naqid, Ibrahim A. and Owen, Jonathan P. and Maddison, Ben C. and Gardner, David S. and Foster, Neil and Tchorzewska, Monika and La Ragione, Roberto M. and Gough, Kevin C. (2015) Prebiotic and probiotic agents enhance antibody-based immune responses to Salmonella Typhimurium infection in pigs. Animal Feed Science and Technology, 201 . pp. 57-65. ISSN 0377-8401

Prebiotic and probiotic agents enhance antibody-based immune responses to *Salmonella* Typhimurium infection in pigs

Ibrahim A. Naqid[a], Jonathan P. Owen[b], Ben C. Maddison[b], David S. Gardner[a], Neil Foster[a], Monika A. Tchórzewska [c,d,1], Roberto M. La Ragione[c,e] and Kevin C. Gough[a*]

[a]School of Veterinary Medicine and Science, The University of Nottingham, Sutton Bonington Campus, College Road, Sutton Bonington, Leicestershire, LE12 5RD. UK.
ibrahim.naqid@nottingham.ac.uk;
david.gardner@nottingham.ac.uk;
neil.foster@nottingham.ac.uk; kevin.gough@nottingham.ac.uk

[b]ADAS UK, School of Veterinary Medicine and Science, The University of Nottingham,
Sutton Bonington Campus, College Road, Sutton Bonington, Leicestershire, LE12 5RD. UK. jonathan.owen@adas.co.uk;
ben.maddison@adas.co.uk

[c]Animal Health and Veterinary Laboratories Agency,
Woodham Lane, New Haw, Addlestone, Surrey. KT15 3NB. UK.

[d] School of Biological Science, Royal Holloway, University of London, Egham, Surrey. TW20 0EX.

[e] School of Veterinary Medicine, DK Building, University of

Surrey, Guildford, Surrey. GU2 7XH. r.laragione@surrey.ac.uk

[1]Present address: School of Veterinary Sciences, The University of Bristol, Langford House, Langford, Bristol. BS40 5DU. UK. m.tchorzewska@bristol.ac.uk

*Corresponding Author: Dr K. C. Gough, School of Veterinary Medicine and Science, The University of Nottingham, Sutton Bonington Campus, College Road, Sutton Bonington, Leicestershire, LE12 5RD. UK. Telephone: +44-115-9516272; FAX: +44-115-9516440;

Email:kevin.gough@nottingham.ac.uk

Abstract

Salmonellosis causes significant economic losses to the pig industry and contaminated pork products are an important source of *Salmonella* for humans. The EU ban on the use of antibiotic growth promoters in pig production, and the emergence of antibiotic resistance has meant there is a pressing need for alternative control strategies for pathogenic bacteria such as *S.* Typhimurium in pigs. Here, we determined the effects of prebiotic, probiotic and synbiotic diet regimes on antibody responses to oral *Salmonella* challenge of pigs. The data demonstrate that the inclusion of the probiotic *Lactobacillus plantarum* B2984 in the diet of piglets (~1 x 10^{10}cfu/animal/day) enhanced serum IgM (P<0.001), IgG (P=0.001) and IgA (P=0.039) responses to *S.* Typhimurium infection including cross-reacting antibodies to *S.* Enteritidis. Similarly, inclusion of the prebiotic lactulose at 1% (w/w) of the feed on a daily basis in the diet enhanced serum IgM (P=0.010), IgG (P=0.004) and IgA (p=0.046) responses to *S.* Typhimurium infection and also cross-reacting antibodies to *S.* Enteritidis. Inclusion of both additives in the synbiotic diet also elicited an enhanced immune response with IgM (P=0.009) and IgG (p=0.046) levels being increased, however a significant interaction of the pre and probiotics was observed when considering the immune responses to *S.* Typhimurium (IgM P=0.004; IgG and IgA, P<0.001 for interaction). The effects of

pre or probiotic administration with respect to immune responses were the same or reduced in the synbiotic diet compared to when used in isolation. The data support the use of *Lactobacillus plantarum* B2984 or lactulose as strategies to contribute to the protection of weaned piglets from zoonotic bacterial pathogens, but caution must be taken when combining dietary supplements as combinations can interact.

Keywords: Prebiotic, Probiotic, Synbiotic, Immune response, *Salmonella*.

1

[1] **Abbreviations:** ELISA, Enzyme-Linked Immunosorbent Assay; cfu, Colony Forming Unit; AHVLA, Animal Health and Veterinary Laboratories Agency; PRE, Prebiotic; PRO, Probiotic; SYN, Synbiotic.

Introduction

Salmonellosis causes significant economic losses to the pig and poultry industries. Pigs and chickens are also a significant source of *Salmonella* for humans, usually transmitted through the consumption of *Salmonella* contaminated chicken and pork products (Thorns, 2000; Boyen *et al*., 2008; Prendergast *et al*., 2009). The most frequently isolated serovars from pigs is *S.* Typhimurium both in the United States and Europe. In pigs, infection with *S.* Typhimurium can result in inflammation in the small and large intestine, and diarrhoea, and more rarely lead to sepsis (Meurens *et al*., 2009). However, infections are commonly asymptomatic and self-limiting. Infection predominantly involves colonisation of the small intestine, invasion of enterocytes and M-cells and bacterial dissemination to lymph nodes and other organs, followed by systemic infection (Fedorka-Cray, 1995).

Antibiotic overuse in food production animals is thought to have contributed to the emergence and proliferation of antimicrobial resistance and resulted in a European wide ban in 2006 on the use of antibiotic growth promoters (regulation [EC] no. 1831/2003). This ban has contributed in part to a growing need for alternative control strategies for bacterial

121 pathogens of food producing animals, including *S.*
122 Typhimurium infection of pigs. Possible strategies include
123 vaccination and the use of prebiotics, probiotics and synbiotics.
124 Probiotics are living microorganisms that are fed to animals to
125 colonise the gut environment to encourage a better microbial
126 balance (Fuller, 1989; Bello *et al*., 2001). Probiotics have been
127 shown to stimulate gut mucosal immunity and systemic
128 immunity, increase protection against toxins created by
129 pathogenic bacteria and inhibit the growth and dissemination of
130 pathogenic microorganisms, they can also increase growth and
131 feed intake (Lessard and Brisson, 1987; Bengmark, 1998; Xuan
132 *et al*., 2001; Mappley *et al*., 2012; Guerra-Ordaz *et al*., 2013).
133 The term prebiotic was defined by Gibson and Roberfroid
134 (1995) as "a non-digestible food ingredient that beneficially
135 affects the host by selectively stimulating the favourable
136 growth and activity of one or a limited number of bacteria in
137 the colon and therefore attempt to improve host health". Also,
138 prebiotics are oligosaccharides, one of the most significant
139 natural macromolecules stimulating immune responses against
140 infection (Swanson *et al*., 2002a, b; Patterson and Burkholder,
141 2003; Searle *et al*., 2010; Kim *et al*., 2011). The term synbiotic
142 describes a combination of probiotic and prebiotic approaches
143 (Gibson and Roberfroid, (1995). An early study by Smith and
144 Jones (1963) demonstrated that a diet supplemented with
145 synbiotics could increase antibody levels and lactate

production, and decrease the growth of harmful bacteria in the host. Feeding a synbiotic diet to pigs can enhance growth and decrease diarrhoea or mortality (Kumprecht and Zobac 1998; Krause *et al*. 2010).

Here, we assessed the ability of probiotic, prebiotic and synbiotic feed regimes to modulate the recognition of *S.* Typhimurium by the porcine B-cell immune response.

Material and Methods

Animal challenge study:

The animal procedures were conducted under the jurisdiction of a UK Home Office project licence (Animals Scientific Procedures Act, 1986 that was amended in January 2013 by Directive 2010/63/EU) and all studies were reviewed by the local AHVLA Ethics Review Committee. The studies conformed to the AHVLA standard quality framework (ISO9001). Twenty-four commercial breed (Large white X Landrace) mixed sex piglets with a mean initial weight of 7.98± 0.7 kg were used for the study. Animals were weaned at 4 weeks of age, faecal samples were collected from sows (n = 3) and piglets and tested for the presence of *Salmonella* before the trial commencement. Piglets were randomly divided into four equal groups of six and housed in a bio-containment facility (CLII). Piglets were faecally sampled *per rectum* to confirm freedom from *Salmonella.* Pigs were housed in

separate pens allocated for each treatment group and acclimatised for 1 week. All staff visiting the pigs were required to wear separate dedicated protective clothing before entering the animal pens. Additionally, the control group animals were visited first, prior to other treatment groups (Tchórzewska 2013).

Following acclimatisation, piglets were then fed a supplemented diet. Each pen was equipped with a feeder and water supply from a water tray and from a nipple. Pens, feeders and water trays were cleaned on a daily basis. Pigs were fed commercial un-medicated pelleted pig feed (mainly based on wheat, soya bean, barley and rapeseed meal; Lillico Attlee, Wm. Lillico & Son Ltd), according to their daily requirements (ASU Unit, AHVLA) and water was provided *ad libitum*. Any un-eaten feed was weighed every morning to determine the feed intake. One group (PRE) was fed the prebiotic lactulose at 1% (w/w) of the feed on a daily basis mixed into the feed. A further group (PRO) was fed probiotic *L. plantarum* B2984 (re-suspended in 0.1 M pH 7.2 PBS) which was resuspended in sterile water and mixed with ~150 g of feed for each pig to receive ~1 x 10^{10} cfu/pig/day. The *Lactobacilli* were found to be viable when cultured from the feed and the full dose was received by the pigs. A third group (SYN) was treated with both the prebiotic and probiotic and a final control group (CTR) had no prebiotic or probiotic treatment.

Following 7 days on the above diets, each piglet in the four groups was orally challenged with *S.* Typhimurium SL1344nal^{r} (~1 x 10^{8} cfu in 10ml of 0.1 M pH 7.2 PBS). Approximately 45 minutes prior to the challenge pigs were orally dosed with 10% (w/v) sodium bicarbonate to neutralise the stomach acid (20 ml). The diet regimes were maintained through to ten days post challenge with *Salmonella*.

Single blood samples were taken from each pig on day 5 of acclimatisation (3 days prior to the diet regime application and 10 days prior to challenge with *S.* Typhimurium). Further single blood samples were taken from each pig 10 days after the *Salmonella* challenge. All blood samples were taken using a non-heparinised vacutainer and then incubated at ambient temperature for 2 hours to allow clotting. Samples were centrifuged at 4300 g to collect the sera which was stored at -20°C until analysis.

Antigen preparation

The strains of *S.* Typhimurium 4/74 or *S.* Enteritidis P125109 were used in this study. Bacteria were cultured for 16 hours aerobically in nutrient broth (NB, Oxoid, UK). Culture (5ml) was transferred into 100ml NB, and grown until the OD reached between 0.5-0.8; cells were then pelleted at 2500 *g*, 4°C, for 20 minutes. The bacterial pellet was re-suspended in 5 ml of PBS and sonicated on ice for a total of 5 minutes at 15 x

10 second pulses at amplitude of 37 (Vibra cell; Sonics & Materials Inc- 500 Watt Ultrasonic processor, Model No. VCX 500, USA). Bacterial lysate was stored at -20°C until use.

ELISA

Enzyme linked immunosorbent assay was used to measure the concentration of *S.* Typhimurium-specific IgG, IgM, and IgA antibodies in porcine serum. Maxisorp-ELISA plates (Thermo Scientific™ Nunc, UK) were coated with 100 µl of neat *Salmonella* lysate as antigen and incubated for 16 hours at ambient temperature. The plates were washed three times with phosphate buffer solution (PBS) and then blocked with 3% (w/v) Marvel PBS (400 µl/ well) for 1hr at room temperature. Sera was diluted in 3% (w/v) Marvel PBS and added to the wells. After 1hr at room temperature, the plates were washed 6 times with PBS + 0.05% (v/v) Tween 20 (PBST) and 6 times with PBS. Bound antibody was detected with 100 µl of goat anti-pig IgG (Source BioScience, UK), IgM or IgA (Laboratories, Cambridge, UK) alkaline phosphatase secondary antibody (1:4000). After 1hr, the plates were washed as before and 100 µl of p-Nitrophenyl Phosphate substrate added to each well. Absorbance at 405 nm was read after 1 hour. For each ELISA plate a minimum of 3 wells were coated with *Salmonella* lysate and detected as above but without any primary sera, the mean of this assay background was then

246 subtracted from all readings for that plate before further
247 analysis of the data.
248 When determining an immune response to *S.* Typhimurium,
249 single samples of pre-challenge and post-challenge sera from
250 individual animals were assayed in duplicate (1:1000 dilution)
251 on the same plate and the signals compared.
252 When determining the effects of diet regime on antibody titres
253 against *Salmonella*, the ELISA assays were carried out using a
254 single post-challenge sera sample from each animal (diluted
255 1:4000). All samples from the CTR group and each of the three
256 diet regime groups were analysed in duplicate wells on the
257 same plate. In addition, to determine the effects of the diet
258 regimes on the titres of antibodies that cross-reacted with *S.*
259 Enteritidis, all samples from the CTR, PRE and PRO groups
260 were analysed in duplicate wells on the same plate.
261
262 **Statistical analysis**
263 To determine *Salmonella*-specific antibody response, sera were
264 taken before and after the immune challenge and data
265 compared by paired Students t-test.
266 To assess the immune responses of challenged animals fed
267 different diet regimes antibody responses from post-challenge
268 animals were analyzed as a 2 (probiotic, yes/no) × 2 (prebiotic,
269 yes/no) factorial ANOVA. If any significant interactions were
270 indicated then further univariate post-hoc comparisons

(unpaired Student's *t*-test) of antibody responses between treatment groups were carried out.

For all analyses, significant differences were considered if the P value was < 0.05.

Results

Clinical disease following challenge with *S.* Typhimurium

Pigs in each diet group had very similar feed intakes over the study duration and there was no significant differences in weight gain between the groups: for pigs in the CTR group average feed intake was 6.02 ±0.52 kg per pen/day, for the PRO group 5.95 ±0.70 kg per pen/day, for the PRE group 6.28 ±0.54 kg per pen/day and for the SYN group 6.04 ±0.67 kg per pen/day. For all challenge groups, animals showed mild diarrhoea and pyrexia at 2 days post challenge, that lasted for 3-4 days. Colonisation of the piglets by *S.* Typhimurium SL1344nal^r, as assessed by selective culture of faeces, indicated that the majority of piglets (5 out of 6 piglets) in all experimental groups on day 1 after challenge were colonised. Shedding thereafter was intermittent and sporadic and on average all treatment groups shed lower numbers of *S.* Typhimurium than the control group (data not shown; Tchórzewska 2013).

Immune responses to *S.* Typhimurium

When considering the cohorts of 6 pigs in each diet regime group, all cohorts had a significant immune response to the pathogen for each of IgG, IgM and IgA (Table 1).

Do probiotic and prebiotic diet regimes interact in the immunomodulation of host responses to *S.* Typhimurium infection?

Titres of each specific antibody isotype (IgG, IgM or IgA) that bound to *S.* Typhimurium were measured for sera collected from animals fed the four different diets. The data for probiotic and prebiotic diet regimes were analysed as a 2 (probiotic, yes/no) x 2 (prebiotic, yes/no) factorial ANOVA, showing a highly significant interaction of the two diet regimes when considering the synbiotic group compared to the probiotic or prebiotic groups alone (Table 2). The data showed that when the prebiotic and probiotic treatments were fed together then the mean antibody responses were, in all cases, either equivalent or less than that observed when they were fed in isolation (Figure 1). Indeed, the IgG and IgA responses with the SYN diet were significantly less than the PRO diet alone. The data therefore showed that the prebiotic and probiotic treatments interacted and the effects seen for each dietary treatment when fed in isolation were the same or greater than when they were fed together (Figure 1).

The effects of a probiotic, prebiotic or synbiotic diets on antibody responses to *S.* Typhimurium infection

When considering the effects of the probiotic treatment compared to the control diet (Figure 1), the IgG, IgM and IgA responses of the host to the bacterial infection were enhanced significantly (P values 0.001, <0.001 and 0.039 respectively). Similarly, when considering the effects of the prebiotic treatment (Figure 1), the IgG, IgM and IgA responses of the host to the bacterial infection were again enhanced significantly compared to animals fed the control diet (P values 0.010, 0.004 and 0.046 respectively). With the synbiotic diet (Figure 1), the IgG and IgM responses was significantly enhanced (P=0.046 and 0.009 respectively) but IgA responses were not increased (P=0.737).

Cross recognition of a distinct pathogenic *Salmonella* serovar

Next, we considered whether the enhanced serum antibody responses seen with prebiotic and probiotic diet regimes upon infection with *S.* Typhimurium also resulted in enhanced cross reaction to a related bacterial infection. Sera taken from piglets subjected to the different diet regimes were analysed for their interaction with *S.* Enteritidis lysate. Similar results were obtained for this related *Salmonella enteric* serovar. Considering the probiotic diet, the IgG and IgM responses were

enhanced significantly compared to animals fed the control diet and the effects on IgA also showed a trend for an increased response (Figure 2). For the prebiotic diet, the IgG and IgA binding to the pathogen was significantly enhanced and IgM levels also showed a trend for an increased response (Figure 2).

Discussion

In the current study, we have evaluated the influence of probiotic, prebiotic and synbiotic diets on the generation of antibodies (IgG, IgM and IgA) to *S.* Typhimurium infection in pigs. The results of our report indicate that supplementation of the *L. plantarum* (B2984) strain into the feed of weaned piglets that were challenged orally with *S.* Typhimurium SL1344nal^{r} resulted in significant increases in the levels of IgG antibody compared to the animals fed a control diet. In addition, the total serum IgM and IgA levels against *S.* Typhimurium were also significantly higher for animals fed this probiotic. These significant increases may be due to the *L. plantarum* persisting in the intestinal tract and acting as immune adjuvant to the humoral immune system and therefore stimulating antibody production against *Salmonella* infection. As pigs in all diet groups had reduced shedding of the pathogen compared to the control group, the increase in circulating pathogen-specific antibodies in the probiotic-fed group is unlikely to be due to an increase in pathogen load in these animals.

When considering previous studies on the effects of probiotics in pigs, a recent study reported a similar influence of *Enterococcus faecium* in the total serum IgM and IgA antibodies of pigs challenged with *S.* Typhimurium, but without any influences on serum IgG levels (Szabo *et al*., 2009). However, this study also noted that the *in vivo* colonisation and shedding of the pathogen was increased in the probiotic-fed group leading to speculation that this increase in pathogen load could result in the increased antibody levels. Pollmann *et al.* (2005) reported in their study that pigs fed *E. faecium* showed reduced natural *Chlamydia* infections and a significant decrease in the frequency of enteropathogenic *Escherichia* coli serovars. Scharek *et al*. (2005) also showed that piglets fed *E. faecium* had reduced enteropathogenic bacterial loads but that this may represent a reduced immunological challenge resulting in an observed reduction in epithelial CD8+ lymphocytes and systemic IgG levels. Studies in pigs have also shown that lactic acid bacteria (a mix of *L. acidophilus* strain LAP5 and *L. reuteri* Pg4) can boost immune responses to *S.* Choleraesuis challenge infections and lead to more rapid clearance of the pathogen (Chang *et al*., 2013) and that *E. faecium* can stimulate the systemic antibody response from a trivalent influenza vaccine (Wang *et al.,* 2014). However, in contrast to these studies, Kreuzer and co-workers (2012) found that *E. faecium* had no beneficial effects on

396 piglets following *S.* Typhimurium infection in terms of growth
397 rate, protection from clinical symptoms, *in vivo* dissemination
398 and shedding of the pathogen; they also observed no increase in
399 serum IgG responses to the pathogen although monomeric cell
400 surface bound IgM levels were enhanced in the probiotic
401 group. It is clear therefore that the benefits of probiotic feed to
402 stimulate immunity in pigs is not universally successful but the
403 data presented here details a precise application of this strategy
404 that does indeed promote an improved immune response
405 against pathogenic challenge that is not due to any increase in
406 pathogen load.
407 Our study clearly also indicated that supplementation with
408 lactulose to the feed of weaned piglets that were challenged
409 orally with *S.* Typhimurium showed significant increases in the
410 levels of IgG antibody responses compared to a control diet
411 group. The total serum IgM levels against *S.* Typhimurium
412 were also significantly higher in the prebiotic group compared
413 to the control group animals. Consistent with the current result,
414 Yin *et al*. (2008) observed that dietary supplementation with
415 prebiotic galacto-mannan-oligosaccharide (GMOS) or chitosan
416 oligosaccharide (COS) resulted in significantly increased serum
417 levels of IgG, IgM and IgA antibodies compared to the control
418 group in weaned piglets. Furthermore, dietary supplementation
419 with Mannan-oligosaccharides (MOS) has been shown to
420 enhance antibody levels in poultry (Cetin *et al*., 2005; Woo *et*

421 *al*., 2007). The mechanisms by which prebiotics (including
422 lactulose) affect the immune system are not fully established; it
423 has been proposed that they may have an indirect action
424 through the alteration of autochthonous microbiota of the
425 intestine and possibly the resulting changes in microbial
426 metabolite production (Gourbeyre *et al.,* 2010). Fermentation
427 of dietary fibre results in the production of short chain fatty
428 acids (SFCAs) such as acetate and propionate (Baldwin *et al*.,
429 1970). These two SFCAs are produced by *Lactobacillus* and
430 when rat mesenteric lymphocytes were cultured with acetate
431 and propionate, production of both IFN-γ and IL-10 was
432 increased (Cavaglieri *et al*., 2003). Relatively little is known
433 about the *in vivo* effect of lactulose fermentation on the
434 immune response in pigs. However, one study has shown that
435 IL-6 is increased in the colon of pigs fed fermentable
436 carbohydrates that included lactulose (Pié *et al.,* 2007). In this
437 latter study IL-6 production was correlated with lactic acid
438 concentration but not with the concentration of SCFAs (acetate,
439 propionate and butyrate) in the colon. Thus suggesting that,
440 feeding pigs fermentable carbohydrates, such as lactulose, may
441 increase lactic acid producing bacteria, such as *Lactobacillus*,
442 which may increase IL-6 expression in the pig colon but not via
443 the production of SCFAs. Lactulose feeding has been shown to
444 cause diarrhoea in pigs (Kien *et al.,* 1999) and it is, therefore,
445 possible that the increased IgM and IgG responses associated

446 with prebiotic lactulose in this study may have been linked to a
447 non-beneficial alteration in microbiota composition. However,
448 this is unlikely since pigs were fed a prebiotic diet 1 week prior
449 to challenge and mild diarrohea was only observed after
450 challenge, suggesting that in our study a prebiotic diet did not
451 cause diarrhoea.
452 The term synbiotic describes a combination of probiotic and
453 prebiotic approaches (Gibson and Roberfroid, 1995). Several
454 reports using rodent models have shown that the use of
455 synbiotics can increase humoral and/or secretory antibody
456 levels (Hosono *et al*., 2003; Roller *et al*., 2004; Frece *et al*.,
457 2009). From limited research on the feeding of synbiotics to
458 pigs; evidence indicates this can enhance growth and decrease
459 mortality or diarrhoea (Kumprecht and Zobac 1998; Krause *et*
460 *al.*, 2010). A very recent study also showed that following
461 challenge of pigs with pathogenic *E. coli* (O149:K91:H10),
462 feeding lactulose could improve weight gain and reduce
463 inflammation; feeding *L. plantarum* promoted lactobacilli
464 growth, modulated fermentative activity, reduced inflammation
465 and promoted an improved membrane barrier function. Within
466 this study, the application of a synbiotic diet resulted in the
467 benefits of both diet regimes being present, a so-called
468 complementary synbiotic (Guerra-Ordaz *et al.,* 2014). In the
469 present study, the supplementation of feed with both *L.*
470 *plantarum* (B2984) and lactulose demonstrated that the

prebiotic and probiotic interacted and whilst the humoral immune responses were enhanced in the synbiotic fed animals compared to the controls the magnitude of the immunomodulation was the same or less than when the probiotic or prebiotic were used in isolation. A recent study has reported that supplementation of the diet with lactulose can increase the number of *L. plantarum* in porcine colon digesta. The observed levels were lower than when *L. plantarum* was added directly to the diet, and with the application of a synbiotic feed the levels of *L. plantarum* were not significantly altered from those seen with the probiotic feed alone (Guerra-Ordaz *et al.*, 2014). In addition, the same study demonstrated that both diets alone and in combination all increased the levels of *Lactobacillus spp.* found in the gut and that the synbiotic and probiotic treatments had similar effects. It is, therefore, unlikely in the current study that lactulose within the synbiotic diet decreased the growth of *L. plantarum* or *Lactobacillus spp.*, which may have explained why the synbiotic treatment was associated with lower serum antibody concentrations compared to the probiotic diet. It may be possible that the synbiotic treatment reduced B cell stimulation resulting in lower plasma cell differentiation and antibody production, however such a mechanism is yet to be determined.

Conclusions

Whilst a range of studies have demonstrated the efficacy of prebiotics and probiotics in improving the host responses and clinical outcomes of infections, the data in the literature shows such efficacy is not universal and the outcome of the application of such feed additives to protect hosts from infection, reduce shedding of bacteria and stimulate host immunological responses may well depend on the host genetic background, the feed additive being studied, the dose and feeding regime used, and difference in strains or species of the pathogenic microorganisms used and possibly the environmental conditions and stress levels of the animals (Jin *et al*.,1998; Kreuzer *et al*., 2012). Here, the use of *L. plantarum* (B2984) and lactulose in weaned piglets clearly demonstrated that humoral immune responses against *Salmonella* infection were enhanced by both treatments but that a combination of the treatments lessened their immunomodulatory effects. This data further support the use of lactic acid bacteria and lactulose as strategies to enhance pig immune responses to zoonotic bacterial pathogens. However, the data also suggests caution should be taken when combining dietary supplements as combinations can interact.

Acknowledgements

Funding was provided for IN by the Ministry of Higher Education and Scientific Research, Kurdistan Regional

Government, Iraq. The prebiotic, probiotic and synbiotic studies were funded by the Food Standards Agency, UK.

References

Baldwin, R.L., 1970. Energy metabolism in anaerobes. Am. J. Clin. Nutr. 23, 1508-13.

Bello, F.D., Walter, J., Hertel, C.,Hammes, W.P., 2001. *In vitro* study of prebiotic properties of levan-type exopolysaccharides from *Lactobacill*i and non-digestible carbohydrates using denaturing gradient gel electrophoresis. Syst. Appl. Microbiol. 24, 232-7.

Bengmark, S., 1998. Immunonutrition: role of biosurfactants, fiber, and probiotic bacteria. Nutrition. 14, 585-94.

Boyen, F., Haesebrouch, F., Maes, D., Van Immerseel, F., Ducatelle, R., Pasmans, F., 2008. Non typhoidal Salmonella in pigs: a closer look at epidemiology, pathogenesis and control. Vet. Microbiol. 130, 1-9.

Cavaglieri, C. R., Nishiyama, A., Fernandes, L. C., Curi, R., Miles E. A., Calder P. C., 2003. Differential effects of short-chain fatty acids on proliferation and production of pro- and anti-inflammatory cytokines by cultured lymphocytes. Life Sci. 73, 1683–90.

Cetin, N., Guclu, B. K., Cetin, E., 2005. The effects of probiotic and mannan-oligosaccharide on some

545 haematological and immunological parameters in turkeys. *J*
546 *Vet Med A Physiol* Pathol. Clin. Med. 52, 263-7.
547 Chang, C.-H., Chen, Y.-S., Chiou, M.-T., Su, C.-H., Chen,
548 D.S., Tsai, C.-E, Yu, B., Hsu, Y.-M., 2013. Application of
549 *Scutellariae radix, Gardeniaefructus*, and probiotics to
550 prevent *Salmonella enterica* serovar Choleraesuis infection
551 in swine. Ev. Based. Compl. Alt. Med. 2013, ID 568528.
552 Frece, J., Kos, B., Svetec, I.K., Zgaga, Z., Beganovic, J., Lebos,
553 A., Suskovic, J., 2009. Synbiotic effect of *Lactobacillus*
554 *helveticus* M92 and prebiotics on the intestinal microflora
555 and immune system of mice. J. Dairy Res. 76, 98-104.
556 Fedorka-Cray, P.J., Kelley, L.C., Stable, T.J., Gray, J.T.,
557 Laufer J.A., 1995. Alternate routes of invasion may affect
558 the pathogenesis of *Salmonella Typhimurium* in swine.
559 Infect. Immun. 63, 2658–64
560 Fuller, R., 1989. Probiotics in man and animals. J. Appl.
561 Bacteriol. 66, 365-78.
562 Gibson, G.R., Roberfroid, M.B., 1995. Dietary modulation of
563 the human colonic microbiota: introducing the concept of
564 prebiotics. J. Nutr. 125, 1401-12.
565 Gourbeyre P., Denery, S., Bodineir, M., 2010. Probiotics,
566 prebiotics, and synbiotics: impact on the gut immune system
567 and allergic reactions. J. Leuk. Biol. 89, 685-695.
568 Guerra-Ordaz, A.A., Molist, F., Hermes, R.G., Gómez de
569 Segura, A., Pérez, J.F., Martín-Orúe, S.M., La Ragione,

570 R.M., Woodward, M.J., Tchorzewska, M.A., Collins, J.W.,
571 2013. Effect of inclusion of lactulose and *Lactobacillus*
572 *plantarum* on the intestinal environment and performance of
573 piglets at weaning. Anim. Feed Science and Technol. 185,
574 160-8.
575 Guerra-Ordaz, A.A., Gonzalez-Ortiz, G., La Ragione, R.M.,
576 Woodward, M.J., Collins, J.W., Perez, J.F., Martin-Orue,
577 S.M., 2014. Lactulose and *Lactobacillus plantarum*: a
578 potential complementary synbiotic to control post-weaning
579 colibacillosis in piglets. Appl. Environ. Microbiol.
580 DOI:10.1128/AEM.00770-14.
581 Hosono, A., Ozawa, A., Kato, R., Ohnishi, Y., Nakanishi, Y.,
582 Kimura, T., Nakamura, R., 2003. Dietary fructo-
583 oligosaccharides induce immune-regulation of intestinal IgA
584 secretion by murine Peyer's patch cells. Biosci. Biotechnol.
585 Biochem. 67, 758-64.
586 Jin, L.Z., Ho, Y.W., Abdullah, N., Jalaludin, S., 1998. Growth
587 performance, intestinal microbial populations, and serum
588 cholesterol of broilers fed diets containing *Lactobacillus*
589 cultures. Poult. Sci. 77, 1259-65.
590 Kien, C.L., Murray, R.D., Qualman, S.J., Marcon, M., 1999.
591 Lactulose feeding in piglets: a model for persistent diarrhea
592 and colitis induced by severe sugar malabsorption. Dig. Dis.
593 Sci. 44, 1476-84.

Kim, G.B., Seo, Y.M., Kim, C.H., Paik, I.K., 2011. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult. Sci. 90, 75-82.

Krause, D.O., Bhandari, S.K., House, J.D and Nyachoti, C.M., 2010. Response of Nursery Pigs to a Synbiotic Preparation of Starch and an Anti-*Escherichia coli* K88 Probiotic. Appl. Environ. Microbiol. 76, 8192.

Kreuzer, S., Janczyk, P., Aßmus, J., Schmidt, M.F.G., Brockman G.A., Nöckler, K., 2012. No beneficial effects evident for *Enterococcus faecium* NCIMB 10415 in weaned pigs infected with *Salmonella enterica* servoar Typhimurium DT104. Appl. Environ. Microbiol. 78, 4816-25.

Kumprecht, I.A., Zobac, P., 1998. Study of the effect of a combined preparation containing *Enterococcus faecium* M-74 and mannan-oligosaccharides in diets for weanling piglets. Czech. J. Anim. Sci. 43, 477-81.

Lessard, M.A., Brisson G.J., 1987. Effect of a *Lactobacillus* fermentation product on growth, immune response and fecal enzyme activity in weaned pigs. Can. J. Anim. Sci. 67, 509-16.

Mappley L.J., Tchórzewska M.A., Nunez A., Woodward M.J., Bramley P.M., La Ragione R.M., 2012. Oral treatment of chickens with *Lactobacillus reuteri* LM1 reduces

Brachyspirapilosicoli-induced pathology. J. Med. Microbiol. 62, 287-296

Meurens, F., Berri, M., Auray, G., Melo, S., Levast, B., Virlogeux-Payant, I., Chevaleyre, C., Gerdts, V., Salmon, H., 2009. Early immune response following *Salmonella enterica* subspecies *enterica* serovar Typhimurium infection in porcine jejunal gut loops. Vet. Res. 40:5.

Patterson, J.A., Burkholder, K.M., 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82, 627-31.

Pié, S., Awati, A., Vida, S., Falluel, I., Williams, B.A., Oswald I.P., 2007. Effects of added fermentable carbohydrates in the diet on intestinal proinflammatory cytokine-specific mRNA content in weaning piglets. J. Anim. Sci. 85, 673-83.

Pollmann, M., Nordhoff, M., Pospischil, A., Tedin, K., Wieler, L.H., 2005. Effects of a probiotic strain of *Enterococcus faecium* on the rate of natural chlamydia infection in swine. Infect. Immun. 73, 4346-53.

Prendergast, D.M., Duggan, S.J., Gonzales-Barron, U., Fanning, S., Butler, F., Cormican, M., Duffy, G., 2009. Prevalence, numbers and characteristics of *Salmonella* spp. On Irish retail pork. Int. J. Food Microbiol. 131, 233-9.

Roller, M., Rechkemmer, G., Watzl, B., 2004. Prebiotic inulin enriched with oligo-fructose in combination with the probiotics *Lactobacillus rhamnosus* and

644 *Bifidobacteriumlactis* modulates intestinal immune
645 functions in rats. J. Nutr. 134, 153-6.
646 Scharek, L., Guth, J., Reiter, K., Weyrauch, K.D., Taras, D.,
647 Schwerk, P., Schierack, P., Schmidt, M.F., Wieler, L.H.,
648 Tedin, K., 2005. Influence of a probiotic *Enterococcus*
649 *faecium* strain on development of the immune system of
650 sows and piglets. Vet. Immunol. Immunopathol. 105, 151-
651 61.
652 Searle L.E.J., Cooley W.A., Jones G., Nunez A., Crudgington
653 B., Weyer U., Dugdale A.H., Tzortzis G., Collins J.W.,
654 Woodward M.J., La Ragione R.M., 2010. Purified galacto-
655 oligosaccharide, derived from a mixture produced by the
656 enzymic activity of *Bifidobacterium bifidum*, reduces
657 *Salmonella enterica* serovar Typhimurium adhesion and
658 invasion *in vitro* and *in vivo*. J. Med. Microbiol. 59, 1428-
659 39.
660 Smith, H.W., Jones, J.E., 1963. Observations on the alimentary
661 tract and its bacterial flora in healthy and diseased pigs. J.
662 Pathol. Bacteriol. 86, 387-412.
663 Swanson, K.S., Grieshop, C.M., Flickinger, E.A., Bauer, L.L.,
664 Healy, H.P., Dawson, K.A., Merchen, N.R., Fahey Jr., G.C.,
665 2002a. Supplemental fructo-oligosaccharides and mannan-
666 oligosaccharides influence immune function, ileal and total
667 tract nutrient digestibilities, microbial populations and

668 concentrations of protein catabolites in the large bowel of
669 dogs. J. Nutr. 132, 980-9.
670 Swanson, K.S., Grieshop, C.M., Flickinger, E.A., Merchen,
671 N.R., Fahey Jr., G.C., 2002b. Effects of supplemental
672 fructo-oligosaccharides and mannan-oligosaccharides on
673 colonic microbial populations, immune function and fecal
674 odor components in the canine. J. Nutr. 132, 1717S-9S.
675 Szabo, I., Wieler, L.H., Tedin, K., Scharek-Tedin, L., Taras, D.,
676 Hensel, A., Appel, B., Nockler, K., 2009. Influence of a
677 probiotic strain of *Enterococcus faecium* on *Salmonella*
678 *enterica* serovar Typhimurium DT104 infection in a porcine
679 animal infection model. Appl. Environ. Microbiol. 75, 2621-
680 8.
681 Tchórzewska, M.A., 2013. An *in vitro* and *in vivo* assessment
682 of *Lactobacillus plantarum* and lactulose as an intervention
683 strategy against *S.* Typhimurium in pigs. Ph.D., Royal
684 Holloway, University of London.
685 Thorns, C.J., 2000. Bacterial food-borne zoonoses. Rev. Sci.
686 Tech. 19:226-39.
687 Wang, Z., Burwinkel, M., Chai, W., Lange, E., Blohm, U.,
688 Breithaupt, A., Hoffman, B., Twardziok, S., Rieger, J.,
689 Janczyk, P., Pieper, R., Osterrieder, N., 2014. Dietary
690 *Enterococcus faecium* NCIMB 10415 and zinc oxide
691 stimulate immune reactions to trivalent influenza

692 vaccination in pigs but do not affect virological response
693 upon challenge infection. PLoS ONE 9(1): e87007.
694 Woo, K.C., Kim, C.H., Paik, I.K., 2007. Effects of
695 supplementary immune modulators (MOS, Lectin) and
696 organic acid mixture (organic acid F, organic acid G) on the
697 performance, profile of leukocytes and erythrocytes, small
698 intestinal microflora and immune response in laying hens.
699 Kor. J. Anim. Sci. Technol. 49, 481-90.
700 Xuan, Z.N., Kim, J.D, Heo, K.N., Jung, H.J., Lee, J.H., Han,
701 Y.K., Kim, Y.Y., Han, In. K., 2001. Study on the
702 development of a probiotics complex for weaned pigs.
703 Asian-Aust. J. Anim. Sci. 14, 1425-8.
704 Yin, Y.L., Tang, Z.R., Sun, Z.H., Liu, Z.Q., Li, T.J., Huang,
705 R.L., Ruan, Z., Deng, Z.Y., Gao, B., Chen, L.X., 2008.
706 Effect of galacto-mannan-oligosaccharides or chitosan
707 supplementation on cytoimmunity and humoral immunity in
708 early-weaned piglets. Asian-Aust. J. Anim. Sci. 21, 723-31.
709
710

711

712 **Table 1:** Immune responses in pigs to *S.* Typhimurium

713 infection measured by ELISA. OD 405nm are shown.

	IgG response[a]			IgM response[a]			IgA response[a]		
	pre	post	Differences of means	pre	post	Differences of means	pre	post	Differences of means
Control diet	0.30	0.72	0.43±0.08	0.23	0.76	0.53±0.22	0.17	0.29	0.12±0.05
Probiotic treatment	0.46	1.23	0.77±0.27	0.14	1.02	0.88±0.27	0.16	0.35	0.19±0.08
Prebiotic treatment	0.38	0.96	0.58±0.21	0.12	0.87	0.75±0.08	0.16	0.41	0.25±0.06
Synbiotic treatment	0.34	1.03	0.69±0.09	0.19	1.09	0.91±0.07	0.15	0.24	0.09±0.02

714 [a]A single sera sample (1:1000) from each animal was assayed

715 in duplicate. Data are presented as average OD readings before

716 and after challenge together with the mean effect size +/-

717 standard error of the differences between means (SED).

718 Assuming a t-distribution, with 5 degrees of freedom in a

719 paired analysis then the 95% CI for the mean effect size may be

720 estimated as 2*SED.

721

722

723

724

725 **Table 2:** Immune responses in pigs fed different diets to *S.*

726 Typhimurium infection measured by ELISA.

Antibody	Probiotic[a]	Prebiotic[a]		P-values[b]			
		-ve	+ve	SEM	Probiotic	Prebiotic	Interaction
IgG	-ve	0.41	0.70	0.09	0.01	0.61	<0.001
	+ve	0.84	0.61				
IgM	-ve	0.41	0.73	0.09	0.009	0.134	0.004
	+ve	0.82	0.70				
IgA	-ve	0.13	0.21	0.05	0.223	0.355	0.004
	+ve	0.30	0.14				

727

728 [a]OD 405nm are shown ±s.e.m.

729 [b]P-values were determined with a 2 (probiotic, yes/no) × 2

730 (prebiotic, yes/no) factorial ANOVA analysis. SEM, standard

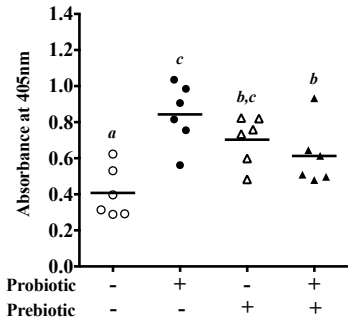
731 error of the mean.

732

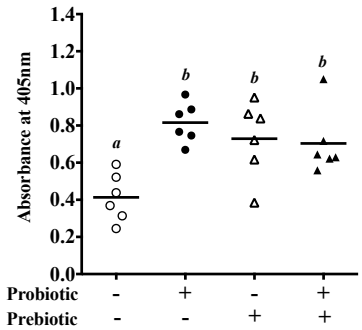
733

734

A



B



C

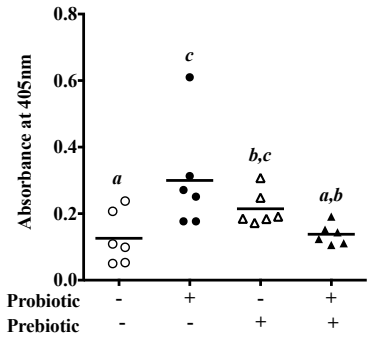
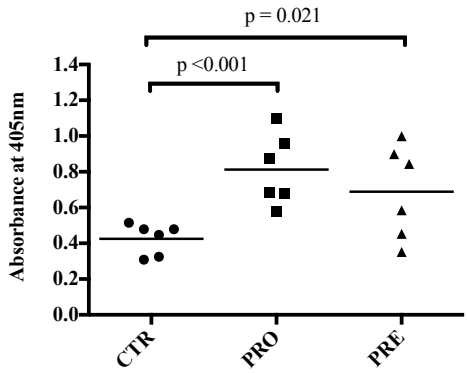


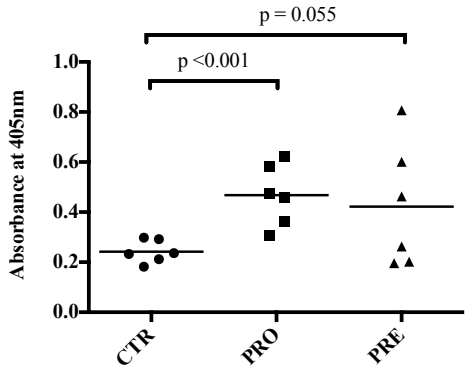
Figure 1: Comparison of IgG (A), IgM (B) and IgA (C) responses in pigs to *S.* Typhimurium infection. Animals were kept on a probiotic, prebiotic or synbiotic diet or had no feed additives (control) and orally challenged with *S.* Typhimurium. Sera was taken 10 days after pathogen challenge and analysed by ELISA against *S.* Typhimurium lysate. Each point represents one serum sample and the horizontal line in each

748 group represents the mean. Antibody levels with all four diet
749 regimes were compared by a factorial ANOVA analysis
750 showing a highly significant interaction of the pro and pre
751 treatments within the synbiotic diet ($P<0.005$). Differing letters
752 above data indicate statistically significant differences ($P<0.05$;
753 ANOVA with individual post hoc comparisons) between
754 treatment groups.
755

A



B



C

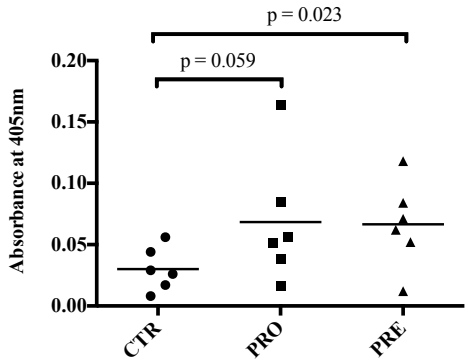


Figure 2: Comparison of IgG (A), IgM (B) and IgA (C) responses in pigs to *S.* Typhimurium infection that cross-react with *S.* Enteritidis. Animals were kept on a probiotic (PRO) or prebiotic (PRE) diet or had no feed additives (CTR) and all

767 animals were orally challenged with *S.* Typhimurium. Sera was
768 taken after pathogen challenge and analysed in ELISA against
769 *S.* Enteritidis lysate. Binding of IgG, IgM and IgA antibody
770 was detected. The immune responses for animals in each of the
771 pre and probiotic diet regimes were compared to the control
772 group responses: statistical analysis was performed using a
773 one-tailed unpaired Student's *t*-test and P values are shown.
774 Each point represents one serum sample and the horizontal line
775 represents the mean.