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

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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*.

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[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

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

with prebiotic lactulose in this study may have been linked to a non-beneficial alteration in microbiota composition. However, this is unlikely since pigs were fed a prebiotic diet 1 week prior to challenge and mild diarrohea was only observed after challenge, suggesting that in our study a prebiotic diet did not cause diarrhoea.

The term synbiotic describes a combination of probiotic and prebiotic approaches (Gibson and Roberfroid, 1995). Several reports using rodent models have shown that the use of synbiotics can increase humoral and/or secretory antibody levels (Hosono *et al*., 2003; Roller *et al*., 2004; Frece *et al*., 2009). From limited research on the feeding of synbiotics to pigs; evidence indicates this can enhance growth and decrease mortality or diarrhoea (Kumprecht and Zobac 1998; Krause *et al.*, 2010). A very recent study also showed that following challenge of pigs with pathogenic *E. coli* (O149:K91:H10), feeding lactulose could improve weight gain and reduce inflammation; feeding *L. plantarum* promoted lactobacilli growth, modulated fermentative activity, reduced inflammation and promoted an improved membrane barrier function. Within this study, the application of a synbiotic diet resulted in the benefits of both diet regimes being present, a so-called complementary synbiotic (Guerra-Ordaz *et al.,* 2014). In the present study, the supplementation of feed with both *L. 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.

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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.

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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.

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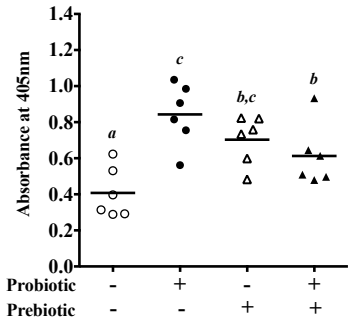
A



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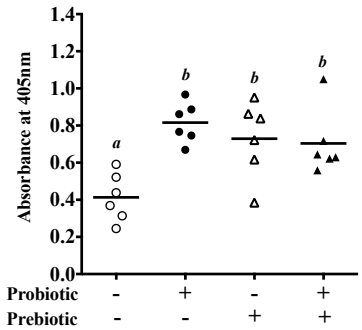

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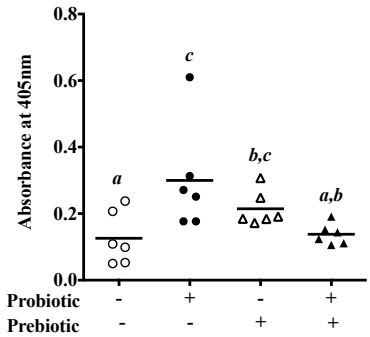
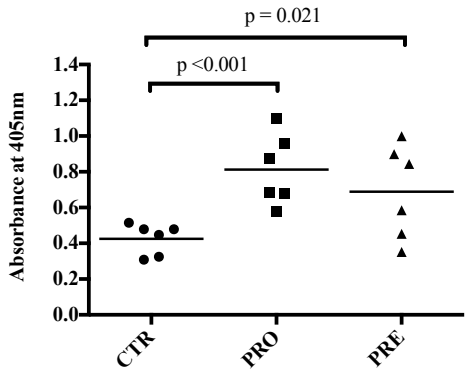


Figure 1: Comparison of IgG (A), IgM (B) and IgA (C) responses in pigs to *S.* Typhimurium infection. Animals were kept on a probiotic, prebiotic or synbiotic diet or had no feed additives (control) and orally challenged with *S.* Typhimurium. Sera was taken 10 days after pathogen challenge and analysed 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



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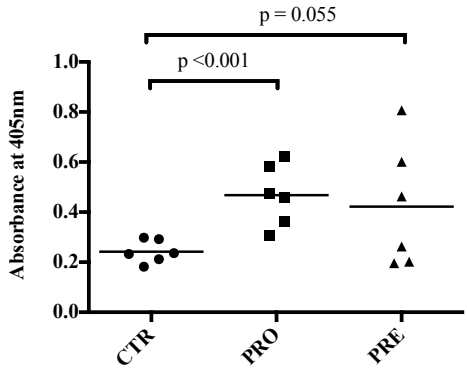

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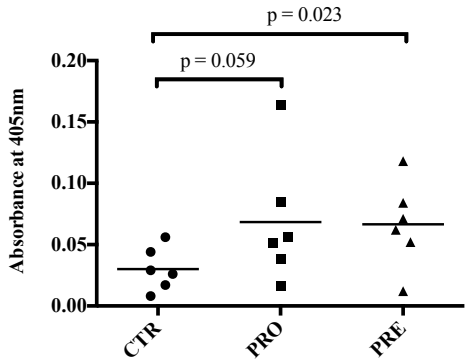

Figure 2: Comparison of IgG (A), IgM (B) and IgA (C) responses in pigs to *S.* Typhimurium infection that cross-react with *S.* Enteritidis. Animals were kept on a probiotic (PRO) or prebiotic (PRE) diet or had no feed additives (CTR) and all

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.