USE OF AN INHIBIN VACCINE TO INCREASE LITTER SIZE IN PIGS

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Final Report prepared for the Australasian Pork Research Institute Limited (APRIL)

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Executive Summary

Introduction

The average litter size (Born Alive) in Australia is 13.2 pigs per litter, which is considerably lower than the US (14.99) and Denmark (18.20). The restricted importation of genetic material in Australia limits the potential for improving this figure. Inhibin, a TGF-beta hormone, regulates follicle-stimulating hormone (FSH) in female mammals. Studies from the 1980s demonstrated that vaccination against an animal's own inhibin could be used to increase ovulation and fecundity in several species, including mice, sheep, cows, goats, and guinea pigs. Preliminary studies in pigs demonstrated a ~35% increase in ovulation and *in utero* development rates using a fusion protein containing residues of bovine inhibin (Brown *et al.*, 1990). However, this study did not extend to live births, leaving a gap in understanding the vaccine's impact on viable piglet births. This study explores the efficacy of an inhibin vaccine, a method shown to increase litter size in other species, in enhancing litter size in gilts.

Methodology

This study was conducted at SAHMRI Pre-clinical, Imaging, and Research Laboratories (PIRL) in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). The trial was approved by the SAHMRI animal ethics committee (ID: SAM-22-088) and involved 44 gilts of 24 weeks of age, divided into two groups: a control group receiving a phosphate-buffered saline solution, and an experimental group receiving an anti-inhibin vaccine. The animals were housed in separate pens under biocontainment conditions and received two doses of their respective treatments at 24 and 28 weeks of age (Day 0 and 28) prior to mating. Gestational weight gain and reproductive performance were monitored from first vaccination, (day 0) through mating, gestation until 6 days after weaning, and blood samples collected to assess the antibody response to inhibin vaccination.

Production outcomes

The data revealed no significant difference in total born and born alive numbers between the control and inhibin-treated animals. However, within those sows who had one or more stillborn piglet the control group exhibited a higher tendency for stillborn piglets (P = 0.055) and a significantly greater number of mummified foetuses (P=0.03).

Serology

The study analysed serum samples from pigs for antibodies to inhibin using two ELISA assays; one with a bacterial expressed antigen and the other with an inhibin peptide.

Bacterial Expressed Antigen ELISA:

- Both immunized and non-immunized pigs had detectable antibodies throughout the experiment, peaking at Day 50 (last day of Regumate) for the immunized group and Day 140 (~87 days gestation)for the control group.
- Immunized pigs showed significantly higher antibody levels at time points; day 28 (second vaccination), day 50 (pre-mating), day 80 (pregnancy confirmation), day 110 (midway through gestation), day 140 (late gestation), day 162 (farrow crate entry) suggesting a specific immune response.
- Unexpectedly, the control group also showed increasing antibody levels, indicating possible exposure to similar antigens.

Inhibin Peptide ELISA:

- Minimal evidence of peptide-specific antibodies was found, with no significant differences between groups.
- There was a large difference in the scale of antibody levels detected in the two assays.

Western Blot:

- The control and immunised pigs show evidence of reactivity to the inhibin preparation at 24 weeks of age (Day 0).
- Pre-mating (Day 50) the immunised pigs had high levels of reactivity.
- This reactivity is likely to be due to bacterial components that have co-purified with the inhibin.

Outcomes

The inhibin vaccination did not elicit the expected response of an increase in the total number of piglets born. The analysis performed to identify the antibody response to inhibin vaccination displayed contradictory results. The disparity between results from the two assays is likely due to the presence of *E. coli* proteins in the bacterial expressed antigen, which elicited an immune response. This cross-reactivity within the ELISA suggested that the response observed is unlikely to be a response to inhibin specifically, but more a response to the *E. coli* proteins present. As a result, the peptide ELISA was performed to validate the response as cross reactivity with *E. coli* is not possible in this assay, the assay being less sensitive but more specific to inhibin. No response was observed in the peptide ELISA results, suggesting that the vaccine did not provide the expected response. It is possible that the mammalian immune system is less responsive to self-antigens like inhibin compared to bacterial components.

Additionally, the study's validation was limited by the absence of a positive control antibody for porcine inhibin. Future studies should use antibodies specific to porcine inhibin for better validation and consider using different antigen expression systems to avoid bacterial contamination.

This executive summary provides an overview of the methodology and findings of the study, highlighting the potential and challenges of using an inhibin vaccine to increase litter sizes in pigs. Although the vaccine was unsuccessful in increasing litter size within the current study, the findings provide insight and knowledge into how to best refine the vaccine development in order to achieve the desired outcome in future studies.

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1. Introduction

Increases in litter sizes have remained modest within Australia since the 1980's compared with other countries (R, Campbell, 8/11/2024), likely due to the closure of the Australian pig herd at this time, restricting the importation of genetic material (Watson *et al.*, 2018). The current average total born in Australia is 13.2 pigs per litter (R, Campbell, personal communication, 8/11/2024), while the United States of America has an average of 14.99 pigs per litter (PigCHAMP Benchmarking 2020) and Denmark an average of 18.20 pigs per litter (Schild *et al.* 2020). The ability to increase the number of piglets per sow per year is attractive to industry as it allows for a greater opportunity to offset costs, improving farm productivity and profitability. It is clear that in order to make improvements in this area and stay competitive, solutions using existing germplasm/genetics need to be evaluated. One such method with demonstrated success for increasing litter size in other species, is vaccination against inhibin.

Inhibin is a TGF beta hormone that in female mammals regulates the production of follicle stimulating hormone (FSH). Studies in the 1980s established that the regulative properties of inhibin could be used, suggesting that vaccination against naturally produced inhibin may increase ovulation rate and therefore fecundity. This has been established in mice, sheep, cows, goats and guinea pigs and other mammals to some degree by increased ovulation rate and in some cases increases in live births (Takedomi *et al.* 2005; Ma *et al.* 2021; Walton *et al.* 2022). Preliminary studies in cross bred gilts reported a ~35% increase in both ovulation rate (Brown *et al.*, 1990) and a significant increase in embryo numbers (44%) 48 hours after insemination in gilts vaccinated with inhibin (Jia *et al.*, 2020). Wheaton *et al.* (1997) administered an inhibin vaccine to the sows the day before piglets were weaned (at 21 days old) and observed an increase in FSH in these sows but no increase in litter size in subsequent litters . More recently, Guo *et al.* (2020) observed a tendency to increase the total number of piglets born and a significant increase in the number of piglets born alive (7.7%) in the following litter when inhibin vaccination was paired with human chorionic gonadotropin and given to sows 7 days prior to weaning. However, these studies did not assess the effect of inhibin vaccination on the reproductive potential of gilts and only one investigated its effect on live born animals.

Therefore, there is a gap in the knowledge regarding the translation of the efficacy of vaccination against inhibin alone in either gilts or sows and its relation to birth of viable piglets.

Despite the increase in knowledge and success in vaccine pilot studies there remains no commercially available inhibin vaccine as a fecundity agent for sows. There is growing demand for methods to increase litter size as the global pig population average steadily increases and the drive for greater reproductive output and thus reproductive efficiency increases. Therefore, this project aimed to test the efficacy of a developed inhibin vaccine on the fecundity of gilts through to farrowing.

2. Methodology

2.1. Animal Component

2.1.1. Animals, experimental design, housing

This study was conducted at SAHMRI Pre-clinical, Imaging and Research Laboratories (PIRL), in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). The trial was approved by the SAHMRI animal ethics committee (ID: SAM-22-088).

A total of 44 gilts were selected and examined over four replicates, farrowing between November 2023 and February 2024.

Two experimental treatment groups were examined:

1. Control: Phosphate Buffered Saline (PBS) vehicle control solution.
2. Anti-inhibin: Porcine inhibin in PBS, expressed in and purified from *Escherichia coli* (500 µ inhibin emulsified with Montanide ISA 2001VG at a 50:50 ratio based on weight).

Animals were transported from a commercial South Australian piggery to SAHMRI-PIRL at 22 weeks of age (~107 ± 2.5 kg) and underwent a two-week acclimatisation period. On arrival, they were ear tagged for individual identification and randomly allocated to one of the two treatment groups described above.

Gilts were housed in group pens of 2-3 per pen from entry, during mating, and throughout gestation. The pens had concrete flooring, and fresh straw was provided twice weekly. They were fed 2.5 kg commercial dry sow diet (Laucke Mills Feed Mill) daily and provided with *ad libitum* access to water. Due to the nature of the anti-inhibin vaccine, the pigs receiving this treatment were housed under Biocontainment Level 1 (BC-1) conditions (DAFF permit approval #2022/091) and deemed quarantine; therefore, the two groups of animals were housed in separate buildings onsite.

All animals received two doses of their respective treatment, at 24 and 28 weeks of age, via intramuscular injection (1 mL each). Additionally at 24 weeks of age, puberty was stimulated via daily fenceline boar contact in small groups for three days. Four days after their second vaccine, all gilts were orally dosed with 5 mL altrenogest (Regumate® Porcine, MSD Animal Health, NSW, Australia) daily for 19 days. At cessation of altrenogest, gilts were 31 weeks of age and mated using conventional artificial insemination, with fence line boar contact. Pregnancy was confirmed via abdominal ultrasound 28 days post-mating. Any non-pregnant animals were removed from the trial, their ovaries collected and the number of corpus albicans (CA), corpora lutea (CL), follicles and dominant follicles (>4 mm) were counted (n = 7).

Gilts were moved into farrowing crates 8.9 ± 0.3 days prior to their expected parturition date. Each crate was on raised slatted flooring with a feeder and nipple drinker. Room temperature was maintained at 18-24°C, and creep areas were set up with heat lamps to provide warmth for piglets. A commercial lactation sow diet (Laucke Mills Feed Mill) was provided at a level of 2.5 kg daily from crate entry until farrowing and then increased with sow appetite up to 8 kg daily during lactation.

Farrowing was monitored between 0800 and 1600 h (Overall farrow rate: 83%). The total number of piglets born, born alive, stillborn and mummified foetuses was recorded for all gilts. All piglets born alive were ear tagged for individual identification and weighed at 1, 3 and 18 days old. Fostering was performed within 3 days of birth and done within treatment group, where the number of piglets exceeded the number of functional teats. Piglets received an injection of iron at 1 day old and their needle teeth were clipped. Any piglet mortalities between birth and weaning were recorded and date/cause noted. Piglets were weaned from the sow at 18.8 ± 0.1 days. Control progeny were sold to a production farm and anti-inhibin progeny were euthanised at weaning using an intramuscular sedative followed by an anaesthetic, with an intracardiac injection of a barbiturate overdose. The sedative was xylazine hydrochloride administered at 0.2 mL/piglet (ilium Xylazin-100, Troy Laboratories Pty Ltd, NSW, Australia), the ketamine was administered at 1 mL per piglet as per quarantine requirements (Ceva Animal Health Pty Ltd, NSW, Australia), and the barbiturate was pentobarbitone sodium at 5 mL per piglet (Lethabarb®, Virbac Pty Ltd, NSW, Australia).

Sows in replicate 1 were weaned into group pens and underwent boar exposure on days 4-6 after weaning to assess return to oestrus (n = 10). After this period, anti-inhibin sows were euthanised, per quarantine requirements, and control sows were sent to the abattoir. Ovaries were collected and the number of corpus albicans (CA), corpora lutea (CL), follicles and dominant follicles (>4 mm) were counted. Due to sickness of one of the stimulant boars, sows in the remaining replicates did not undergo boar exposure in the return to oestrus period, sows in replicate 2 were maintained for the 6 days after weaning (n = 10), and sows in replicates three and four were not maintained past weaning (n = 11).

Euthanasia for sows was as follows: xylazine hydrochloride at 2 mg/kg (ilium Xylazil-100, Troy Laboratories Pty Ltd, NSW, Australia) via intramuscular injection, then ketamine at 10 mg/kg (Ceva Animal Health Pty Ltd, NSW, Australia) was administered 10 minutes later to induce anaesthesia. Once anaesthetised, pentobarbitone sodium at 160 mg/kg 40 mL per sow (Lethabarb®, Virbac Pty Ltd, NSW, Australia) was administered via intracardiac injection to euthanise the animals.

2.1.2. *Animal Measurements*

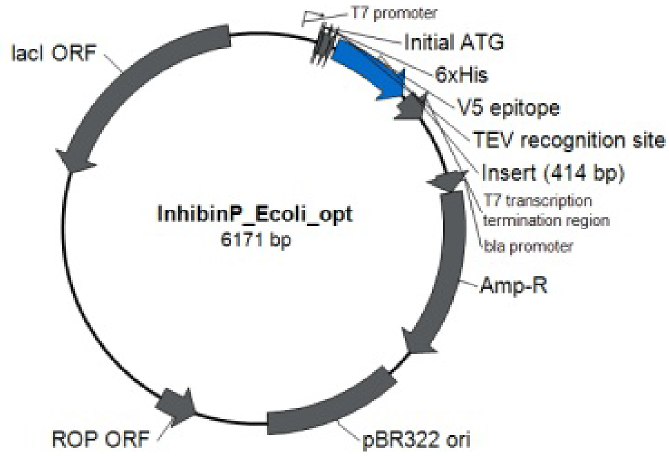
All gilts were weighed, and blood samples collected at 8 timepoints over the trial period: day 0 (first vaccination), day 28 (second vaccination), day 50 (pre-mating), day 80 (pregnancy confirmation), day 110 (midway through gestation), day 140 (late gestation), day 162 (farrow crate entry) and day 190 (weaning). Blood samples were collected via jugular venepuncture using an 18G 1.5" needle and 10 mL clot activator vacutainer tube (BD Vacutainer, BD, Belliver Industrial Estate, Plymouth, UK). The samples were left to clot for 60-90 min at room temperature and then centrifuged at 1300 RCF for 15 min to separate the serum. Serum was collected and divided into three aliquots, then stored at -20°C for later analysis.

2.1.3. Vaccine development *Plasmid Transformation Procedure*

The hypothesis tested in this experiment was that immunisation of pigs with porcine inhibin would stimulate antibodies with higher avidity to porcine inhibin compared to those generated by immunisation with bovine inhibin. Therefore, improving on the results using bovine inhibin as reported by Brown et al. (1990).

The porcine Inhibin A open reading frame (ORF) was synthesised and cloned into the *Escherichia coli* expression vector, pET151/D-TOPO, by the manufacturer (Thermo Fisher Scientific GENEART GmbH) using their proprietary methods. The manufacturing processes were certified to be free of BSE and did not use any animal derived production. The expression plasmid map and sequence of the ORF are shown in Figure 1. The codon usage of the inhibin ORF was modified for optimal expression in *E. coli*.

A



B

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     M  R  R  S  T  A  P  L  P  W  P  W  S  P  A  A  L  R  L  L  Q  R  P
1.   ATGCGTCGTAGCACAGCACCGCTGCCGTGGCCGTGGTCACCAGCAGCACTGCGTCTGCTGCAGCGTCCG
     P  E  E  P  A  V  H  A  D  C  H  R  A  S  L  N  I  S  F  Q  E  L  G
70.  CCTGAAGAACCGGCAGTTCATGCAGATTGTCATCGTGCAAGCCTGAATATTAGCTTTCAAGAATTAGGT
     W  D  R  W  I  V  H  P  P  S  F  I  F  H  Y  C  H  G  G  C  G  L  P
139. TGGGATCGCTGGATTGTTCATCCGCCTAGCTTTATCTTTCATTATTGTCATGGTGGTTGTGGTCTGCCG
     T  L  P  N  L  P  L  S  V  P  G  A  P  P  T  P  V  Q  P  L  L  L  V
208. ACACTGCCGAATCTGCCGCTGAGCGTTCCGGGTGCACCGCCTACACCGGTTCAGCCGCTGCTGTTAGTT
     P  G  A  Q  P  C  C  A  A  L  P  G  T  M  R  S  L  R  V  R  T  T  S
277. CCTGGTGCACAGCCGTGTTGTGCAGCCCTGCCTGGCACCATGCGTAGCCTGCGTGTTCGTACCACCAGT
     D  G  G  Y  S  F  K  Y  E  T  V  P  N  L  L  T  Q  H  C  A  C  I  *
346. GATGGTGGTTATAGCTTCAAATATGAAACCGTTCCGAACCTGCTGACCCAGCATTGTGCATGTATTTAA
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Figure 1. Schematic of the porcine inhibin open reading frame (ORF) used in this study. A) Plasmid map of the pET151/D-TOPO expression vector with the inhibin ORF (blue arrow). Major features of the plasmid are also illustrated. B) The inhibin ORF nucleotide sequence is illustrated with the inhibin amino acid sequence shown above first nucleotide for each codon.

The expression plasmid (5 ng) was used to transform chemically competent OneShot™ *E. coli* strain BL21 Star(DE3) (ThermoFisher Scientific) according to the manufacturer's instructions. The cells were allowed to recover in 1 mL of VLB-Broth Miller (Formedium) at 37°C for 1 hr. Aliquots (100 µL) were plated on VLB agar plates containing 100 µg/mL ampicillin, and then incubated at 37°C for 12-14 hr.

A starter culture was established by transferring a single well isolated colony to 5 mL of VLB-Broth Miller containing 100 µg/mL ampicillin, followed by incubation at 37°C for 12-14 hr with vigorous shaking. An aliquot of the starter culture (500 µL) was used to inoculate 10mL VLB-Broth Miller containing 100 µg/mL ampicillin, followed by incubation at 37°C with vigorous shaking. Aliquots of culture (1 mL) were removed every hour for 6 hr. The aliquot was centrifuged at 10,000 g for 10 min and culture media carefully removed. The bacterial cell pellet and media were stored at -20°C until required.

For large scale expression of Inhibin, an aliquot of a fresh starter culture (1 µL) was used to inoculate 50 mL VLB-Broth Miller containing 100 µg/mL ampicillin, followed by incubation at 37°C with vigorous shaking. The optical density at 600 nm (OD600) was monitored on an hourly basis until it reached approximately 0.5 ± 0.05. Protein expression was induced through the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM. After 4hr the bacterial cells were covered by centrifugation at 10,000 g for 10min and culture media carefully aspirated and discarded. The bacterial cell pellet was stored at -20°C until required.

2.1.4. *Purification of Inhibin*

The bacterial cell pellet was thawed, and the inclusion bodies recovered as described by Cavallaro *et al.* (2011). Following purification, the inclusion bodies were dissolved in SDS-PAGE reducing buffer for analysis on SDS-PAGE as described below.

2.1.5. *Dose formulation*

Following solubilisation in sterile PBS containing 1% Tween20, the Inhibin (200 µg/mL) was formulated with the adjuvant Montanide ISA 201VG (SEPPIC) according to the manufacturer's instructions. Once formulated, each dose delivered 100 µg of Inhibin in a volume of 2 mL.

2.1.6. *Fusion Protein Extraction*

The frozen cell pellet derived from the procedure outlined in section 2.1.3 was thawed on ice for 10 min and resuspended in 50 µL of lysis buffer, then incubated for 1 min at room temperature before a 5-minute centrifugation step at 17,000 × g. The supernatant, which is the soluble cellular fraction, was then transferred into new microcentrifuge tubes for SDS-PAGE analysis. The insoluble cell pellets were resuspended in 50 µL of 2% SDS solution in TRIS HCl at room temperature, centrifuged again, and then placed back on ice. This supernatant was collected as the insoluble cellular fraction for SDS-PAGE evaluation.

2.1.7. *SDS-PAGE*

Samples (10.4 µL) for SDS-PAGE analyses were 4 µL of 4×LDS sample buffer and 1.6 µL of 10× reducing agent and heated at 100°C for 10 min. Insoluble material was pelleted at 12,000 g for 5min and12 µL of solution was loaded onto a Bolt™ Bis-Tris Plus mini protein gel, 4-12%, 1.0 mm, WedgeWell™ (Thermo Fisher Scientific) in the appropriate apparatus as described by the manufacturer. The polypeptides were resolved at 200V for 40 min in MOPS buffer (Thermo Fisher Scientific). At least one SeeBlue™ Plus2 Pre-Stained protein molecular weight standard (10 µL, Thermo Fisher Scientific) was included on each gel to enable molecular weight estimations.

2.2. Serology

2.2.1. Western Blot Analysis

For Western blotting, a Mini-PROTEAN® 3 (BioRad) was used to transfer the protein samples of interest from the SDS-PAGE gel to a nitrocellulose membrane (pre-wetted) 1× transfer buffer containing 20% (v/v) methanol according to the manufacturer's instructions. Post-transfer, the membrane was stained with Ponceau S staining solution and membrane imaged. The stain was removed with multiple water washes, and the membrane blocked with 5% non-fat dry milk in TBS-T20 for 1 h at room temperature on a shaking platform. It was then placed overnight at 4°C in a primary antibody dilution of 1:5000. After three 10-min washes in the Tris buffer saline-Tween 20, the membrane was incubated for 1 h at room temperature in a secondary antibody dilution at 1:5000. Following another three 10-min washes in the buffer, the membrane was incubated with the Pierce™ ECL Plus Western blotting chemiluminescence Substrate for 1 min. The membrane was subsequently imaged using the iBright1500 with varying exposure times.

2.2.2. Enzyme Linked Immunosorbent Assay (ELISA) - Inhibin.

Maxisorb ELISA plates (Nunc) were coated with Inhibin by adding 100 µl of a 1 ng/µL solution in bicarbonate buffer each well of the plate, following overnight incubation at 4°C. Plates were washed twice with PBS-T (PBS, containing 0.1% Tween20. Nonspecific antigen binding was blocked by adding 200 µl of PBS containing 1% w/v BSA, 1% (1 w/v) skim milk powder. Followed by incubation for 1hr at room temperature. After blocking, the plate was washed three times with PBS-T. Serial dilutions of each serum sample in PBS starting at 1:200 were prepared and 100 µL added to the appropriate wells. The plate was incubated for 1 hr, with gentle shaking, at 37°C. The plate was subsequently washed three times with PBS-T. The secondary antibody (Rabbit anti-swine-HRP) was added to each well, 100 µl of a 1:20,000 dilution, and the plate incubated for 1 hr, with gentle shaking, at 37°C. The plate was washed three times with PBS-T. The TMB substrate (100 µL) was added to each well and the plate incubated for 10 min in the dark. The reaction was stopped with 100 µl of 1N HCl and the optical density read at OD450 nm.

2.2.3. Enzyme Linked Immunosorbent Assay (ELISA) - Peptide.
ELISA were also performed as described in Section 2.2.2., except the plates were coated with a peptide, TAPLPWPWSPAALRLLQRPPEEPAVHAD (Auspep Pty Ltd), at 1ng/mL.

2.3. Statistical Analysis

Data were analysed using the statistical software R (https://www.r-project.org/, version 3.6.1). Farrowing factors (Total born, born alive, stillborn, mummified) and wean number were analysed using an ANOVA model (type 3 error), accounting for replicate as a random factor and total born as covariate in born alive, mummified, stillborn and wean number models. Farrowing performance analysis was performed twice, once with all sows included in the model (n = 36) and a second time excluding three outliers (n = 33). Outliers were determined as sitting two or more standard deviations from the mean (sows with 4 (tmt group 1), 5 and 18 (tmt group 0) piglets born in total). Follicle-related factors were not statistically analysed due to the variation in ovary collection days due to abattoir scheduling and small sample size. Statistically significant differences were assessed against a significance level of 0.05.

Data for the serological analyses were analysed using unpaired t-tests with Welch correction, assuming individual variance for each group with a two-stage step-up (Benjamini, Krieger, and Yekutieli) in GraphPad Prism (Version 10.2.2).

3. Results

3.1. Animal data

Mean and standard error of the mean for weight and farrowing data with or without outliers are presented in Tables 1, 2 and 3. Mean gestation length (control: 113.7 ± 0.6, inhibin: 114.1 ± 0.4; *P* = 0.890) and average total placental weight (kg; control: 3.3 ± 0.2, inhibin: 2.8 ± 0.2; *P* = 0.296) did not differ significantly between treatments.

Table 1. Mean ± standard error of the mean for gilt weights (kg) at 7 timepoints for control and inhibin treated animals.

	Day						
Treatment	0	28	50	80	110	140	162
Control	115.6 ± 1.6	134.2 ±1.9	151.3 ± 2.4	163.7 ± 2.1	182.7 ± 2.4	200.6 ± 2.4	210.5 ± 3.2

Inhibin	117.7 ± 1.8	135.5 ± 2.0	145.5 ± 2.1	160.6 ± 2.2	180.6 ± 2.5	196.9 ± 2.3	203.9 ± 3.4

Table 2. Mean ± standard error of the mean for farrowing data for inhibin and control treated animals (n = 36).

	Control	**Inhibin**	***P*-value**
Total Born	12.5 ± 0.8	11.3 ± 0.7	0.253
Born Alive	12.1 ± 0.8	11.0 ± 0.7	0.768
Stillborn	0.5 ± 0.2	0.4 ± 0.1	0.768
Mummified	1.1 ± 0.3	0.3 ± 0.2	0.024
Wean Number	9.9 ± 0.6	9.1 ± 0.3	0.388

Table 3. Mean ± standard error of the mean for farrowing data for inhibin and control treated animals with outliers removed.

	n	Control	Inhibin	*P*-value
Total Born	33	12.7 ± 0.7	11.7 ±0.6	0.289
Born Alive	33	12.1 ± 0.7	11.4 ± 0.6	0.595
Still Born	33	0.5 ± 0.3	0.3 ± 0.1	0.595
Mummified	33	1.2 ± 0.4	0.3 ± 0.2	0.150
Wean number	33	9.9 ± 0.6	9.1 ± 0.4	0.306

[#] Sows who had one or more stillborn piglets. [+] Sows who had one or more mummified piglets.

There were no significant differences between total born and born alive numbers for analysis where all data was included or with outliers removed ($P > 0.05$; Table 2 and 3). The number of sows that gave birth to one or more stillborn piglets was similar across treatment groups (Control = 4, Inhibin = 5: P=0.595).). A greater number of control sows gave birth to mummified foetuses (Control = 9 sows, Inhibin = 3 sows) however this was not significantly different (Table 3; P = 0.150). There was no significant difference between the number of piglets weaned per sow (Tables 2 and 3; $P > 0.05$).

The mean number of corpora albicans, follicles, follicles greater than 4 mm and corpora lutea were counted and are reported in Table 4.

Table 4. Mean number of follicles, follicles > 4mm, corpora lutea and corporal albicans ± standard error of the mean, for sows not pregnant (NP), at weaning and 6 days post weaning (PW) with (boar)or without boar exposure.

Treatment	Stage	n	CAs	Follicles	Follicles >4mm	CL
Control	NP	4	6.2 ± 2.3	33.5 ± 5.9	1.9 ± 0.8	10.1 ± 1.4
	Wean	3	8.5 ± 0.3	43.2 ± 3.1	0	0
	6d PW	5	8.5 ± 1.3	12.0 ± 4.0	1.4 ± 0.6	7.4 ± 1.9
	6d PW boar	5	10.5 ± 2.3	10.9 ± 6.9	2.1 ± 1.7	7.5 ± 1.9
Inhibin	NP	2	11 ± 2.0	25.0 ± 11.5	3.5 ± 0.5	4.8 ± 4.7
	Wean	8	7.6 ± 0.9	50.6 ± 10.2	0.1 ± 0.1	0
	6d PW	5	11.5 ± 0.8	5.9 ± 1.7	0.8 ± 0.6	8.6 ± 1.1
	6d PW boar	5	6.1 ± 1.2	8.5 ± 2.5	6.2 ± 2.5	4.4 ± 1.9

3.2. *Serological analysis*

The serum samples from each of the gilts were analysed for antibodies to inhibin using western blot analysis and two ELISA assays, using either the same bacterial expressed antigen used to immunise the pigs or an inhibin peptide.

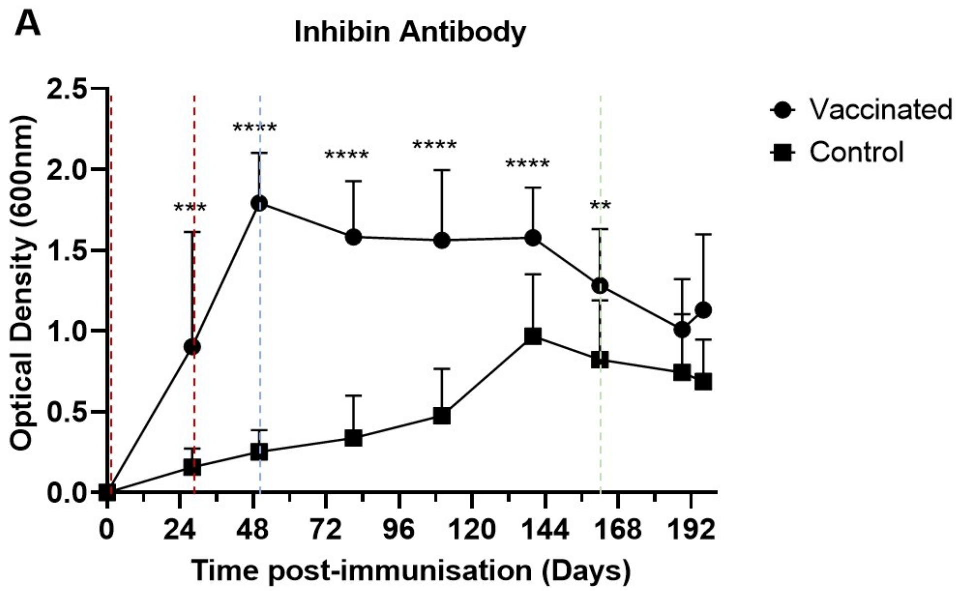
Five animals were excluded from the dataset shown in Figure 2, as no antibody responses were detected in these animals and hence, they were excluded from the analyses. There was no evidence for any antibody responses to either inhibin or the bacterial contaminants that the remaining immunised pigs appeared to react to. This strongly suggests that for reasons that are not apparent, the vaccination failed completely in these animals resulting in no detectable antibody. Including these animals in the analyses would have skewed the results.

For the ELISA using the bacterial expressed antigen, both the inhibin immunised, and the non-immunised gilts had detectable levels of antibody for the duration of the experiment, peaking prior to mating and midway through gestation (Day 50 and Day 140 post first injection), respectively (Figure 2A). Significantly higher levels of antibody were detected on Day 28, Day 50, Day 81, Day 110, Day 140, and Day 162 in the inhibin immunised group (Figure 2A).

The serological data for the immunised pigs suggest a classical response to the levels of antibody peaking prior to mating, at Day 50 following the first immunisation (Figure 2A). The results suggest that the gilts in this group did mount a specific immune response to the immunisation formulation.

However, it was unexpected that the antibody levels in the control group increased from second vaccination until late gestation (Day 0 to Day 140) in a similar way, albeit at significantly lower levels. These results suggest that the pigs in the control group were exposed to an antigen(s) during the course of the experiment.

In contrast the ELISA with the inhibin peptide as the substrate suggests minimal evidence for the presence of peptide-specific antibody and no significant differences were detected between the treatment groups (Figure 2B). Note, there is an order of magnitude difference between the scales on the y-axis of Figure 2A and 2B.



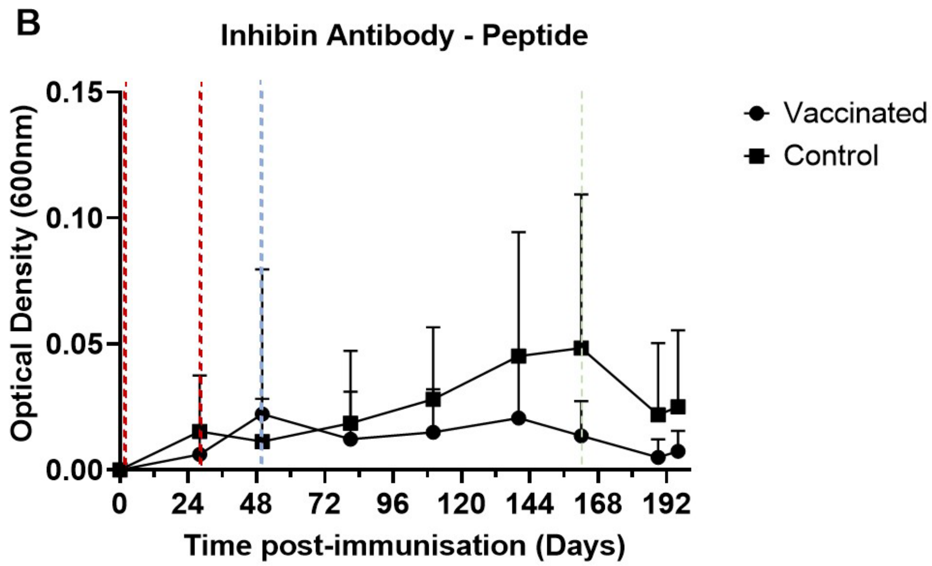


Figure 2. Analysis of the serological responses of pigs immunised with inhibin using ELISA. Panel A: Antibody levels for the immunised pigs (Vaccinated, n = 14, the data for five pigs was removed from the dataset) and control pigs (Control, n = 13) using the bacterial expressed inhibin as the substrate in the ELISA. Panel B: Antibody levels for the immunised (Vaccinated, n =19) and control pigs (Control, n = 13) using an inhibin peptide as the substrate in the ELISA. Data points represent the mean absorbance for each group at each time point. Error bars represent one standard deviation. Asterisks designate timepoints where statistically significant differences were detected between the means of the two groups, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Red dotted line = vaccination administration, blue dotted line = mating and green dotted line = farrowing. Note different scales on the Y-axes of these figures.

To further investigate the serological responses of the sera from control immunised pigs, Western blotting analyses were undertaken. While Western blotting is a more laborious procedure and low throughput assay compared to ELISA, it enables cognitive assessment of background reactivity to be assessed. As shown in Figure 3A and 3C, the control and immunised pigs show evidence of reactivity to the inhibin preparation at Day 0. As discussed for the ELISA results, this reactivity is likely to be due to bacterial components that have co-purified with the inhibin. Similarly, at Day 50, for the immunised pig high levels of reactivity were observed (Figure. 3D). This likely to be a result of the adjuvant eliciting strong immune responses to these bacterial components.

That the reactivity observed on the Western blot was a result of these co-purifying contaminants is further illustrated by Figure 3E and Figure 3F. When the bacterially expressed inhibin was replaced on the Western blot by the synthesised inhibin peptide, no reactivity was observed (Figure 3E). Furthermore, when the Western blot was probed with an antibody specific to the 6His-tag (added to the inhibin to enable purification), the only reactive band was at the expected size for inhibin (Figure 3F). These results also suggest that the immune response in the immunised gilts was to the bacterial contaminants.

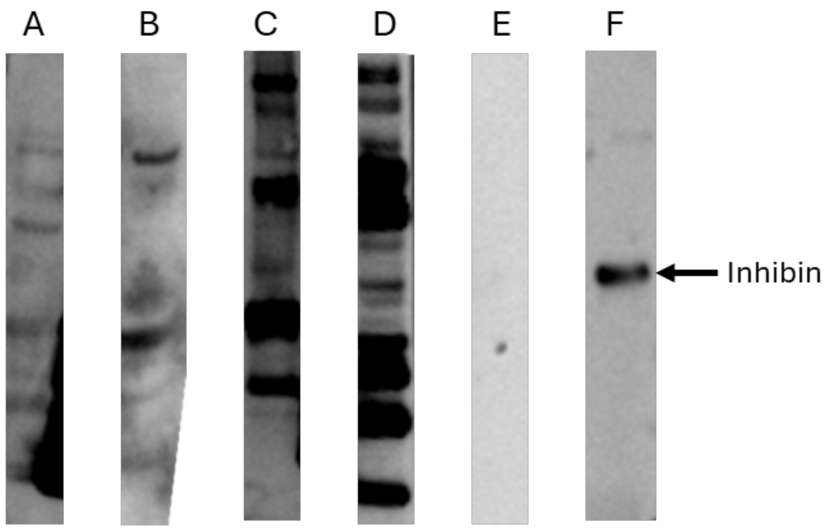


Figure 3. Western blotting analyses of the serological responses of a control gilt and a gilt immunised with the inhibin. Panel A Control pig (PV43), Day 0; Panel B Control pig (PV43), Day 50; Panel C Immunised pig (PV10) Day 0; Panel D Immunised pig (PV10) Day 50; Panel E Immunised pig (PV10) Day 50 with the inhibin peptide blotted to the membrane; Panel F Primary antibody 6His monoclonal replacing pig sera.

4. Outcomes

The hypothesis that vaccination against inhibin would increase the total number of liveborn piglets is not supported within this study, and is likely the result of an inadequate response to vaccination. In order to identify whether vaccination elicited the expected response two ELISAs (inhibin and inhibin peptide) and a western blot were performed. It is difficult to reconcile the results for the inhibin and inhibin-peptide ELISAs. A plausible explanation is that most of the antibodies detected in the inhibin ELISA are not specific to inhibin, rather they are to *E. coli* proteins that have co-purified with the inhibin during the expression and purification processes. While every effort was made to ensure that inhibin was as pure as possible, it is very difficult to reach 100% purity and still have enough antigen for the planned experiments. As a result, it is likely that the inhibin preparation used for the immunisations also contained bacterial proteins that would have also elicited immune responses in the pigs. The results of the western blot analysis further corroborate this. For the control group, it is possible that during the production cycle they were exposed to bacteria that were antigenically similar to the *E. coli* used to express inhibin. Indeed, a similar exposure for the immunised group cannot be ruled out either.

This hypothesis would also help explain why results for the inhibin-peptide ELISA were negative. Bacterial derived contaminants in the inhibin are likely to be highly immunogenic as the mammalian immune system has evolved to detect and respond to such threats. In contrast, the stimulation of immune responses to inhibin requires overcoming the -self-recognition barrier which the mammalian immune is programmed to prevent, to enable robust detection of pathogens and stop the development of autoimmune diseases. Thus, it is likely that the immune systems of the immunised pigs would react more robustly to any bacterial components in the immunisation formulations compared to inhibin.

The overall validation of both ELISAs was hampered by the lack of a positive control antibody for porcine inhibin to clearly define what a positive result should look like in these assays. Any future studies could address this by producing antibodies specific for porcine inhibin in a model species (e.g. rabbits or mice) to enable robust validation of the inhibin ELISAs. It would also be useful to have the inhibin used in the ELISA, expressed in a different system to prevent any co-purification contaminants in the inhibin immunisation formulation confounding the results.

Another potential improvement to the study reported here would be the re-evaluation of the antigen used to immunise the gilts. As discussed above, one of the issues with using porcine inhibin is the need to overcome the barrier posed by -self-recognition by the immune system. The original study of Brown *et al.* (1990) utilised immunisation with bovine inhibin to demonstrate increased follicle formation in pigs. Bovine and porcine inhibin are closely related, sharing greater than 85% amino acid sequence similarity. Thus, when bovine inhibin is used to immunize pigs there is sufficient difference to prevent self-recognition preventing an immune response, while the sequence similarity is high enough to enable antibodies to bovine inhibin to recognise porcine inhibin to produce the outcome reported by Brown *et al.* (1990).

The hypothesis underpinning the current study that antibodies specific to porcine inhibin would give a more robust effect is still valid as those antibodies would have higher specificity and avidity. However, for porcine inhibin to be the immunogen it may need to be presented differently to the pig's immune system to improve its immunogenicity to overcome the self-recognition barrier. One approach would be to use inhibin peptides conjugated to a carrier molecule (e.g., keyhole limpet hemocyanin) to enable the stimulation of strong immune responses. Alternatively, the expression construct for inhibin could be modified to include a molecular adjuvant to improve its immunogenicity.

5. Application of Research

This research provides insights into the potential application of inhibin vaccination in enhancing reproductive efficiency in pigs. While the vaccine did not significantly increase the total number of piglets born, there was also no difference in stillborn and mummified piglets indicating no detrimental effects on litter quality . Serological analysis to assess the vaccines efficacy at establishing an appropriate response suggests that alterations to the vaccine during development are necessary in order to have the desired outcome of increased litter size. This research is important as it provides insight into the changes necessary to reach this desired outcome.

6. Conclusion

While the inhibin vaccine did not significantly increase litter size in this study as was originally hypothesised, the outcomes observed provide foundational knowledge for the ability to improve upon and develop an effective inhibin vaccine for improving fecundity in pigs. Another interesting outcome was inhibin's potential in reducing foetal mortality rates and should be investigated in subsequent studies.

7. Limitations/Risks

The primary limitation to the study was the inability to appropriately validate the ELISAs as there was no positive control antibody for porcine inhibin. Future studies should address this by producing antibodies specific for porcine inhibin in a model species first.

Additionally, the quarantine requirements for the anti-inhibin group meant that animals of both treatments were housed in separate buildings, this may have introduced some variability.

The hypothesis tested in this experiment was that immunisation of pigs with porcine inhibin would stimulate antibodies with higher avidity to porcine inhibin compared to those generated by immunisation with bovine inhibin. However, it was not anticipated that it would be so difficult to overcome the self-self-recognition barrier when using porcine inhibin as the antigen in pigs. In hindsight, the use of bovine inhibin or additional immunisations with porcine inhibin were required to elicit inhibin specific responses. Conjugated inhibin porcine peptides were not used in the current study, as while they may overcome the self-self-recognition barrier, the high costs associated with the production of these types of antigen would only be cost-effective at an experimental scale. Consequently, if conjugated inhibin porcine peptides proved to increase ovulation and conception rates, an alternative antigen would have needed to be developed to move the vaccine through to a commercialisation pipeline.

8. Recommendations

Future research should focus on assessing whether using bovine inhibin or conjugating inhibin peptides to a carrier molecule will enhance immunogenicity to elicit a larger response. Additionally, modifications to the expression construct to include a molecular adjuvant would also be beneficial.

There is a need to explore alternative adjuvants and optimize the vaccine dosage to enhance the vaccine's efficacy. In the first instance it would be recommended to assess the vaccine’s efficacy on the number of follicles and subsequent embryos as Brown *et al.* (1990) did to ensure an appropriate response. Thereafter, larger-scale trials that result in liveborn piglets and assess the long-term impact of inhibin vaccination on pig reproductive performance are needed.

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