Slutrapport

Supplementing piglets with probiotics: assessing effectivity, feasibility and economics

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Part 1: Swedish abstract
Vid kommersiell grisuppfödning finns ett stort behov att hitta lösningar som kan leda till minskad stress och främja hälsa och välbefinnande hos grisar. Forskning som genomförts på gnagare har visat att intag av probiotika tidigt i livet kan skydda mot störningar och påverka en individs sociala beteende och förmåga att hantera stress. Denna studie syftade därför till att undersöka om tillskott av probiotika tidigt i livet påverkade grisens hälsa, tillväxt och stresstolerans. Två studier har genomförts: i den första studien utvärderades om oralt tillskott av två bakteriestammar av arterna, *Lactobacillus reuteri* och *Lactobacillus plantarum,* från tre dagars ålder och tre gånger/vecka fram till avvänjning, påverkade grisens stressrespons i ett stresstest (mätning av cortisol och ACTH före och efter hantering) och i ett novel object test. Detta genomfördes på sju kullar, där 5 grisar/kull fick probiotikatillskott och 5 grisar fick placebo, men tillskottet gav inte utslag på några av de tester som utvärderades. I den andra studien, administrerades samma probiotikastammar, men där utvärderades istället olika sätt att administrera probiotikan; oralt till suggan, (alla vardagar från 1 vecka före och under hela diperioden), oralt till smågrisen vid normal hantering (två tillfällen, dag 1 och 3-4 efter födseln), via sprayning av juvret (alla vardagar under diperioden) eller via torv som distribuerades i smågrishörnan (alla vardagar från födsel fram till två veckor efter avvänjning). Som kontroller inkluderades grisar helt utan probiotikatillskott och grisar som fick tillskott av probiotika via torv, men först efter avvänjning. Totalt inkluderades 58 kullar med totalt 736 smågrisar i studien och de parametrar som utvärderades var tillväxt, foderåtgång, hälsa, tarmflora och beteende. Dessutom gjordes en ekonomisk kalkyl för att jämföra kostnader, tidsåtgång och lönsamhet. Utvärderingen av data visade att de smågrisar som fått probiotika under diperioden hade 10% högre vikt vid 70 dagars ålder jämfört med kontrollgrisarna utan att foderåtgången ökat. Denna effekt syntes inte för den grupp där probiotikan gavs till suggan eller då probiotikan bara administrerades efter avvänjning. Data visade även att probiotikatillskottet påverkade en välfärdsparameter (tårflöde) i positiv riktning, men inga av de övriga utvärderade parametrarna

påvisade några skillnader. Den ekonomiska analysen visade att det orala tillskottet till smågrisen var det mest ekonomiskt gynnsamma administreringssättet. Enligt kalkylen tjänade lantbrukaren i studien 5.71 kr/smågris i denna behandlingsgrupp jämfört med kontrollgruppen som inte fick probiotika och därför växte sämre. Sammantaget så visar studien att probiotikatillskott kan bidra till en ökad tillväxt och en förbättring av vissa välfärdsparametrar om det ges vid tidig ålder men fler studier behövs för att utforska de underliggande mekanismerna.

Part 2: Report

1. Introduction

In nature, the normal exploratory behaviour of a piglet means it is likely to be exposed to a range of different bacteria common in the environment. A normal development of the gut microbiota is therefore almost guaranteed. However, our attempts to protect piglets from disease, by cleaning and disinfecting the pen and even the whole building at regular intervals, may be limiting exposure to the bacteria necessary for a healthy well-functioning gut microbiota. This can have long term consequences for pig health and performance [1, 2]. In particular during stressful periods such as weaning, there is a risk of developing an unbalanced gut-microbiota. These rapid changes in diet and environment combined with high stress can lead to reduced food intake, growth rates, and increased risks of health and welfare problems. With the phasing out of zinc oxide and pressure to reduce the use of antibiotics, there is a need for alternative solutions to promote growth, health and welfare in commercial pig production.

Emerging evidence suggests that the gut microbiota plays key roles in early programming of the stress system (Hypothalamus-pituitary-adrenal- (HPA) axis) [1] and in the development of the immune system [3]. Germ-free raised mice showed exaggerated corticosterone and adrenocorticotrophic hormone (ACTH) responses to a mild restraint stressor compared to control mice [4]. Germ-free mice also showed reduced anxiety-like behaviours [5]. Reconstitution of the gut microbiota normalized the stress response and anxiety-like behaviours when this was done early in life [4, 5]. These findings suggest that there is a critical developmental window where the gut microbiota influences the central nervous system and modulates stress responses as well as behaviour.

One method to promote a healthy gut development is to supplement the diet with living beneficial bacteria that normally inhabit the gut, also called probiotics. Several *Lactobacillus* strains have shown promising effects on parameters related to gut health and behaviour [6]. *Lactobacillus* supplementation reduced anxiety- and depression-like behaviours and stress responses in rats [7], and restored disturbed social behaviour in mice with an unbalanced gut-microbiota [8]. In pigs, it has been shown that probiotics after weaning can promote growth and food intake and reduce diarrhoea [9, 10], even though not all studies have shown consistent results [11]. However, there is little knowledge on how probiotics supplementation affects the behaviour in pigs, or other markers indicative of stress such as tear staining and skin lesions resulting from aggression. Furthermore, there is insufficient knowledge on the effectives of probiotics when given in early in life and whether it is effective under commercial conditions. Finally, it is important to find the administration method that minimizes labour but leads to maximal growth and welfare in commercial farms.

There are three main objective in this study:
I. to investigate how probiotic supplementation influences behavioural and physiological stress responses in piglets under controlled experimental conditions,
II. to determine an optimal route of administration of probiotics under commercial conditions and assess the effects on growth, food intake and general health, and
III. to perform a cost-benefit analysis on the economic feasibility of administering probiotics on pig farms. We hypothesise that probiotic supplementation reduces behavioural and physiological stress responses, and promotes growth, health and welfare.

2. Material and methods

Ethical note: This project was carried out in accordance with the recommended European Guidelines for the accommodation and care of animals as well as the Swedish animal welfare codes. The project was approved by the Animal Research Ethics Committee in Uppsala (DNR. C105416/16, DNR. 5.8.18-01998/2018 and DNR. 5.8.18-01218/2018). No detrimental health effects of probiotics supplementation were observed over the course of this experiment.

2.1. Part I. Concept development: effects of probiotic supplementation on behaviour and stress reactivity

Seven sows and their litters were selected for this experiment. Five piglets in each litter were orally supplemented with *Lactobacillus reuteri* and *Lactobacillus plantarum* (dose 10^8 bacteria/occasion) 3 times a week starting at 3 day of age until weaning, and five piglets per litter were given a placebo.

2.1.1. Assessment of stress reactivity:

Between 40 and 42 days of age, 24 control and 27 probiotics supplemented piglets were placed on their backs and restrained for 5 minutes to induce stress. Blood samples were collected immediately at the start (0 min) and at 15 and 30 min after the stressor. Samples were centrifuged within 1 hr after collection and plasma was stored at -80°C until analysis. Plasma concentrations of the stress hormones cortisol and ACTH were assessed by ELISA essays (DRG instruments, GmbH, Marburg, Germany) that have been validated for pigs [12].

2.1.2. Assessment of fearfulness:

Between 70 and 75 days of age, 19 control piglets and 21 probiotics supplemented piglets' reactions to a novel object that they had not previously seen (beach ball) were recorded (Novel Object Test, NOT). Animals were let into a familiar environment in which a bucket with a known food reward (marshmallow) was placed and when the animal was within approx. 1 m of the food bucket, the beach ball (attached by a rope to the ceiling) was dropped so that it ended up approx. 20 cm above the bucket. The latency to approach the ball, latency to eat the reward and the likelihood to approach the ball first (as opposed to the reward) were assessed as measures of fearfulness.

2.2. Part II. Optimization of probiotics administration on a commercial farm

Part II took place on a commercial farm near Västerås (Tå Gård). Three replicates of sows and their piglets were used between April and July 2019. Selection of the sows was based on the sow being currently healthy and having a litter with more than 10 healthy piglets. In total, 58 sows and 736 individually marked piglets were included in the study.

Sows were randomly assigned to one of six different routes of probiotics administration (a mixture of *L. reuteri* and *L. plantarum*):

- Treatment 1. The probiotics ($5{,}8\times10^9$–$3{,}06\times10^{10}$ cfu/dose) were sprayed directly onto the sow's udder five times a week starting at 1 day of age until weaning (Udder group).
- Treatment 2. The probiotics ($5{,}8\times10^9$–$3{,}06\times10^{10}$ cfu/dose) were mixed five times a week into the clean peat from farrowing until two weeks after weaning (Peat group).
- Treatment 3: The probiotics ($5{,}8\times10^9$–$3{,}06\times10^{10}$ cfu/dose) were mixed five times a week into the clean peat from weaning until two weeks after weaning (Control-peat group).

- Treatment 4. The probiotics ($4{,}8\times10^9$–$5{,}5\times10^{10}$ cfu/dose) were administered orally to each individual piglet at day 1 and again around day 4, when piglets are normally handled for standard farm procedures (Oral group).
- Treatment 5. The probiotics ($9{,}7\times10^9$–$1{,}1\times10^{11}$ cfu/dose) were supplemented orally to the sow during the lactation period (Sow group).
- Treatment 6. No probiotics (Control group)

2.2.1 *Faecal samples*:
Faecal samples from 35 piglets were collected at 49 days of age to assess the impact of the different treatments on the amount of lactobacilli and enterobacteria with the aim to study which of the probiotics supplementation methods were the most effective in minimizing the populations of *Enterobacteriaceae*, that includes opportunistic pathogens such as *E. coli*. The quantification of these bacteria were performed using selective culture based techniques. In addition, we collected rectal swabs from eight piglets/treatment group at 28 and 49 days age to assess the overall influence on the microbiota composition.

2.2.2. *Performance and welfare measures:*
Live weights were assessed at days 15, 36, 43, 49 and 70 days of age, and post-weaning food intake was measured weekly by the automated feeding system. General health (score 0-3), skin lesions (score 0-3), tear staining (score 0-3) and fecal score (0-3) - where zero was the best score and three the worst score - were measured at days 15, 36, 43, 49 of age.

2.2.3. Cost-benefit analysis
Based on the weight and food intake registrations, together with the feed price (in SEK per kg), the feed cost in SEK per kg of growth and per pig was calculated. The feed cost in the different treatments was compared with the control treatment. The time (working hours) taken for the stockperson to administer the probiotics in the different treatments were also recorded.

2.3. Statistical analysis
All data were analysed in R (version 3.6.2) and checked for normally and homoscedasticity assumptions by visual inspection of the residuals. If these assumptions were not met, data were log- or square root- transformed were appropriate. Outliers with residuals more than 2.5 were excluded from the analysis. For part I, mixed models were used to analyse the data using packages lme4, lmertest, and LMERConvenienceFunctions. For the NOT, Probiotics treatment, sex, and their interaction were fitted as fixed effects and pig ID nested within litter as a random effect. The cortisol and ACTH data were first analysed with Probiotics treatment, sex, time, and their interaction were fitted as fixed effects and pig ID nested within litter as a random effect. A second ANOVA analysis was performed on the peak values (highest value for each individual irrespective of time) with treatment and sex as fixed effect and the baseline value at time 0 as a covariate. For the weight gain in part II, the nmle and car packages were used to fit probiotics treatment, sex, age, and their interactions as fixed effects and a continuous autoregressive covariance structure with piglet age and pig ID nested within litter as random effects. The welfare scores were analysed using logistic mixed models using the glmer function with probiotics treatment, time, and their interaction were fitted as fixed effects and pig ID nested within litter as a random effect.

3. Results

3.1. Part I: Concept development: effects of probiotic supplementation on behaviour and stress reactivity

3.1.1. Novel object test

The probiotics group was slightly more likely to approach the ball first, rather than the reward (probiotics group 0.73 ± 0.1 and control group 0.53 ± 0.1), but this difference was not statistically significant. The males were quicker to approach the novel object than the females ($P < 0.001$), but there was no effect of treatment on latency to approach the object (probiotics group 10.9 ± 2.5 s and control group 10.25 ± 1.9 s). The latency to eat the reward was not affected by treatment or sex (probiotics 17.1 ± 3.6 s and control 25.1 ± 4.6 s)

3.1.2. Stress test

The plasma cortisol responses to restraint were not affected by treatment or sex, but changed significantly over time ($P < 0.001$). In the second analysis, the peak concentrations tended to be higher in the probiotics group ($P<0.1$), while baseline concentrations were not different (Fig 2).

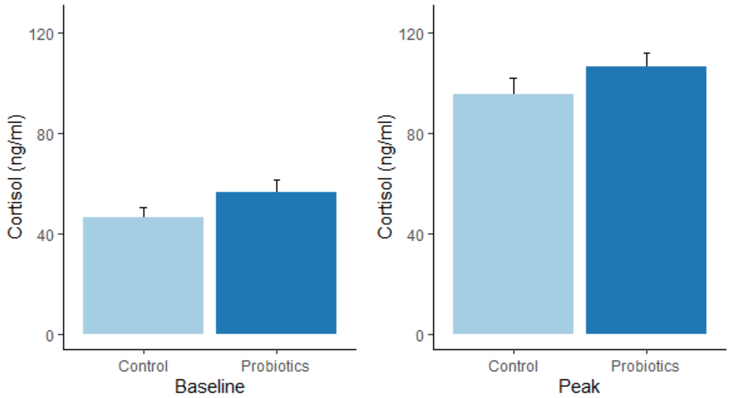

Figure 2. Means ± SEM baseline and peak cortisol concentrations in response to 5 min restraint.

The plasma ACTH responses to restraint were not affected by treatment or sex, but changed significantly over time ($P < 0.001$). In the second analysis, peak concentrations of ACTH were not affected by treatment (Fig 3).

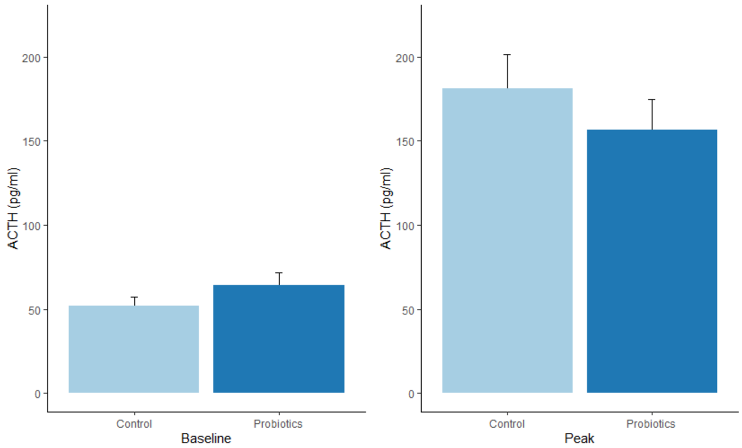

Figure 3. Means ± SEM baseline and peak ACTH concentrations in response to 5 min restraint.

3.2. Part II. Optimization of probiotics administration on a commercial farm

3.2.1. Weight gain

Weight gain from birth until 70 days of age increased over time (P<0.001) and was significantly affected by a treatment by time interaction (P<0.001), with the piglets that received probiotics on the udder, in the peat from birth until two-weeks post-weaning and orally being about 10% heavier than the control group at day 70. Piglets that received the probiotics only after weaning and via supplementation of the sow were of similar weight as the control group (Fig 4).

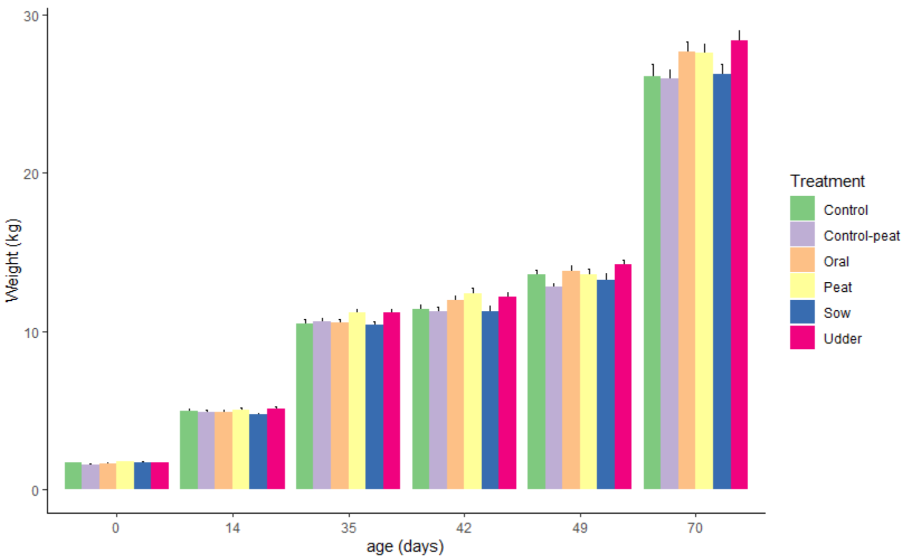

Figure 4. Mean ± SEM weight for the different treatments

3.2.2. Food intake

Post-weaning food intake increased significantly over time (P < 0.001) but was not affected by treatment.

3.2.3. Welfare measures

The proportion of piglets that had a tear staining score of at least 1 significantly reduced with age (P < 0.001) and there was no effect of treatment. Piglets in the oral supplementation group were less likely to have tear staining score 2 or more (e.g., almost half as likely) compared to the control, control-peat and udder (Figure 5).

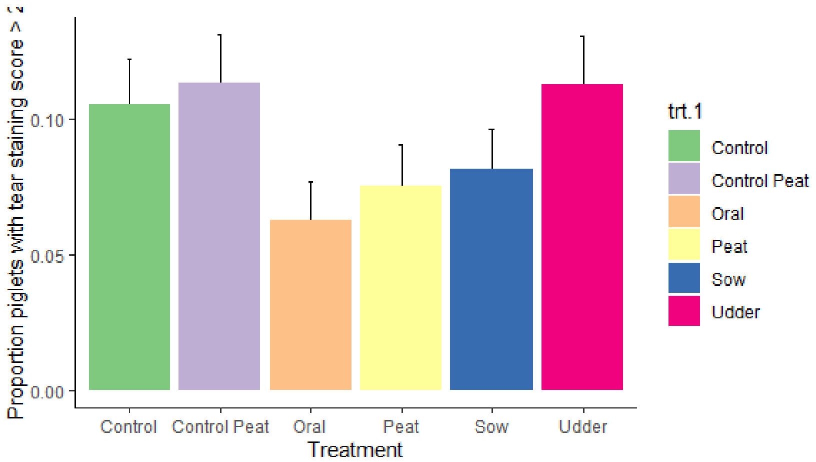

Figure 5. Mean ± SEM proportion of piglets with a tear staining score of more than 2 on a scale 0 (best) to 3 (worst).

The proportion of piglets with a skin lesion score of more than 1 changed significantly over time (P < 0.001), and there was a significant time x treatment interaction (P < 0.02, fig 6). We cannot say whether the differences between treatments that seem to be emerging at 49 days of age would have been maintained or changed if observations had continued to later ages.

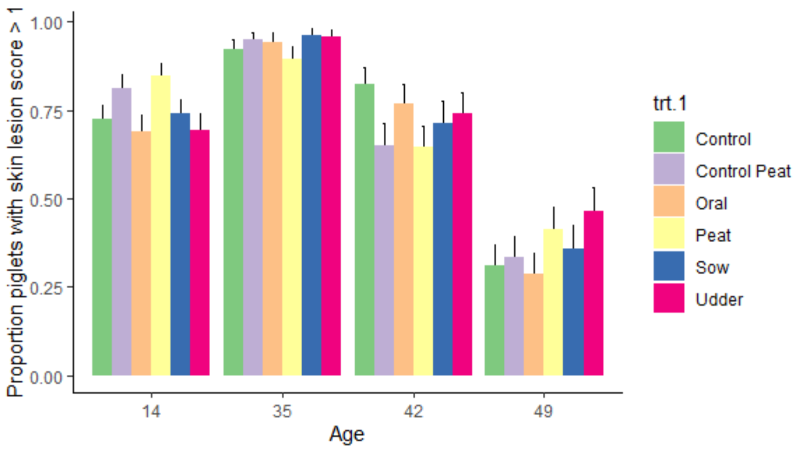

Figure 6. Mean ± SEM proportion of piglets with a skin lesion score of more than 1 on the scale 0 (best) and 3 (worst).

Only 1.6 ± 1% of the piglets was scored as having diarrhoea at the time of the individual observations, and this number was too low to conduct a statistical analysis. Tail biting, and mastitis in the sow were also rarely observed and therefore not analysed.

3.2.4. Data from bacterial analyses

The culture data revealed no significant impact of the treatments, neither on the levels of Lactic acid bacteria nor on the *Enterobacteriaceae* (Fig. 7). The collected rectal swabs have been prepared for 16S amplicon sequence analysis, but the data from the analysis have not been received yet so it is not yet possible to conclude if the probiotic supplementation had any effects on the microbiota overall.

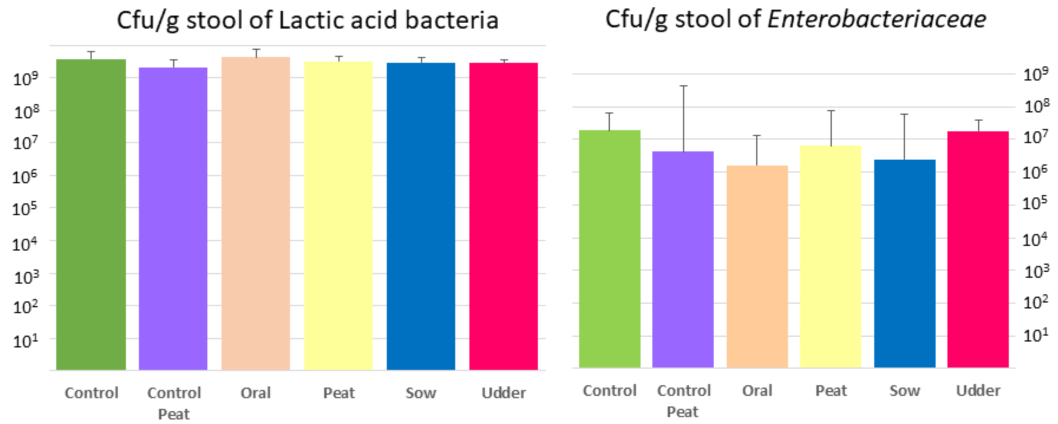

Figure 7. Mean ± SEM for bacterial count data of Lactic acid bacteria and *Enterobacteriaceae*

3.2.5. Economic analysis.

The piglets in the oral supplementation group had the lowest feed cost per kg of growth and per pig, and also had the highest labour consumption per pig for the administration of probiotics. The hourly labour cost has been estimated at SEK 250. Even taking into account the higher labour cost per pig to administer the probiotic orally, the faster growth for the pigs in this treatment results in it being in the oral treatment that there is the greatest financial margin to pay for the probiotic cost (Table 1).

Table 1: Financial margin for probiotic administration taking into account feed and labour costs

treatment	Growth from weaning to 70 days	Feed: SEK per kg growth	Difference SEK per kg growth compared to the control group	Difference SEK per pig compared tothe control grou	Labor cost per pig for probiotica admin.	Economic margin per pig for probiotic cost
Control	15.59	7.55	0	0		
Control-peat	15.34	7.93	0.38	5.78	0.21	-5.99
Oral	17.09	6.97	-0.58	-9.88	4.17	5.71
Peat	16.44	7.34	-0.22	-3.56	0.21	3.77
Sow	15.80	7.54	-0.01	-0.22	0.46	0.68
Udder	17.15	7.38	-0.17	-2.86	1.29	1.57

4. Discussion:

There is currently much interest in novel strategies that promote highly productive animals while at the same time maintaining good welfare, without the use of antibiotics and zinc oxide. Several studies have shown beneficial effects of probiotics on growth, food intake and stress-induced disease susceptibility (such as post-weaning diarrhoea) under controlled experimental conditions in pigs [13, 14]. But to determine whether probiotics could be useful for pig farming, it is important to assess the effectivity under commercial conditions.

Our results showed that the groups receiving the probiotics before weaning (peat, udder and oral supplementation groups) were about 10% heavier than the control group at day 70, while food intake was similar between the groups. Piglets that received the probiotics post-weaning or via the lactating sow (control-peat and sow groups) were of similar weight as the control group. Therefore, probiotics should be given before weaning and directly to the piglet. Other recent studies have also shown that probiotics increased body weight and promoted the development of intestine mucosal system and maintain intestinal mucosal barrier in new-born piglets [15, 16]. In the economic cost-benefit analysis, the oral supplementation group was found to be the most economically beneficial. The farm staff also reported that they thought that the oral supplementation route was the easiest method to use.

Good welfare entails both the physical and mental health of animals. As an indicator of stress and welfare we also recorded tear staining [17, 18]. Our results suggest that the oral group was less likely to have a score of more than 2 compared to the control, control peat and udder groups, indicating that the oral supplementation group may have experienced less stress. We also recorded skin lesions scores, which is a validated proxy of social aggression [19]. However, we did not observe a clear pattern in our study: the control-peat and peat groups seemed less likely to have skin lesions at day 42. However, the peat and udder groups were slightly more likely to have skin lesions at day 49 of age. In rodents, abnormal social behaviour following a maternal high fat diet could be reversed by probiotics supplementation [8], suggesting a beneficial effect of probiotics on social behaviour. In hens, highly aggressive behaviours were linked to lower levels of lactobacilli in in the gut [20]. It is not clear why the probiotics did not have a clear impact on aggression in our study. Alternatively, it may be that the differences in skin lesions observed at days 42 and 49 are a reflection of altered rates of skin healing rather than new lesions. It may be interesting to observe the pigs also at older ages.

Previous research on rodent models have shown that probiotics can reduce anxiety-like behaviours and enhance stress resilience, but whether this is also the case in pigs has not been studied [1, 21]. Our results showed no effects of probiotics on fear towards a novel object and only minor effects on HPA-axis responses to restraint stress. However, we assessed stress responses one week, and fear responses several weeks, after the end of supplementation period, and it is possible that the effects of the supplementation did not last long enough. Our results are in agreement with a study in humans, in which *Lactobacillus* probiotics did not alter stress responses or self-reported anxiety and stress levels [22].

5. Summary and conclusion

Probiotics supplemented pre-weaning directly to the piglets led to a 10% higher weight at 70 days of age, without increasing food intake. As a result, supplementing probiotics orally at days 1 and 4 had the greatest economic benefits, even when the time taken to administer it was taken into account. Probiotics supplemented orally at days 1 and 4 also appeared to have a positive effect on tear staining. However, probiotics did not have a clear impact on skin lesions resulting from social aggression nor did they alter anxiety-like behaviours and HPA-axis responses. Therefore, we conclude that supplementing probiotics early in life has a beneficial impact on growth and some measures of welfare in a commercial farm.

6. Recommendations to the industry

Supplementing probiotics early in life has a beneficial impact on growth and some measures of welfare in a commercial farm. Even taking into account the higher labour cost per pig to administer the probiotics orally, the faster growth for the pigs in this treatment resulted in it being the treatment with the greatest financial margin. Administering probiotic orally at 1 and 4 days of age gave an economic margin for the probiotic cost of 5.71 kr per pig.

7. References

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Part 3: dissimination of results

Vetenskapliga publiceringar	Manuscript in preparation: Dicksved et al., Effects of probiotic treatment on cognition, stress responsiveness and the gut-microbiota in piglets. Intended for 'Scientific Reports', 2021.
	Manuscript in preparation: Verbeek et al., Effects of different routes of probiotics administration on the gut-microbiota, performance, and welfare in piglets. Intended for 'Animals', 2021.
Övriga publiceringar	**SLU website:** **https://www.slu.se/en/departments/animal-environment-health/research/research-project/impact-of-early-gut-microflora/**
	Research gate project page: **https://www.researchgate.net/project/Supplementing-piglets-with-probiotic-assessing-effectivity-feasibility-and-economics** We will add published articles and results once they are available
	A popular science article will be written for Grisföretagaren in the autumn of 2020
	SLU newsletter report: following the publication of a peer-reviewed article, expected 2021
	GD newsletter report: following the publication of a peer-reviewed article, expected 2021
Muntlig kommunikation	Verbeek et al., Effects of probiotics on cognition and HPA-axis functioning in piglets Beneficial microbes, 22-24 March 2021, Amsterdam, The Netherlands (conference postponed due to COVID-19)
	An oral presentation will be given at The Swedish Veterinary Meeting in 2021.
	Presentation to veterinarians at Gård och Djurhälsan titled 'Impact of probiotic supplementation on growth, HPA-axis responses and behaviour in piglets' on 17-6-2020
	Reference group meetings (held on 25-9-2018, 24-1-2019 and 25-5-2020)
Studentarbete	Sara Hammarberg, 2019. Different methods of administration of probiotics to piglets and their effect on growth, health and microbiota. Masters thesis, SLU.
	Amaury Arnaud, 2019. Effects of different probiotic administration routes on piglets behaviour and health. Internship report, IUT de Laval, France.
	Maria Rodriguez, 2020. Can probiotics have an impact on piglets behaviour? Masters thesis, SLU – *in progress*.