Semen evaluation and *in vivo* fertility in a Northern Italian pig farm: can advanced statistical approaches compensate for low sample size? An observational study

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Abstract

The evaluation of sperm functionality and morphology allows discerning between high and low quality ejaculates, but does not give detailed predictive information regarding *in vivo* fertility. The current developments in statistical modeling have helped in carrying out reproductive studies, but their biggest limitation is in the size of the dataset to be used. The aim of the present observational study was to evaluate whether advanced statistical approaches, such as mixed effects regression models and bootstrap resampling, can help in assessing the predictive ability of semen parameters in terms of *in vivo* fertility (farrowing rate and litter size), on a small/medium farm with a limited number of animals.

Data regarding 33 ejaculates, including viability, subjective motility and acrosome reaction, were collected. Two hundred and thirty-five sows were inseminated with an outcome of 167 deliveries and 1734 newborn piglets. In order to evaluate the relationships among the parameters measured and fertility, mixed effects regression statistical models were used. Once the covariates to be included in the final models were identified, non-parametric bootstrapping was used. The results showed that the farrowing rate was highly associated with the total number of spermatozoa and subjective motility, while litter size was associated with percentage of acrosome reaction. In conclusion, the proposed statistical approach seemed to be suitable for studies regarding reproduction and fertility, even for relatively small sample sizes. Nonetheless, larger data sets are still preferable and required in order to achieve higher reliability.

Keywords: Swine semen parameter; in vivo fertility; farrowing rate; litter size; mixed effects regression model.

Introduction

The majority of the swine reproductive industry currently uses artificial insemination (AI) (Rodríguez-Gil and Estrada, 2013) in order to reduce disease transmission and increase zootechnical indices. To optimize AI, it is essential to evaluate the fertility of semen (Foote, 2003) since management errors, frequency of sampling, environmental temperature alteration, season and disease can influence spermatogenesis and thus fertility (Wolf and Smital, 2009). The evaluation of sperm functionality and morphology allows discerning between high and low quality ejaculates, without giving detailed predictive information regarding *in vivo*

fertility (Jung et al., 2015; Schulze et al., 2013, 2014); it needs to be constantly performed since drops in quality are quite common. Several studies have investigated the ejaculate parameters in order to try and identify the best performing boars, but the conclusions are often in disagreement (Lee et al., 2014; McPherson et al., 2014; Popwell and Flowers, 2004; Tardif et al., 1999). Overall, sperm motility appears to be the most important quality index (Broekhuijse et al., 2012a) and one of the best *in vivo* fertility predictor when analyzed both subjectively (Tardif et al., 1999) and objectively (Ruiz-Sánchez et al., 2006). Other parameters, such as the concentration of the used insemination doses, related to both semen and boars, can influence pig field fertility (Broekhuijse et al., 2012a).

The current developments in statistical regression modeling have helped in carrying out reproductive studies, allowing for in-depth investigations regarding fertility, its predictive parameters, and their correlation (Broekhuijse et al., 2012b; Bucci et al., 2014; Didion, 2008; Gadea et al., 2004; Quintero-Moreno et al., 2004; Turba et al., 2007). In particular, appropriate specifications of these models can provide estimates of the joint effects of the characteristics recorded regarding *in vivo* fertility outcomes. Unfortunately, studies which aim to analyze predictive values in the reproductive field have found their biggest limitation in the size of the dataset to be used, thus the need for specific, and accurate national and international databases (Broekhuijse et al., 2012a).

The aim of the present observational study was, therefore, to evaluate whether advanced statistical approaches such as mixed effects regression models and bootstrap resampling could help in assessing the predictive ability of semen parameters in terms of *in vivo* fertility (farrowing rate and litter size) on a small/medium farm with a limited number of animals.

Materials and Methods

Animals and sampling

Data regarding thirty-three ejaculates (n=33) were collected from 9 boars (6 Duroc and 3 Large White; age 11.5 to 31.2 months) bred according to the Italian Welfare laws at the Montrone breeding farm (Valsallustra; BO; IT).

All the samples included in the study derived from ejaculates collected using the hand-glove technique during the spring season (from March to May). The semen was routinely collected twice a week from each

boar within the facility, but the samples analyzed in the present study were only the ones used for the insemination of the sows bred within the same facility (approximately every 21 days).

Health status was assessed on the basis of clinical examination and rectal temperature, carried out by the farm veterinarian together with a standard clinical chemistry panel carried out on the serum samples collected from the jugular vein at the beginning of March from each animal.

Semen preparation and insemination

Immediately after collection, total semen volume was measured with a sterile graduated cylinder at 28°C, and then diluted 1/1 (v/v) with Swine Fertilization Medium (SFM) (Lavitrano et al., 2002) at the same temperature.

Concentration was assessed in the farm laboratory using an optical densitometer accurately calibrated each morning using a Thoma hemocytometer. The total spermatozoa count (Tot spz) was subsequently calculated multiplying the concentration by the total volume of the ejaculate.

Subjective motility (SM) was immediately assessed by the farm veterinarian using contrast phase microscopy by loading 50 µl of each sample on a heated glass slide; ejaculates showing SM ≥85% were classified as optimal, those showing SM <85% as low motile ejaculates according to the literature (McGlone and Pond, 2003).

Insemination doses were prepared by diluting 3 x 10^9 spermatozoa in SFM for a final volume of 100 ml, and stored in a refrigerated thermostat at 16°C (±0.5) for a maximum of 72 hours. Multiparous Large White sows (n=235) were then inseminated by the farm personnel, upon post-weaning heat detection, twice within 12 hours.

Semen analyses and *in vivo* fertility data collection

For each ejaculate, an aliquot of 5 ml (diluted 1/1 v/v) was delivered to the laboratory of the Department of Veterinary Medical Sciences (University of Bologna) for morph-functional assessment consisting of viability (V) and acrosome reaction (AR).

Viability. An aliquot of 25 µl of semen was incubated with 2 mL of a 300 mM solution of SYBR green-14 and propidium iodide (PI) for 5 minutes at 37° C in dark conditions (Huo et al., 2002; Silva and Gadella, 2006). Ten microliters of suspension were then placed on a microscope slide and analyzed using an

epifluorescence microscope (Nikon Eclipse E600; Nikon Corporation, Tokyo, Japan) with a double-band-pass filter for green and red fluorescence. A minimum of 200 cells were counted and evaluated in order to obtain the percentage of viability; green heads were considered as live sperm and red ones as dead.

Acrosome reaction. Spermatozoa acrosomes were evaluated by Brilliant Blue G 250 (Sigma-Aldrich Corp. St. Louis, MO, USA) staining as described by Larson and Miller (Larson and Miller, 1999). Acrosomes stained blue were considered normal while the unstained ones were considered as reacted or lost. The percentage of AR was based on a minimum of 200 cells (Bacci et al., 2009). The percentage of AR was classified as appropriate when ≤ 5%, and as not appropriate when > 5% (Huo et al., 2002).

Bacteriological evaluation. The bacteriological evaluation was carried out by the General Diagnostic Section of the "Istituto Zooprofilattico Sperimentale of Lombardia and Emilia Romagna Bruno Umbertini (IZSLER)" by seeding a sample of each ejaculate on agar plates which were then incubated overnight at 37°C in normal air with 5% CO_2. The isolated microorganisms were identified using biochemical tests.

In vivo fertility. Data were collected directly at the farm. The number of deliveries and the number of newborn piglets were registered 114 days (± 2 days) after insemination. The two parameters analyzed were: farrowing rate (FR=number of deliveries/number of inseminated sows) and litter size (LS=total number of newborn piglets/number of deliveries).

Statistical analysis

Descriptive statistics of the recorded parameters were calculated. Continuous variables were expressed as mean and standard deviation, while the categorical variables as absolute and percentage frequencies.

In order to evaluate the relationships among the recorded characteristics and the fertility parameters, linear and generalized-linear mixed effects statistical models were used (Goldstein, 2011; Roy, 2013). The incorporation of a random effect term in the models gave the possibility of taking into account correlations among ejaculates of the same boar.

Regarding FR analysis, mixed effects binomial regression models were used (Hosmer et al., 2013), setting the dependent variable as the log-odds of delivery (Eq.1). Here, the number of deliveries was viewed as the number of "successes" out of a given number of "trials" (inseminated sows), allowing for direct assessment of the probability of delivery, which is at the basis of FR calculation. The results of FR analysis were reported as Odds Ratio (OR) of delivery with associated 95% Confidence Interval (95% CI) and p-value.

Equation 1. Random effects binomial regression model used in FR analysis

$$logit(p_{ij}) = \left(X_{ij}\beta\right) + \varepsilon_{ij} + u_j$$

$$y_{ij} \sim Bin\left(n_{ij}, p_{ij}\right);\ \varepsilon_{ij} \sim N(0, \sigma_\varepsilon^2);\ \ u_j \sim N(0,\ \sigma_u^2)$$

Notes to Eq.1:

β is a vector of regression parameters and X is the design matrix for the independent variables. For the i-th ejaculate related to the j-th boar: y_{ij} is number of deliveries, n_{ij} is number of inseminated sows, p_{ij} is the probability of delivery, ε_{ij} are error terms and X_{ij} is the ij-th row of X, while u_j is the random effect term related to the j-th boar.

With respect to Litter Size analysis, mixed effects linear regression models were used, excluding the two ejaculates which did not fertilize any sow, leading to a total number of 31 ejaculates included (n=31).Here, the dependent variable was the total number of born piglets (TNBP), while the other component of LS calculation - number of deliveries - was treated as a fixed covariate (Eq.2).Results of the LS analysis were reported as Mean Difference (MD) in TNBP with associated 95% CI and p-value.

Equation 2. Random effects linear regression model used in LS analysis

$$y_{ij} = \left(X_{ij}\beta\right) + \varepsilon_{ij} + u_j$$

$$\varepsilon_{ij} \sim N(0, \sigma_\varepsilon^2);\ \ u_j \sim N(0,\ \sigma_u^2)$$

Notes to Eq.2:

β is a vector of regression parameters and X is the design matrix for the independent variables. For the i-th ejaculate related to the j-th boar: y_{ij} is TNBP, ε_{ij} are error terms and X_{ij} is the ij-th row of X, while u_j is the random effect term related to the j-th boar.

In both FR and LS analyses, mixed models were fitted by using a variance components correlation structure, while estimation was performed with maximum likelihood method.

For each of the two analyses, a model selection procedure consisting of three steps was carried out. In the three phases, the following models were fitted:

- "basic" models: in FR analysis, all simple models (i.e. with one independent variable); in LS analysis, all the multivariable models including number of deliveries and one other independent variable;
- "multivariable" model: the multivariable model including all recorded variables;
- "final" models: two multivariable models, with covariates selected according to backward-stepwise ($p<0.05$ for staying in the model) and forward-stepwise ($p<0.05$ for entering in the model) variable selection procedures, respectively (Hastie et al., 2009).

Once the covariates to be included in the final models were identified, non-parametric bootstrapping was performed. Bootstrapping is a resampling technique which turned out to be robust and effective in estimating statistics of interest, giving more reliable estimates and confidence intervals (Efron and Tibshirani, 1993; Fox, 2002). In our case, two thousands cluster-bootstrapped samples (i.e. samples consisting of all ejaculates from nine boars randomly drawn with replacement from the original data) were generated and the structure of the final model was fit onto each of them. Bootstrapped regression estimates were the average estimated coefficients across bootstrapped samples. The confidence intervals for regression parameters were the 2.5^{th} and 97.5^{th} percentiles of the bootstrapped estimates, while p-values were calculated as described by Efron & Tibshirani (Efron and Tibshirani, 1993).

Finally, the Variance Partition Coefficient (VPC) index was calculated on the final models (Goldstein et al., 2002) in order to assess the rate of variance of the outcomes attributable to differences between the boars. The VPC for the linear model was calculated as the variance of random effects divided by the sum of the variances of random effects and error terms (σ_u^2 and σ_ε^2 in Eq.2, respectively) while, for the binomial model, it was calculated according to the latent variable approach described by Goldstein et al. (Goldstein et al., 2002) and already applied within the FR analysis by Iida & Koketsu (Iida and Koketsu, 2016).

Analyses were performed using R 3.0.3 statistical software (The R Foundation for Statistical Computing) at a confidence level equal to 95%.

Results

All the boars enrolled in the study were considered healthy. The clinical examinations did not show any alteration and the mean rectal temperature (38.42 ± 0.25°C) was within the physiological range (Reece, 2015).The blood work results (Supplementary Table S1) were normal.

The seminal, bacteriological and in vivo fertility parameters are reported in Table 1.

Semen evaluation. Sperm morphology analyses did not show any important alterations in the percentage of abnormal forms (20% ± 8.5), and only 5 samples showed the presence of few spermatic aggregates. Regarding subjective motility, 78.8% of samples (n=26) showed SM ≥85% (optimal), and the remaining 21.2 % (n=7) showed SM <85% but never asthenospermia.

Bacterial contamination was detected in 87.9% of the samples. The species isolated and their frequencies are reported in Table 1. Simultaneous isolation of two different species was only observed in two ejaculates; one sample showed the presence of E. coli and Proteus spp., and another showed E. coli and Polymorph bacteria flora. In 12.1% of the cases, none of the bacterial species investigated were detected.

In vivo fertility. Fertility data (Table 1) were collected at the farm: 167 sows, out of the 235 inseminated, gave birth to a TNBP of 1734. The average FR was 67.8 ± 27.8 and the born/delivery ratio (LS) was 9.7 ± 2.9.

Farrowing rate analysis.

The results obtained from mixed effect binomial regression models between the FR analysis outcome and the principal semen parameters are reported in Table 2. Sperm volume was not included in the models due to its high correlation with total spermatozoa count. Variables related to Pseudomonas spp., Proteus spp. and Staphylococcus spp. were also not considered due to the low number of positive samples (Table 1).

Both backward and forward selection procedures chose the same final model (Table 2); its results showed that FR was associated with the total number of spermatozoa (Tot spz) (OR= 1.7, *p value*= 0.0004, 95%CI = 1.2; 2.4) and also to subjective motility (OR= 0.21, *p value*= 0.0049, 95%CI = 0.09; 0.49), as was also observed in the basic and multivariable models. Acrosome reaction was associated with probability of delivery only in the basic model, but was not in the multivariable and final models. No other parameter showed association with probability of delivery.

According to our estimated final model, considering an ejaculate with average values of the random effect term and total count of spermatozoa parameters – zero and 101.4, respectively - the baseline odds (i.e. for an ejaculate with SM > 85% and Tot spz = 101.4) was 3.42 while the estimated delivery probability was 77.4%. Delivery probability was similarly calculated for ejaculates which showed low SM, and was equal to 41.9%.

Variability attributable to the boar within the final model, measured with the VPC index, explained 0.9% of the total variation in FR analysis.

Bootstrapped analysis gave similar results (Tot spz OR = 1.8, *p value*= 0.0158, 95% CI = 1.1; 3.7, SM<85% OR = 0.16, p value < 0.0001, 95%CI = 0.06; 0.46) and confirmed the findings of the final model.

Litter size analysis.

The results obtained from mixed effect linear regression analysis considering total number of born piglets and the principal semen parameters are reported in Table 3. As in the previous analysis, some of the parameters were not included (refer to the section on farrowing rate).

In light of the fact that the deliveries were treated as a fixed covariate in the LS analysis, as expected, its value was always highly significant (all, $p < 0.0001$).

In basic models, association was found only between TNBP and AR (MD=-6.1, *p value*= 0.0355, 95% CI = -11.4; -0.7) while, in the multivariable model, a relationship between TNBP and the age of the boar was also observed. Nonetheless, both our final models proved to be identical to the AR basic model.

Variability attributable to the boar was 7% of the total variation of the outcome.

Bootstrapped analysis confirmed the final model findings, in particular regarding the relationship between TNBP and AR (MD=-6.9, *p value*< 0.0001, 95% CI = -12.8; -3.1).

Discussion

In the present study, the farrowing rate and litter size of a small/medium Northern Italian pig farm were analyzed using statistical regression models. In order to compensate for the relatively small dataset, the models were improved by means of bootstrapping. Indeed, when the focus of the analyses was the prediction of the relationship between several variables, large sample sizes are required to achieve good reliability.

It should also be noted that the aim of the latest trends in reproductive and fertility studies is to discover new molecular biomarkers, using mostly proteomic and genomic approaches (Kwon et al., 2015; Rahman et al., 2013; Zannoni et al., 2017). These techniques can be extremely sensitive and accurate, but require time and economic investment, making them more suitable for the research field and larger genetic facilities than for zootechnical production.

The present study only focuses on male performance, but the fertility outcomes also depend equally on the female reproductive status. The sows used in this study were healthy multiparous animals of proven fertility; in fact, the inseminations were performed upon post weaning heat detection. Overall, any analysis regarding *in vivo* fertility would be more complete if both males and females were taken into account, but it has to be stated that, within the reality of small pig farms, it is always easier to gain more in-depth information regarding boars since spermatozoa are, however, always collected and analyzed.

As previously stated, the boars were also considered healthy on the basis of a clinical chemistry panel, but despite the recent interest in setting specific age-related reference intervals for pigs (Ventrella et al., 2017), data regarding boars are still lacking. For the interpretation of the blood analysis results of the present study, non-age-specific reference intervals were used, and this might explain the small fluctuations between our data and the published standards. A good example representative of this issue can be find in the levels of Alkaline phosphatase (ALP), an enzyme directly related to osteoblast activity in younger, growing animals (Ventrella et al., 2017): the values of all the analyzed boars are indeed consistently and significantly lower than the reference interval ones. Moreover, it has to be acknowledged that the others parameters that slightly differ from the reference intervals, such as Albumin and Globulins concentrations, are coherent and similar within the entire group of animals, further validating the hypothesis of the authors rather than subclinical pathological statuses.

Several attempts to analyze the relationship between semen quality evaluation and in vivo fertility have already been carried out for the porcine species, with extremely variable results (Broekhuijse et al., 2012b; Didion, 2008; Gadea et al., 2004; Graham et al., 1990; Lee et al., 2014; Lovercamp et al., 2007; McPherson et al., 2014; Moretti et al., 2005; Schulze et al., 2015; Tardif et al., 1999).

Despite it being one of the most important parameter, our statistical analyses did not report any correlation between the outcomes and spermatozoa viability. This finding seemed to agree with what was reported by Gadea and colleagues (Gadea et al., 2004).

The microbiological analyses carried out in this paper demonstrated how the most representative bacterial species are consistent with those formerly described for swine ejaculates (Kuster and Althouse, 2016). The overall amount of bacterial contamination of the samples was extremely low, proving the good health state of the boars and that the specimens were sampled and handled in the best way possible. A negative correlation between the presence of bacteriospermia and semen quality has previously been reported in pigs

(Schulze et al., 2015), but a direct correlation between FR and the presence of bacteria has never been demonstrated. On the other hand, LS seems to be affected by the presence of sperm agglutination, which is directly related to the amount of E. coli within the ejaculate (Maroto Martín et al., 2010). The results of the heretofore used statistical models did not demonstrate any association between the main species of bacteria isolated and either of the outcomes (FR and LS). One of the reasons why could be the fact that, despite its presence, the bacterial contamination found in the ejaculates used for this study was too low to influence any parameter in a significant manner.

Subjective (Tardif et al., 1999) or the objective (Broekhuijse et al., 2012a, 2012b) sperm motility in boars is the most important parameter for evaluation of the ejaculate quality and one of principle predictors of *in vivo* fertility. Our results suggested that motility analysis, using a subjective method, may have a good predictive value for the farrowing rate, as has already been described and confirmed by the existing literature (Gadea et al., 2004). It has to be stated that an objective analysis of the motility by computer-assisted sperm analysis (CASA) is, in fact more reliable and reproducible, but not always possible; the instrumentation itself and slides still represent an important economic investment which not every farm can afford. Therefore, it is still quite common among smaller facilities to rely on well-trained experienced operators to subjectively analyze this parameter.

The multivariable binomial regression model (Table 2) highlighted how the only two parameters associated with FR are the total count of spermatozoa and subjective motility, with major emphasis on SM.

In fact, the data obtained showed that, the TotSpz being equal (referable to the mean value), the probability of completing the pregnancy for a sow inseminated with an optimal ejaculate (SM ≥85%) was 77.38%,which decreased to 42% for those inseminated with ejaculates having subjective motility <85%. The model and the statistical analyses were confirmed by the bootstrapping method.

This finding might have extremely important implications for the zootechnical swine industry since the analysis of subjective motility is simple and does not require advanced instrumentation as has previously been stated.

Regarding the analysis of litter size (Table 3), the only significant parameter in the final model was acrosome reaction, classified as appropriate when ≤5%, and not appropriate when >5%. This result seemed to confirm what has already been described by the existing literature for other species, including humans

(Lee et al., 2014; Moretti et al., 2005). As for farrowing rate analysis, bootstrapping did confirm the results of the final model, giving more strength and robustness to our findings.

Regarding intra-boar correlations, variations in FR and LS outcomes were only minimally dependent on the boar (respectively 0.9% and 7%, as measured by VPC), confirming the fact that the quality of semen is the result of a multifactorial interaction which depends minimally on the individual (Broekhuijse et al., 2012b).

The use of statistical models applied to our data highlighted some of their potentialities and limitations. In the former category, the ability of simultaneously controlling for the effect of several explicative parameters should be taken into consideration as well as flexibility in the specification of the model. Here, the specification of the model was suggested by the mathematical structure of both the FR and LS parameters. Similarly, the use of a binomial model, expressed in terms of log-odds of delivery, was also reported by Gadea et al. (Gadea et al., 2004) and Iida & Koketsu (Iida and Koketsu, 2016) while the use of linear regression within the LS analysis using TNBP as a dependent variable was also considered by Brooekhuijse et al. (Broekhuijse et al., 2012b). By using mixed effects models, researchers also have the possibility of accounting for differences among boars within model fitting, but also analytical variability partition indicators, such as VPC, can be derived.

Overall, despite the relatively low sample size, this study seemed to confirm how the most important parameters for the evaluation of *in vivo* fertility, were spermatozoa motility and appropriate acrosome reaction (i.e. < 5%) for the farrowing rate and litter size, respectively. Bootstrapping proved to be useful in improving the performance of the regression model used, by confirming the results of multivariable models and giving more strength to their findings

In conclusion, the proposed statistical approach seems to be suitable for studies regarding reproduction and fertility, even for relatively small sample sizes. Nonetheless, larger data sets are still required to achieve higher reliability.

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Conflict of interest statement

The Authors have no conflict of interest to declare.

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Table 1. Descriptive statistics for semen quality parameters and *in vivo* fertility (sample n=33).

***In vivo* fertility parameters**		
Inseminated sows (n)	mean (SD)	7.1 (3.7)
Deliveries (n)	mean (SD)	5.1 (3.5)
Born piglets (n)	mean (SD)	52.6 (36.6)
Farrowing rate (%)	mean (SD)	67.8 (27.8)
Litter size (born piglets/deliveries)	mean (SD)	9.7 (2.9)
Seminal parameters		
Volume (ml)	mean (SD)	218.1 (84.3)
Total number of spermatozoa (x 10^9)	mean (SD)	101.4 (37.3)
Concentration (spz x 10^6/ml)	mean (SD)	490.9 (161.7)
Viability (%)	mean (SD)	78.5 (11.6)
Acrosome Reaction, ≤5%	n (%)	21 (63.6)
Subjective Motility, ≥85%	n (%)	26 (78.8)
Bacteria Species		
Polymorph bacteria flora, positive	n (%)	12 (36.4)
Escherichia coli spp., positive	n (%)	11 (33.3)
Pseudomonas spp., positive	n (%)	4 (12.1)
Proteus spp., positive	n (%)	3 (9.1)
Staphylococcus spp., positive	n (%)	1 (3.0)

Table 2. Odds Ratio (95% CI) between the main semen parameters and the Farrowing Rate, calculated using mixed effects binomial regression models (Eq 1).

Parameters	Basic Models	Multivariable Model	Final Model (Backward)	Final Model (Forward)	Bootstrapped Final Model
Boar breed (LW vs. D)	0.76 (0.59; 1.42) p = 0.5560	0.49 (0.24; 1.02) p = 0.0568	/	/	/
Boar age (months)	0.91 (0.31; 1.89) p = 0.6900	0.93 (0.62; 1.38) p = 0.7056	/	/	/
Tot spz (standardized Gaussian scaled)	1.77 (1.20; 2.62) p = 0.0039**	1.86 (1.27; 2.72) p = 0.0013**	1.68 (1.17; 2.41) p = 0.0004***	1.68 (1.17; 2.41) p = 0.0004***	1.82 (1.10; 3.67) p = 0.0158*
Viability (%)	1.01 (0.98; 1.04) p = 0.4510	1.01 (0.98; 1.04) p = 0.6249	/	/	/
Acrosome Reaction (>5% vs. ≤5%)	0.39 (0.21; 0.74) p = 0.0040 *	1.01 (0.33; 3.07) p = 0.9865	/	/	/
Subjective Motility (<85% vs. ≥85%)	0.23 (0.09; 0.54) p = 0.0008***	0.23 (0.07; 0.80) p = 0.0201*	0.21 (0.09; 0.49) p = 0.0049**	0.21 (0.09; 0.49) p = 0.0049**	0.16 (0.06; 0.46) p = 0.0000***
Polymorph bacteria flora (positive vs. negative)	1.55 (0.82; 2.97) p = 0.1709	1.70 (0.84; 3.43) p = 0.1415	/	/	/
E. coli spp. (positive vs. negative)	0.73 (0.39; 1.35) p = 0.3140	1.02 (0.46; 2.30) p = 0.9458	/	/	/

Backward = backward-stepwise selection ; Forward = forward-stepwise selection ; LW= Large White; D= Duroc; Tot spz= Total spermatozoa count. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 3. Mean Differences in total newborn piglets (95% CI) between the main semen parameters calculated using mixed effects linear regression models (Eq 2).

Parameters	**Basic Models**	**Multivariable Model**	**Final Model (Backward)**	**Final Model (Forward)**	**Bootstrapped Final Model**
Deliveries (units)	10.30 (9.57;11.02) p = 0.0000***	9.80 (9.07;10.53) p = 0.0000***	10.03 (9.32;10.75) p = 0.0000***	10.03 (9.32;10.75) p = 0.0000***	10.04 (9.32;10.72) p = 0.0000***
Boar breed (LW vs. D)	3.63 (-1.44;8.70) p = 0.1479	2.1 (-2.51;6.70) p = 0.2637	/	/	/
Boar age (months)	-0.77 (-3.40;1.87) p = 0.3350	-0.02 (-0.04;-0.00) p = 0.0192*	/	/	/
Tot spz (standardized Gaussian scaled)	0.02 (-0.05;0.08) p = 0.3387	0.03 (-0.03;0.09) p = 0.2223	/	/	/
Viability (%)	0.18 (-0.02;0.37) p = 0.0848	0.06 (-0.14;0.25) p = 0.3332	/	/	/
Acrosome Reaction (>5% vs. ≤5%)	-6.08 (-11.37;-0.71) p = 0.0355*	-8.64 (-14.85;-2.42) p = 0.0128*	-6.08 (-11.37;-0.78) p = 0.0355*	-6.08 (-11.37;-0.78) p = 0.0355*	-6.91 (-12.76;-3.11) p = 0.0000***
Subjective Motility (<85% vs. ≥85%)	-2.89 (-10.04;4.26) p = 0.2868	3.35 (-3.94;10.64) p = 0.2613	/	/	/
Polymorph bacteria flora (positive vs. negative)	-0.49 (-5.32;4.35) p = 0.3878	1.44 (-3.04;5.92) p = 0.3225	/	/	/
E. coli spp. (positive vs. negative)	2.28 (-2.75;7.31) p = 0.2646	4.07 (-1.23;9.38) p = 0.1280	/	/	/

Backward = backward-stepwise selection; Forward = forward-stepwise selection; LW= Large White; D= Duroc; Tot spz= Total spermatozoa count. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.