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A cross-sectional study to understand the correlation among *Salmonella enterica* infection and *Lactobacillus reuteri* concentration in fecal samples of post weaning piglets and their association in causation of diarrhea

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ABSTRACT

Interaction among host gut microbiome and disease-causing organisms play an important role in the establishment of infection. Considering it, the relationship between the concentration of *Lactobacillus reuteri* and the level of *Salmonella* infection was assessed in the feces of post weaning piglets, in reference to diarrheic (sick) and healthy piglets. Eighty fecal samples from piglets (40-50 days of age) of eight different pig farms were examined. Piglets were classified as sick (diarrhea) and healthy (norm) by Indikal indicator paper method. Fecal samples were processed for the extraction of genomic DNA of *Lactobacilli* and *Salmonella* and subjected to real-time PCR to detect concentration of microorganisms in the sample. The results indicated that *Salmonella* infection did not directly affect the development of post weaning diarrheal syndrome in piglets. Positive correlation between the presence of *L. reuteri* and the PCR prevalence of salmonellosis in piglets was puzzling, since this contradicts a lot of literature data. The relationship between opportunistic intestinal microbiota and *Lactobacilli* of normal microflora is more complex under conditions of long-term coevolution. The probiotics based on *L. reuteri* may be more effective, provided that there was no long-term adaptation of *Salmonella* to *Lactobacilli* of this species.

Keywords: Diarrheal syndrome, *Lactobacilli reuteri,* Post-weaning, Piglets, *Salmonella*

Currently, a very promising direction in improving the gut health is the use of probiotics and prebiotics, which contribute to the normalization of the intestinal microflora. *Lactobacillus reuteri* (*L. reuteri*) is one of the dominant species of piglet gut microbiome. *L. reuteri* is a heterofermentor and possesses specific inherent enzymatic processes which produce short-chain fatty acids: acetic acid and lactic acid, while releasing carbon dioxide and ethanol, creating a low pH in the intestinal lumen. (Afonyushkin *et al*. 2013, Ovcharova and Petrakov 2018). *L. reuteri* can inhibit the growth of pathogenic microorganisms in humans and animals, by inducing the production of butyric acid, which has antitumor properties, and is also a source of energy for enterocytes (Zakharova *et al*. 2018). The protective functions of *L. reuteri* are directly related to the production of reuterin which have an inhibitory activity towards many bacteria including *Escherichia, Salmonella, Shigella, Proteus, Pseudomonas, Clostridium, Staphylococcus, Streptococcus,* and *H. pylori*. (Dobrogosz *et al*. 1989, Dobrogosz and Lindgren 1996, Casas and Dobrogosz 2000).

Post weaning is a critical phase in which piglets are at risk of variety of enteric infections. This may be due to substantial change in gut microbiome including significant reduction in the number of *Lactobacilli* (Frese *et al*. 2015, Bernad-Roche *et al*. 2021). Therefore, during these disturbed states of gut microbiota, piglets are more susceptible to enteric pathogens like Salmonella (Gresse *et al*. 2021). In pigs, *Salmonella* infections are frequently asymptomatic, along with continuous or intermittent fecal shedding over long periods of time, regardless of clinical infection (Soliani *et al*. 2023, Drumo *et al*. 2016). Understanding the changes occurring in the host gut microbiome at the post

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weaning stage due to *Salmonella* infection is of paramount importance with respect to understand the gut health, feed utilization, and development of piglets. Considering the knowledge gap in this aspect, the present study was conducted to investigate the relationship between the level of *Salmonella* infection in piglets and the prevalence of *Lactobacillus reuteri* with reference to development of post weaning diarrhea development in piglets.

MATERIALS AND METHODS

Place of work: The work was carried out at molecular biology sector-based institution the Federal State Budgetary Institution of Science Siberian Federal Scientific Center for Agrobiotechnology of the Russian Academy of Sciences (630501, Novosibirsk region, Novosibirsk district, Krasnoobsk work settlement Tsentralnaya str., 7). The objects of the study were piglet feces provided by eight different pig farms located in the south of the Russian Federation (warm weather conditions, temperate continental climate).

Sampling methods: 80 fecal samples (10 from each farm) of piglets aged 40-50 days (a period of development of the post-weaning diarrheal syndrome), were examined. Feces were collected using a sterile disposable spatula into a disposable sterile vial at the time of natural defecation or directly from the rectum. Fecal samples were taken separately from sick (43) and healthy (37) piglets following their clinical status (Supplementary table 1)

Assessment of fecal moisture in piglets: The assessment of the moisture content of the feces was carried out using an original method based on indicator paper, Indikal. The technique utilizes a filter paper coated with a finely dispersed powder of a water-soluble dye coated with a polymer on an adhesive basis (Parlyuk *et al*. 2019).

When a fecal sample (100 µg) was applied on indicator paper, a wet spot formed on the surface of the paper. The volumetric moisture index was calculated by the formula:

$$X = \frac{S}{m}$$

X: Volumetric moisture index,
S: Spot area, (mm^2),
m: Mass of the feces sample, (g)

DNA extraction and Real time PCR: The isolation of genomic DNA was carried out by the silica-sorption method (Boom *et al*. 1990). Information of Primers and Taqman probe used to detect *L. reuteri and S. enterica* and reaction mixture condition are mentioned in the supplementary Table 2. (Afonyushkin *et al*. 2011).

The PCR reaction mixture was comprised of primers (0.30.3 µM), Taqman probe (0.1µM), Tris-HCL (pH 8.5 at 25°C) (60 mM), $MgCl_2$ (1.5 mM), 2-mercaptoethanol (10 mM), Triton X -100 (0.1%), HS-Taq, DNA polymerase (Biolabmix) (1 U) and dNTP (0.2-0.3 mM). Amplifications were carried out in 25 µL volumes in a CFX96 Touch Real-Time PCR Detection System with the cycling conditions for *L. reuteri* includes-denaturation at 95°C for 3 min, followed by 42 cycles 95°C for 15 sec and 61°C for 35 sec and for *S. enterica* first denaturation at 95°C for 6 min, followed by 45 cycles 95°C for 12 sec and 66°C for 40 sec. To quantify the content of *L. reuteri*, in genomic equivalents, the DNA of the *L. reuteri* culture, was initially characterized for the CFU content in the initial bacterial suspension. The sensitivity of the test system was 10^3 CFU/mL *L. reuteri.* The test showed linearity in the concentration range of *L. reuteri* $1x10^7$-$1x10^3$CFU/mL ($r_2 = 0.992$).

Statistical data processing: The accumulation, correction, systematization of the initial information, and the visualization of the results were carried out in Microsoft Office Excel 2016 spreadsheets. Statistical analysis was carried out using the STATISTICA 13.3 program (developed by StatSoft.Inc). The results of *S. enterica* prevalence between clinical status and *L. reuteri* abundance were compared by the Odds Ratio (OR) and 95% confidence intervals were calculated using MedCalc Software. The Pearson correlation coefficient using *L. reuteri* concentration per g. feces and Salmonellosis cases data was calculated in the MS Excel software. Differences were considered significant at $p<0.05$. Chi-square tests were employed to evaluate differences in clinical status of piglets and *Salmonella* prevalence in fecal sample.

RESULTS AND DISCUSSION

Fecal moisture assessment of piglets: The equal weights of fecal samples were placed on 70 mm square of indicator

Table 1. Group wise (sick and healthy) prevalence of *L. reuteri* and *S. enterica* in fecal samples of piglets and relationship between the prevalence of *Salmonella* and the mean concentration of *L. reuteri* in the group of sick and healthy animals

Name	Number of positive samples %					
	L. reuteri		*S. enterica*			
	Sick, %	Healthy, %	Sick		Healthy	
			%	M (*L.reuteri*), Log_{10} GE/5 mkl±SD	%	M (*L.reuteri*), Log_{10} GE/5 mkl±SD
Pig farm 1	88.8	88.8	33,3	6.20±0.69	33,3	6.39±0.26
Pig farm 2	85.7	71.4	42,9	6.88±0.77	57.1	5.93±0.42
Pig farm 3	80.0	100.0	0,0	5.48±1.13	20,0	6.13±0.37
Pig farm 4	100.0	90.0	0,0	5.24±2.0	0.0	6.20±2.54
Pig farm 5	60.0	46.7	28.5	7.31±0.38	6.66	7.48±0.53
Pig farm 6	80.0	70.0	60.0	3.95±0.44	90.0	3.62±1.75
Pig farm 7	20.0	100.0	80.0	5.04±2,71	40,0	3.13±0.85
Pig farm 8	100,0	50,0	85.7	3.74±0.85	80.0	4.13±1.98

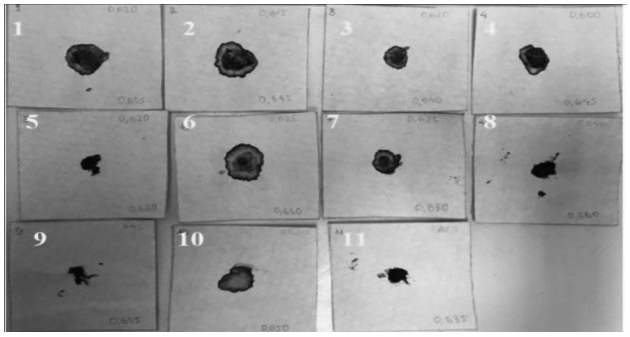

Fig. 1. Indicator paper showing dyed spot due to fecal samples

paper "Indikal", as shown in Fig. 1. As per volumetric moisture index, the highest moisture content is observed in the feces of sick animals (supplementary Table 03). The average value of the index in sick animals was 6 times higher than that of healthy animals so instrumentally and visually, they were classified as diarrhea (sick) and norm (healthy).

Note: Feces of normal consistency (sample 4, 5, 8, 9, 11), feces showing signs of diarrhea (samples no. 1, 2, 3, 6, 7, 10) and the calculated volumetric index of these samples are mentioned in the Supplementary Table. 4

Assessment of Salmonella and L. reuteri prevalence and their association: Positivity of *Salmonella* infection in piglets can be grouped as lower infection (0 to 28.5% positivity, in three farms) and higher infection (in remaining five pig complexes ranged from 33.3% to 95%). The majority of piglets included in both the healthy and the sick groups have *L. reuteri* in the gastrointestinal tract. One pig farm (Farm 7) found to have comparatively fairly low level of *Lactobacilli* in the group of animals infected with *Salmonella*. Assessment of the presence of a relationship between the average concentration of *L. reuteri* and the level of their infection with *Salmonella* was also carried out separately for each group of animals (sick pigs, healthy pigs) (Table 1).

Analysis of the relationship between the Salmonella positivity and the occurrence of the diarrheal syndrome: Out of eight investigated pig farms, three farms (farm 5, 7 and 8) showed positive association between *Salmonella* positivity and the occurrence of diarrheal syndrome in piglets) (Table 2).

Analysis of the influence of the concentration of Lactobacilli on Salmonella prevalence (%): Both healthy and the sick groups had *L. reuteri* in the fecal samples. Data from one of the pig farms indicate a fairly low level of *Lactobacilli* only in the group of animals infected with *Salmonella* (Table 2).

Assessment of presence of a relationship between the average concentration of *L. reuteri* and the level of their infection with *Salmonella* separately was carried out in the group of sick and healthy animals by calculating the Spearman's rank correlation coefficient. A direct non-linear relationship between the mean *L. reuteri* concentration and the *Salmonella enterica* infection rate of sick piglets was

Table 2. Association between diarrheal syndrome in piglets and the presence of *Salmonella*, odds ratio (OR, CI 95%) in different pig farms

Pig Farms	OR	S	Lower bound 95% CI	Upper bound 95% CI
Pig farm 1	1	1.0	0.141	7.099
Pig farm 2	0.56	1.08	0.68	4.67
Pig farm 3	0	-	0.00	-
Pig farm 4	0	-	0.00	-
Pig farm 5	5.6	1.19	0.541	57.95
Pig farm 6	1.17	0.82	0.234	5.81
Pig farm 7	4	0.55	0.55	29.09
Pig farm 8	2.25	1.32	0.17	29.77

seen with the Spearman's correlation coefficient (r_s) 0.899. We unexpectedly discovered, relationship between the studied traits, the closeness of the relationship according to the Chaddock scale was high. In the group of healthy animals, moderate closeness of the relationship between the rates of infection with *Salmonella* and the concentration of *Lactobacilli*, was indicated by Spearman's correlation coefficient (r_s) i.e. 0.595 along with a noticeable tightness of communication on the Chaddock scale.

The obtained result indicated that during post weaning period with an increase in the number of *L. reuteri* in the gastrointestinal tract of piglets, the level of infection with *Salmonella* decreases. An obvious conclusion is that the higher the concentration of *Lactobacilli*, the lower the infection rate in the piglets, this picture is expressed in most of the pig complexes.

To assess the strength of the connection and the significance of the differences between the two compared indicators - the concentration of *Lactobacilli* in the fecal samples and the occurrence of the diarrheal syndrome and the relationship between the level of *Salmonella* infection and the occurrence of piglet diarrheal syndrome in the post-weaning period in piglets in the post-weaning period, we calculated the criteria for assessing the significance of differences in outcomes.

When comparing the obtained values of the chi-squared test, chi-squared test with Yates's correction, Fishers' exact tests with the critical ones, it was found that the relationship between *Salmonella* infection and the occurrence of piglet diarrheal syndrome has no statistical significance.

Odds ratio, analysis indicated that the lack of probability of the influence of the presence of *Lactobacilli* in piglets on the level of infection with *Salmonella* and the strength of the connection was insignificant.

The data obtained indicated that the likelihood of exposure to such a factor as the absence of *Lactobacilli* in the feces does not affect the increase in the level of *Salmonella* infection in piglets.

L. reuteri attracts the attention of many researchers based on its specific enzymatic processes. *Lactobacilli* can inhibit the growth of pathogenic microorganisms of the gastrointestinal tract of animals and humans. This

bacterium is distinguished by its ability to synthesize reuterin, which, in turn, is capable of inhibiting the growth of a large number of opportunistic, pathogenic and other microorganisms, as well as fungi.

Visual and instrumental examination of fecal samples showed highest moisture content in the group of sick animals. Similarly, the index of the ratio of spot area on the paper to the mass of feces, showed 6 times higher value in sick compared to the index of healthy ones.

Based on the data obtained, the obtained samples were divided according to the characteristics of «diarrhea», «norm». The results showed good congruence with the visual assessment of the clinical status in piglets. The advantage of the proposed method can be a quantitative assessment of fecal moisture, which has prospects for analyzing the influence of various factors on the course of the diarrheal syndrome in piglets.

The analysis of the existence of the relationship - the odds ratio (OR) between the infection of piglets with *Salmonella* and the occurrence of diarrheal syndrome showed the presence of such relationship in three piglet farms, out of eight. Nevertheless, in three piglet farms, where OR> 1, the infection of piglets with *Salmonella* did not significantly increase the risk of developing the diarrheal syndrome.

Piglets are the reservoir of many *Salmonella* serotype which are zoonotic so the presence of *Salmonella*, depending on the serotype, can have a sanitary significance, since with a high level of infection of piglets with *Salmonella*, the risk of contamination of meat and offal increases significantly.

Evaluating the existence of a relationship between the presence of *L. reuteri* in the feces of piglets and the level of *Salmonella* infection, it was found that at one of eight piglet farms, the frequency of occurrence of *Lactobacilli* in the group of healthy animals was 100%, while in the group of sick it was only 15%. Based on these data, one can assume the existence of a direct relationship between the presence of *Lactobacilli* and *Salmonella* infection. However, at another piglet farm, the opposite picture is observed, and at the other six, there is no clear connection at all.

Salmonella infection of sick and healthy piglets did not differ in all pig farms; therefore, *Salmonella* might not be the cause of diarrhea in these pig farms. The fecal positivity and recovery concentration may vary widely in post weaning piglets as number of factors may contribute to the health status of piglets (Ainslie-García *et al*. 2018, Nair *et al*. 2020). Postweaning diarrhea is a multifactorial issue and other than microbial causes there are factors including housing factors, environmental temperature, stocking density, feeding regimen, and parity also play important role. (Hou *et al*. 2015, Rhouma *et al*. 2017). However, only some of weaned piglets develop this disorder and that highly dependent up on faecal microbiota transplantation as it positively or negatively affects the postweaning diarrhea development (Karasova *et al*. 2021).

Thus, based on the results obtained in both groups studied, it can be stated that the average concentration of *Lactobacilli* may influences the level of *Salmonella* infection. But the odds ratio results indicated no probability of the influence of the presence of *Lactobacilli* in piglets on the level of infection with *Salmonella*. Guerra-Ordaz *et al.* 2014 observed no significant effect of dietary supplementation *L. plantarum* in diet of weaned piglets on reduction of incidence of diarrhoea and colonic *E. coli* counts (Guerra-Ordaz *et al*. 2014).

The fact that salmonellosis in piglets can be asymptomatic makes their diagnosis difficult and demotivates owners to fight this disease. (Bonardi 2017). On the other hand, the lack of relationship between the presence of *Salmonella* and diarrheal syndrome disregards the persistent infection, but the transient feed infection. In the latter case, the reason for the lack of antagonistic activity of *L. reuteri in vivo* can be explained. At the same time, the positive correlation between the presence of *L reuteri* and the PCR prevalence of salmonellosis in piglets is puzzling, since this contradicts a lot of literature data. It seems obvious that *Salmonella*, which is constantly present in pig herds, must be resistant to normal intestinal microflora, including *L. reuteri*. The wide spectrum of antagonistic activity of *L. reuteri* should imply a high probability of suppressing other bacteria that are antagonistic to *Salmonella* (Afonyushkin *et al*. 2016). Many studies support the effect of probiotics in modifying or replenishing the gut microbe for prevention and control of infection and growth promotion (Yu *et al.* 2019). However, the inefficacy is also not uncommon as many studies indicated no effect of probiotic including *Lactobacillus rhamnosus* GG supplementation in the prevention of development of diseases (Bruzzese *et al*. 2018, Prantera *et al.* 2002).

The relationship between opportunistic intestinal microbiota and *Lactobacilli* of normal microflora is more complex under conditions of long-term coevolution. It is possible that the use of probiotics based on *L. reuteri* may be more effective, provided that there is no long-term adaptation of *Salmonella* to *Lactobacilli* of this species. In the surveyed pig farms in the Russian Federation, *Salmonella* infection did not significantly affect the possibility of post-weaning piglet syndrome. A positive correlation has been established between the average concentration of *L. reuteri* and the level of *Salmonella* infection in the group of sick piglets, which should be considered as an important factor in reducing *Salmonella* infection in piglets. The probiotics based on *L. reuteri* may be more effective, provided that there was no long-term adaptation of *Salmonella* to *Lactobacilli* of this species.

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