Quantitative comparison of some faecal bacterial communities in groups of Mangalica and commercial pigs

Károly Tempfli[1*], *Emil Balázs* Herceg[1], *Klaudia* Szalai[1], *Loretta* Szabó-Sárvári[1], *László* Pongrácz[1], and *Ágnes* Bali Papp[1]

[1]Department of Animal Sciences, Faculty of Agricultural and Food Sciences, Széchenyi István University, 2 Vár square, 9200 Mosonmagyaróvár, Hungary

Abstract. Different housing technology, breed, age and nutrition can contribute to changes in the composition of microbial communities in pigs. Faecal samples from groups of Mangalica and commercial pigs were collected and analysed by qPCR in order to identify changes and differences regarding the quantity of total faecal bacteria, *Prevotella* genus, *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp. and the family *Enterobacteriaceae*. In both Mangalica and commercial pig samples, quantities of total faecal bacteria increased from weaner pigs to lactating sows. The relative quantity of total bacteria was larger ($p<0.05$) in Mangalica growers and lactating sows compared to commercial pigs. The ratio of *Prevotella* genus in total bacteria was higher ($p<0.05$) in Mangalica growers and lower in Mangalica lactating sows compared to respective commercial groups. The ratio of *Lactobacillus* spp. was largest ($p<0.05$) in samples of Mangalica boars, whereas ratios of *Bifidobacterium* spp. were greater ($p<0.05$) in Mangalica weaners, growers, and boars. Faecal samples of Mangalica growers contained a higher ratio of *Enterobacteriaceae* in total bacteria, whereas *Enterococcus* spp. was more prevalent in commercial weaner pigs and boars ($p<0.05$). Considerable changes in faecal bacteria communities were observed in association with different age and utilization.

1 Introduction

Despite the popular quote attributed to Hippocrates that ‘all disease begins in the gut’ the significance of bacterial composition of the gastrointestinal tract (GIT) was overlooked for centuries, primarily because early research mainly focused on pathogenic microbes, furthermore, because available technological conditions were lacking in processing capacity required for the analysis of such complex systems. The microbiota and especially bacteria of the GIT are now recognized for their roles in the development of the immune system, the susceptibility to infections and inflammations, obesity, allergies or asthma, deeply intertwining with lifelong health [1].

* Corresponding author: tempfli.karoly@sze.hu

Studies investigating the microbial composition of the GIT of farm animals multiply rapidly and results are gaining practical importance since an increasing amount of evidence confirms that gut microbiota plays crucial parts in animal health development and production. A notable proportion of the microbiota offers nutritional, developmental, and immunological benefits to the animal, while on the other hand commensal microbes are in competition with the host for resources [2].

In the present study five bacterial genera were selected in order to investigate their potential associations with age and utilization type in different Mangalica and commercial pig groups.

The *Prevotella* genus is widely prevalent in the large intestine, and moderately prevalent in the small intestine of pigs. The *Prevotella* enterotype is one of the most common pig enterotypes. In piglets after weaning, a *Prevotella*-dominant enterotype has been associated with advantages in animal health and production. Abundance of *Prevotella* in pig GIT correlated with increased daily feed intake and average daily gain; however, feed efficiency was lower in *Prevotella*-dominant pigs [3].

Several strains of the *Lactobacillus* genus are gaining wider acceptance in farm animal nutrition and medicine as feed additives that promote both health and production. *Lactobacillus* species have been shown to improve GIT homeostasis and feed efficiency, and moderate the colonization of pathogenic bacteria. Supplementation can be especially beneficial during the weaning period when piglets are exposed to considerable stress and GIT dysbiosis [4].

Beside *Lactobacillus* species, the *Bifidobacterium* genus is also widely applied as animal-feed probiotics. [5] found that *Bifidobacterium* was more prevalent in wild boars compared to commercial animals, and suggested that domestication and grain-based nutrition may have resulted in the predominance of *Lactobacillus* over *Bifidobacterium* in commercial pigs.

The *Enterobacteriaceae* family consists of non-pathological commensal species and pathogens. Several species and strains of this family (e. g. *Escherichia coli*) are associated with diarrhoea and various inflammations (e. g. mastitis) in pigs. The composition and quantity of the *Enterobacteriaceae* family greatly differs between individual pigs [6].

The *Enterococcus* genus involves species that are commonly used as probiotics (e. g. *E. faecium*) and improve feed conversion and growth rate in pigs [7], whereas the emergence of antibiotic-resistant *Enterococci* causes concerns in human infections.

While the sampling of different fractions of the GIT often requires the sacrifice of experimental animals, faecal sampling provides a non-invasive method for the analysis of bacterial communities; therefore, the associations between faecal and GIT microbiota are intensively studied in several animal species [8]. Faecal microbiota profile showed definite similarity (75%) to the large intestine and moderate similarity (38%) to the small intestine in Large White pigs [9]. Sampling and monitoring of stool microbial communities is a prerequisite of faecal microbiota transplantation, an emerging procedure in the pig industry to improve performance and disease resilience through modulating or restoring GIT homeostasis.

The indigenous Hungarian Mangalica is considered one of the fattest pig breeds in the world that lags behind modern commercial meat-type pigs regarding growth and lean meat content; nonetheless, Hungarian Mangalica production is increasing due to superior meat quality and breed conservation efforts.

The aims of this preliminary study were (1) to identify potential differences in the quantity of selected bacteria between Mangalica and commercial pigs under general conditions of production, furthermore, (2) to detect age-related changes in faecal bacteria quantities between various age groups of pigs.

2 Materials and methods

2.1 Animals and sample collection

Four different age or utilization groups of healthy Blonde Mangalica and commercial pigs were sampled. The groups included weaner (15-20 kg body weight; n=10 Mangalica, n=10 commercial pigs) and grower pigs (60-80 kg; n=10-10), lactating sows (n=10-10), and boars (n=5-5). Commercial groups involved Hungarian Large White × Hungarian Landrace F_1 sows and Duroc boars; whereas commercial weaner and grower pigs originated from F1 sow and Duroc boar crosses. Commercial sows routinely received amoxicillin treatment via intramuscular injection (Betamox LA, Norbrook, UK) within one day after farrowing. Commercial weaner pigs were delivered oral amoxicillin supplementation (Amoxyveto, V.M.D., Belgium) via drinking water for five days after weaning. Commercial grower pigs and boars, and Mangalica groups were not administered antibiotics.

Faecal samples were freshly collected after defecation in 2 ml cryogenic tubes (DNase-free). Approximately 500 mg of each sample was washed and centrifuged in 1.5 ml Phosphate buffered saline (PBS) followed by homogenization in lysis buffer by TissueLyser LT (Qiagen, Germany) with 2 mm bashing beads (Zymo Research, USA). Microbial genomic DNA was isolated using Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) following the manufacter's instructions. DNA quantity was determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

2.2 Molecular biology techniques and analysing bacterial communities

A total of 100 ng DNA was used in subsequent quantitative PCR (qPCR) on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) with Maxima SYBR Green Master Mix (Thermo Fischer Scientific, USA). The reactions composed of 12.5 µl master mix, 1-1 µl of forward and reverse primers (0.3-0.3 µM concentration) (Table 1), 2 µl DNA template, and nuclease-free water up to 25 µl total volume. The PCR condition was as follows: initial denaturation of one cycle at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at specific temperature (Table 1) for 30 s, and extension at 72 °C for 30 s. The reactions were finished by melting analyses: the samples were exposed to gradually increasing temperature between 65 and 95 °C in order to determine the specific melting temperature where the fluorescent signal decreases substantially. Identical melting temperature of the PCR products indicates high specificity of the applied primers. Each reaction included no template controls (NTC). The $2^{-\Delta Ct}$ method was applied for the comparison of relative total bacteria quantity of the analysed groups.

PCR products were purified by Wizard SV Gel and PCR Clean-up System (Promega, USA) and diluted tenfold in order to obtain standards. Diluted standards provided information for PCR efficiency and facilitated the calculation of copy numbers. The formula published by [10] was used to calculate the copy number of standards.

The calculation of the copy number of standards facilitated the quantification of unknown samples. The ratio of *Prevotella*, *Lactobacillus* spp., *Bifidobacterium* spp., *Enterobacteriaceae*, and *Enterococcus* spp. in total bacteria was calculated based on quantities of copy numbers.

The SPSS Statistics for Windows v.20.0 (IBM Corp., USA) package was applied for statistical analysis. Normal distribution was determined by Shapiro–Wilk tests. The Mann–Whitney test was run on relative quantity values for total bacteria, whereas the Mann–Whitney or the independent samples t-test was applied for the detection of significant

($p<0.05$) differences between group means of *Prevotella*, *Lactobacillus* spp., *Bifidobacterium* spp., *Enterobacteriaceae*, and *Enterococcus* spp. ratio in total bacteria.

Table 1. Primers employed in this study and qPCR efficiency for the target bacterial groups.

Target	Primer sequence (F, R; 5'-3')	Annealing (°C)	Length (bp)	Efficiency (%)
Total bacteria [11]	GTGSTGCAYGGYYGTCGTCA, ACGTCRTCCMCNCCTTCCTC	55	147	98.2±5.0
Prevotella [12]	CACRGTAAACGATGGATGCC, GGTCGGGTTGCAGACC	59	121	92.7±3.5
Lactobacillus [13]	AGAGGTAGTAACTGGCCTTTA, GCGGAAACCTCCCAACA	59	391	91.9±5.3
Bifidobacterium [14]	CGCGTCCGGTGTGAAAG, CTTCCCGATATCTACACATTCCA	59	126	95.1±4.4
Enterobacteriaceae [15]	ATGGCTGTCGTCAGCTCGT, CCTACTTCTTTTGCAACCCACTC	59	385	90.9±4.7
Enterococcus [16]	CCCTTATTGTTAGTTGCCATCATT, ACTCGTTGTACTTCCCATTGT	59	144	91.5±5.1

3 Results and discussion

3.1 Relative quantity of total bacteria

The quantity of total bacteria in faecal samples increased with age in both Mangalica and commercial pigs (Figure 1). Highest quantities were observed in the lactating sow group in Mangalica, and in the commercial boar group. The relative quantity of total bacteria was significantly ($p<0.05$) higher in Mangalica growers and lactating sows than in the same commercial groups.

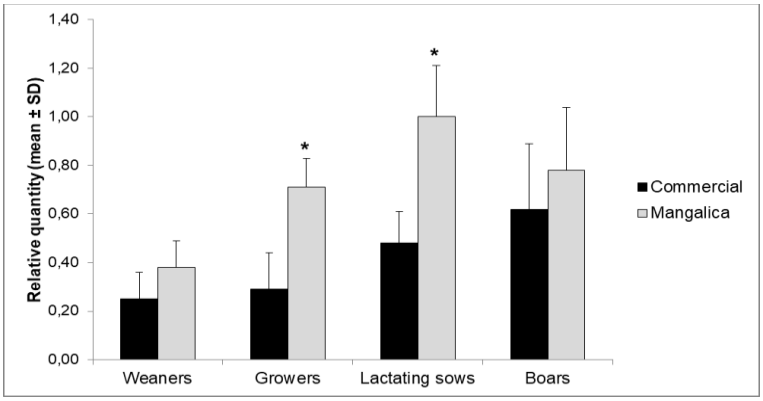

Fig. 1. Relative quantity of total bacteria in different groups of Mangalica and commercial pigs.

Notes: * indicates that means within the group differ significantly ($p<0.05$)

Larger total bacteria quantities were observed in commercial domesticated pigs compared to wild animals (e. g. wild boars and Red River hogs) in a former study [5]. Taxonomic richness increased with the age of pigs during the first month of life [17], furthermore, faecal microbiota richness significantly increased from 25 to 240 days of age [18]. Based on the present results it can be suggested that increasing age-related richness associates with increased total bacteria quantity, as well. In this study, Mangalica and

commercial pigs were sampled at comparable body weight; however, Mangalica weaners and growers were notably older at this stage. Overall, Mangalica requires remarkably more time to reach the slaughter weight of 110-130 kg (8-10 months for Mangalica, 5-6 months for commercial pigs) due to characteristic physiological differences.

Regarding that the relative number of total bacteria differed considerably between age and utilization groups the comparison of subsequent genera was done by calculating their ratio (%) in total bacteria.

3.2 *Prevotella* ratio

The ratio of *Prevotella* in total bacteria was largest in Mangalica growers and lowest in Mangalica boars. The ratio of *Prevotella* was significantly ($p<0.05$) higher in Mangalica compared to commercial grower pigs, whereas samples of commercial lactating sows showed higher ($p<0.05$) ratio compared to Mangalica sows (Figure 2). The ratio of *Prevotella* did not differ ($p>0.05$) between Mangalica and commercial weaner pigs or boars.

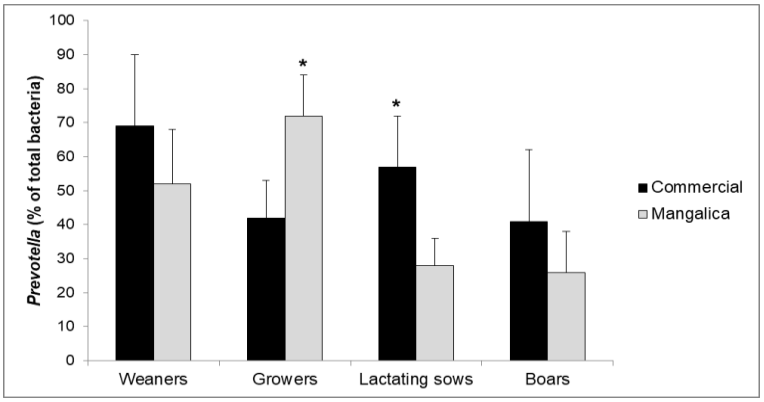

Fig. 2. Ratio of *Prevotella* genus (percentage of total bacteria; mean ± SD) in various groups.

* indicates that means within the group differ significantly ($p<0.05$)

Along the GIT of pigs the microbiota was roughly divided into three parts by [19]: the duodenum-jejunum section was dominated by *Lactobacillus* and *Bacteroides*, *Fusobacterium* and *Escherichia* were most prevalent in the ileum, while *Prevotella* dominated the cecum-rectum section. The abundance of *Prevotella* changes considerably with age: during the suckling and the finishing period *Prevotella* ratio is moderate around 10% in the GIT, whereas pigs around weaning show the highest ratio (25-50%) of Prevotella in the GIT [3]. In accordance with these observations, faecal samples of commercial weaner pigs produced the highest ratio of *Prevotella*; however, the ratio in the Mangalica group was highest in growers potentially indicating different breed-related patterns. In German Landrace and Pietrain crossbred barrows, *Prevotella* was exclusively associated with polysaccharide breakdown in pigs and was abundant in faecal samples irrespective of nutritional treatments with high-fat/low-fiber or low-fat/high-fiber diets [20]. A higher ratio of *Bacteroidetes* phylum – generally dominated by the *Prevotella* genus – was associated with obesity in human twin studies and mice [21], consistent with the present observation that *Prevotella* ratio was significantly ($p<0.05$) higher in Mangalica compared to commercial lean-type growers; although the ratio in sows showed an opposite pattern.

3.3 *Lactobacillus* ratio

The ratio of *Lactobacillus* spp. varied between 0.24±0.12 and 1.30±0.39 % of total bacteria. Overall, Mangalica faecal samples contained larger ratios of *Lactobacillus* spp. compared to relevant commercial groups; however, significant ($p<0.05$) difference was only detected for boars (Figure 3).

The abundance of the *Lactobacillus* genus showed gradual increase with age in the first month during the suckling period [17]. Negative correlations were detected between *Lactobacillus* and *Prevotella* in different age groups of a previous publication [22], consistent with the results obtained in the commercial groups of the present study (Figure 2 and 3); however, this association was not observed in Mangalica. We detected in this study that the relative quantity of *Lactobacillus* increased from weaners to growers, but decreased in older groups, which was consistent with the findings of a previous study [18] that reported that *Lactobacillus* abundance in faecal samples of crossbred pigs (crosses of Western and Chinese breeds, e. g. Landrace, Large White, Duroc and Erhualian, Bamaxiang, and Laiwu) greatly increased from 25 to 80 days of age, then decreased in subsequent age groups.

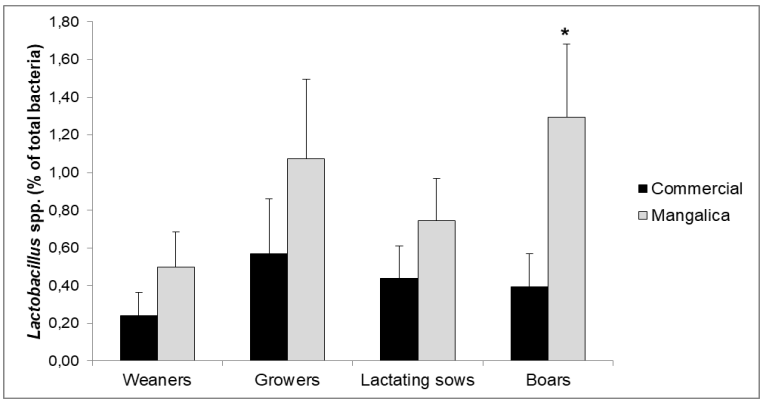

Fig. 3. Ratio of *Lactobacillus* spp. (percentage of total bacteria; mean ± SD) in various groups.

Notes: * indicates that means within the group differ significantly ($p<0.05$).

3.4 *Bifidobacterium* ratio

Similarly, to total bacteria, *Bifidobacterium* spp. ratio showed an increasing trend with age in both Mangalica and commercial groups (Figure 4). The *Bifidobacterium* spp. ratio was observed between 0.021±0.006 and 1.175±0.353 % of total bacteria. Higher ($p<0.05$) ratios occurred in Mangalica weaners, growers and boars compared to respective commercial groups, whereas lactating sows did not differ ($p>0.05$) despite the administration of antibiotics in the commercial group.

The *Lactobacillus* and *Bifidobacterium* genera were suggested as potential biomarkers of intestinal health. Dietary fibres have been associated with GIT health promotion and beneficial effects on the ratio of these genera, as also suggested by present results: an increasing dietary crude fiber content from weaners to sows resulted in increasing *Bifidobacterium* ratio in both Mangalica and commercial groups. A high-fat/low-fiber diet resulted in a remarkable decrease of the *Bifidobacterium* ratio compared to the low-fat/high-fiber group [20].

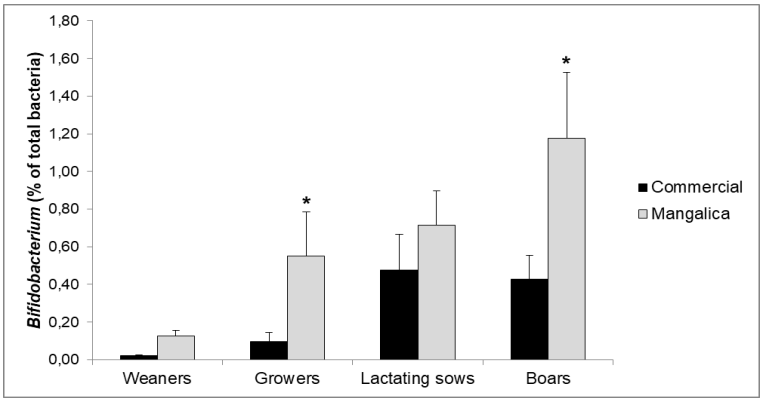

Fig. 4. Ratio of *Bifidobacterium* spp. (percentage of total bacteria; mean ± SD) in various groups.

* indicates that means within the group differ significantly ($p<0.05$)

3.5 *Enterobacteriaceae* ratio

A gradual increase was observed from commercial weaners to boars regarding *Enterobacteriaceae*, whereas Mangalica ratios did not follow an age-related pattern. Samples of Mangalica growers contained a higher ($p<0.05$) ratio of *Enterobacteriaceae* compared to faecal samples of the commercial animals (Figure 5).

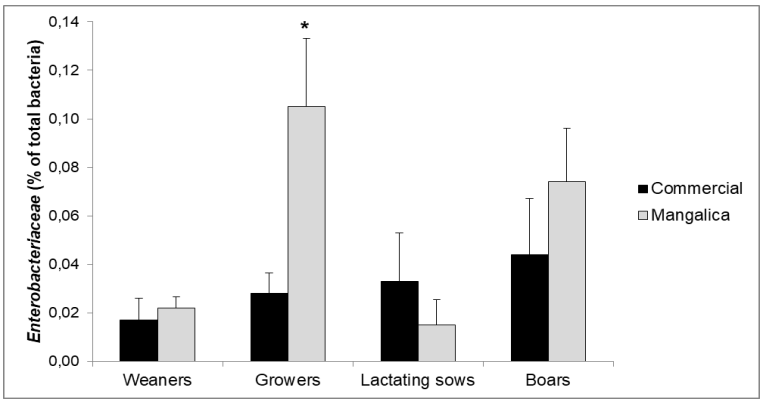

Fig. 5. Ratio of *Enterobacteriaceae* (percentage of total bacteria; mean ± SD) in various groups.

Notes: * indicates that means within the group differ significantly ($p<0.05$).

Overall, *Enterobacteriaceae* ratio was generally higher in Mangalica samples in every group excluding lactating sows. A diet lower in fat and high in fibres resulted in the decrease of *Enterobacteriaceae* ratio in pig faecal samples that indicates the health-promoting role of dietary fibres and the mitigative effects on pathogenic bacteria colonisation [20]. *Enterobacteriaceae* was one of the most prevalent families in the small intestine of Large White pigs, and showed a decreasing ratio in the consecutive segments of the GIT [9]. The faecal samples of free-roaming Gabonese pigs contained higher abundance of *Enterobacteriaceae* compared to commercial pigs and domesticated wild boars, and domestication may have potentially increased the prevalence of the genus [5]. A higher ($p<0.05$) *Enterobacteriaceae* ratio in faecal samples of Mangalica growers contradicts the

conception that Mangalica shows moderate consequences of domestication and is closer to wild boars than commercial pigs regarding GIT bacteria composition.

3.6 *Enterococcus* ratio

The ratio of *Enterococcus* spp. was highest ($p<0.05$) in commercial weaner pigs, while other groups did not differ ($p>0.05$). Commercial weaner and boar samples were characterized by a higher ($p<0.05$) *Enterococcus* spp. ratio compared to the same Mangalica groups (Figure 6).

A previous study [9] found that *Enterococcus* was one of the most differentiating genera between faecal and intestinal microbiota of pigs, and suggested that this genus plays roles in the digestion, primarily in the small intestine.

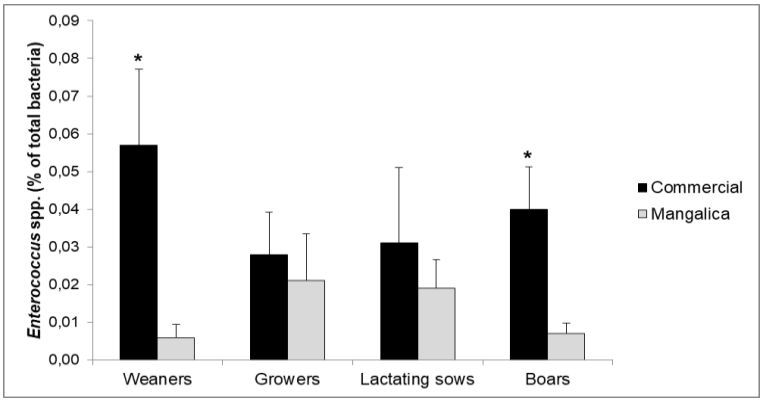

Fig. 6. Ratio of *Enterococcus* spp. (percentage of total bacteria; mean ± SD) in various groups.

Notes: * indicates that means within the group differ significantly ($p<0.05$)

4 Conclusion

Overall, Mangalica and commercial pigs demonstrated remarkably similar age-related patterns in the experimental groups of weaner and grower pigs, lactating sows and boars regarding total bacteria, *Lactobacillus* and *Bifidobacterium* communities. Discrepancies in the trends of *Prevotella*, *Enterobacteriaceae* and *Enterococcus* ratios between Mangalica and commercial groups indicate potential breed-related characteristics in the changes of GIT composition. Further studies with modified experimental design (e. g. identical housing technology, nutrition, and sampling age) are needed to clarify reasons behind these observations. In the last decade the research on pig microbiome has expanded remarkably; however, the majority of relevant experiments mainly focus on commercial pig breeds, whereas the present study provides novel information regarding the faecal quantity of selected microbial communities in the indigenous Hungarian Mangalica pig breed.

Acknowledgements

This work was supported by the EFOP-3.6.3-VEKOP-16-2017-00008 project. The project is co-financed by the European Union and the European Social Fund.

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