

ORIGINAL ARTICLE

Effect of mixed rearing of barrows and gilts on backfat thickness and serum metabolite profiles of the Kagoshima-Kurobuta (Berkshire) pig

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Abstract

We investigated the effect of mixed rearing of barrows and gilts on the backfat thickness and the serum metabolite profiles of Kagoshima-Kurobuta (Berkshire) pigs. A total of 149 pigs with an average body weight of 35 kg were divided into the following groups: 100%, 90%, 70%, 50%, 30%, 10%, and 0% groups consisting of 10 barrows (1 pen), 9 barrows + 1 gilt (3 pens), 7 barrows + 3 gilts (2 pens), 5 barrows + 5 gilts (3 pens), 3 barrows + 7 gilts (2 pens), 1 barrow + 9 gilts (3 pens), and 9 gilts (1 pen), respectively. All pigs were raised to a shipping weight of 120 kg. Mixed rearing significantly reduced ($p < 0.001$) backfat thickness, and the optimum mixing ratio of barrows and gilts was 7:3 (the 70% group). Four types of circulating sex steroids were found in both the barrows and gilts in the 50% group but were not detected in barrows from the 100% group. These results indicated that mixed rearing of barrows and gilts was effective for reducing the backfat thickness of barrows, and induced sex steroid hormones may influence the backfat thickness of barrows in mixed-reared groups.

KEYWORDS

backfat thickness, Kagoshima-Kurobuta pig, mixed rearing, serum metabolites, sex steroids

1 | INTRODUCTION

“Kagoshima-Kurobuta” is the trademarked name of Berkshire pigs raised with a dedicated diet containing sweet potato meal for the finishing stage, in Kagoshima, Japan. Kagoshima-Kurobuta pork is highly regarded by consumers for its superior quality. Berkshire species have a thick subcutaneous fat layer (backfat) compared with other species (Ministry of Agriculture Forestry and Fisheries, 2015). It was also reported that the backfat thickness of barrows is thicker than that of gilts in a survey of 2663 carcasses of Berkshire species (Harada et al., 2006). A pig carcass with excessively deposited backfat will have a lower evaluation score, leading to a decreased profit for pig farm production. It is generally recommended that Kagoshima-Kurobuta

barrows and gilts be raised separately in production farms for general management reasons and for improved growth and pig carcass characteristics. In a previous study, we reduced excessive backfat thickness of barrows by adjusting feeding management (Ohkoda et al., 2017). Our findings suggested that the mixed rearing of barrows and gilts, switching feed early, and lowering the total digestible nutrients (TDN) content of feed for the finishing stage, were effective management methods to raise the percentage of top-grade carcasses of Kagoshima-Kurobuta pigs, by suppressing excessive backfat thickness. The most effective method was the mixed rearing of barrows and gilts in the same pig pen at a ratio of 5:5. However, it is unclear why the mixed rearing caused changes in the backfat thickness of barrows, and the underlying physiological and biochemical mechanisms remain

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unknown. In addition, it is still unknown which rearing ratio of barrows and gilts is the most effective at suppressing backfat thickness of barrows. In this study, we examined the optimal rearing ratio of barrows and gilts in a pig pen to improve the backfat thickness of Kagoshima-Kurobuta barrows. Therefore, first, in this study, we examined the optimal rearing ratio of barrows and gilts in a pig pen to improve the backfat thickness of Kagoshima-Kurobuta barrows.

Metabolomic methods have been widely applied to swine research in recent years (Goldansaz et al., 2017), although they have mainly examined differences in blood biomarkers between swine breeds (Bovo et al., 2016), to study changes in blood composition in response to nutritional conditions (He et al., 2009), and to investigate the taste components of pork (Muroya et al., 2014; Ohkoda et al., 2020). Second, in this study, we applied metabolomic analysis to investigate whether the metabolites of barrows are altered by mixed rearing with gilts. The current study is the first to examine metabolites in the blood of barrows compared with those in gilts.

Our analysis of serum metabolites indicated that mixed rearing altered sex steroid biosynthesis and metabolites related to lipid metabolism in barrows. These findings expand our knowledge on the effects of castration on pork quality. Surgical castration is thought to have a significant effect on the behavior and meat quality of male pigs.

2 | MATERIALS AND METHODS

2.1 | Animal care and grouping

All experimental protocols and procedures were conducted according to the guidelines for the proper conduct of animal experiments by the Science Council of Japan (2006) and under the supervision of a veterinarian. Animal experiments were conducted in an integrated production farm where 450 Berkshire sows were constantly raised. A total of 149 Berkshire pigs (average body weight of 35 kg) were selected and divided into the following seven groups: 100%, 90%, 70%, 50%, 30%, 10%, and 0% groups, consisting of 10, 30, 20, 30, 20, 30, and 9 pigs, respectively. The mixing numbers of barrows and gilts per pig pen for the 100%, 90%, 70%, 50%, 30%, 10%, and 0% groups were 10 barrows ($\times 1$ pen), 9 barrows + 1 gilt ($\times 3$ pens), 7 barrows + 3 gilts ($\times 2$ pens), 5 barrows + 5 gilts ($\times 3$ pens), 3 barrows + 7 gilts ($\times 2$ pens), 1 barrow + 9 gilts ($\times 3$ pens), and 9 gilts ($\times 1$ pen), respectively. Male piglets were surgically castrated at 3 to 7 days of age. For the growing stage (35 to 80 kg body weight [BW]), pigs were fed a commercial grower diet (13.9 MJ/kg ME and 16.0% crude protein [CP]). Then, for the finishing stage (80 to 120 kg BW), pigs were fed a commercial finisher diet (13.5 MJ/kg ME and 13.5% CP) containing 10% sweet potato meal (Table 1). The days to reach 120 kg (market age), carcass weight, backfat thickness, and the percentages of top-grade and lower grade carcasses were investigated. Backfat thickness was measured at the thin part of subcutaneous adipose tissue just above the 9th to 13th thoracic vertebral joints. The carcass scores were evaluated by the professional graders in accordance with the standards of the Japan Meat Grading Association (2018).

TABLE 1 Composition of commercial diets

	Grower diet (35 to 80 kg BW)	Finisher diet (80 to 120 kg BW)
Ingredients (%)		
Cereals (corn etc.)	68	66
Vegetable oil cakes (soybean oil residue etc.)	24	19
Sweet potato meal	0	10
Crushed bran	3	2
Other	5	3
Analytical values (%)		
Crude protein	16.0	13.5
Ether extract	3.0	2.5
Crude ash	8.0	8.0
Crude fiber	5.0	5.0
Calcium	0.5	0.5
Phosphorus	0.4	0.4
ME (MJ/kg)	13.9	13.5

Abbreviations: BW, body weight; ME, metabolizable energy (calculated value) = $4.41 \times \text{TDN} (\%) / 100 \times 0.96$.

2.2 | Analysis of serum metabolites

At 200 days of age, blood samples were collected from the jugular vein of the following nine pigs: three barrows from the 100% group and three barrows and three gilts from the 50% group. These pigs were randomly selected from each pen in each treatment group. Blood was centrifuged at 5900 g for 10 min, and serum was stored at -80°C . The backfat thickness of three barrows in the 100% group, three barrows in the 50% group, and three gilts in the 50% group were 3.6 ± 0.1 , 3.3 ± 0.5 , and 2.2 ± 0.1 cm, respectively.

Analysis of serum metabolites was performed by Human Metabolome Technologies, Ltd. (HMT, Tsuruoka, Japan). Ionic metabolites were analyzed by capillary electrophoresis and time-of-flight mass spectrometry (CE-TOFMS), and fat-soluble metabolites were analyzed by liquid chromatography-TOFMS (LC-TOFMS). For sample pretreatment for CE-TOFMS, 50 μl of serum was added to 450 μl of a methanol solution containing internal standards (10 μM final concentration); then, 500 μl of chloroform and 200 μl of Milli-Q water were added, and samples centrifuged (2300 g, 5 min). After centrifugation, 400 μl of the water phase was transferred to an ultrafiltration tube (Ultra Free MC PLHCC, HMT, centrifugal filter unit 5 kDa) and centrifuged (9100 g, 120 min). The filtrate was dried and dissolved in 50 μl of Milli-Q water. For sample pretreatment for LC-TOFMS, 500 μl of serum was added to 1500 μl of 1% formic acid-acetonitrile solution containing internal standards (6 μM final concentration) and centrifuged (2300 g, 5 min). Phospholipids in the supernatant were removed by solid phase extraction, and the filtrate was collected, dried, and dissolved in 100 μl of 50% (v/v) isopropanol. In CE-TOFMS analysis, the measurement was performed in the cation and anion modes,

and the peaks detected were identified and analyzed for substances registered in the metabolite library of HMT and the Known-Unknown peak library. In LC-TOFMS analysis, the measurements were performed in the positive and negative modes, and the peaks were identified and analyzed for substances registered in the metabolite library of HMT. The peak area value was converted to the relative area value.

2.3 | Statistical analysis

Data obtained from animal experiments were analyzed by two-way analysis of variance (ANOVA) after calculating the mean value for each treatment group. Mixed rearing and sex were set as factors, and the individual effect of factors and an interaction were examined. *p* values under 0.05 were considered statistically significant. All analyses were performed using R (version 4.0.2 for Windows, R Foundation for Statistical Computing, 2020).

3 | RESULTS

3.1 | Growth and carcass performance

The results of growth and carcasses performances are shown in Table 2. The age (days) to reach the target slaughter weight of 120 kg was different (237 to 246 days) among the groups. Significant effects of mixed rearing ($p < 0.05$) and sex ($p < 0.001$) on the age of final were observed. However, this difference was within the standard shipment day-of-age range (230 to 270 days) for Kagoshima-Kurobuta pigs. The

carcass weights showed different values among the groups, but there was no effect of mixed rearing. A significant effect of sex ($p < 0.05$) was observed on the carcass weight. The backfat thickness gradually decreased from 2.9 cm in the 100% group to 2.1 cm in the 0% group, except for 2.4 cm in the 70% group, as the percentage of mixed-reared gilts increased. Highly significant effects of mixed rearing ($p < 0.001$) and sex ($p < 0.001$) were observed on the backfat thickness. The percentages of top-grade carcasses for the 100%, 90%, 70%, 50%, 30%, 10%, and 0% groups were 20%, 40%, 65%, 57%, 50%, 57%, and 78%, respectively, and the percentages of lower grade carcasses for those groups were 70%, 33%, 15%, 17%, 25%, 7%, and 11%, respectively. Significant effects of mixed rearing ($p < 0.05$) and sex ($p < 0.001$) were observed on the carcass grades. There was no interaction between mixed rearing and sex for all measures.

The data in Table 3 show the carcass performance summarized separately for the barrows and gilts in each group. For the barrows in the 70% group, the backfat thickness was thinnest, the percentage of top-grade carcass was highest, and the percentage of lower grade carcass was lowest among the groups. The changes in backfat thickness and carcass grades of gilts were smaller than those of barrows.

3.2 | Serum metabolite profiles

In this study, 185 metabolites and compounds were detected by CE-TOFMS, and 112 metabolites and compounds were detected by LC-TOFMS, in the porcine serum samples. Of these, 28 metabolites were differentially detected in serum samples from barrows of the 100% group and in serum samples from barrows and gilts of the 50% group.

TABLE 2 Effect of mixed rearing on growth and carcass performance of Kagoshima-Kurobuta (Berkshire) pigs

Group ^a					Carcass traits			
Naming	Ratio of barrow to gilt in a pen	Numbers of pens and pigs	Final body weight (kg)	Age at final (days)	Weight (kg)	Backfat thickness (cm)	Grades	
							Top (%)	Lower (%)
100%	10:0	1, 10	120 ± 5	241 ± 7	78 ± 3	2.9 ± 0.5	20	70
90%	9:1	3, 30	119 ± 5	237 ± 10	77 ± 4	2.7 ± 0.5	40	33
70%	7:3	2, 20	119 ± 6	239 ± 11	76 ± 5	2.4 ± 0.3	65	15
50%	5:5	3, 30	120 ± 7	241 ± 10	78 ± 5	2.5 ± 0.5	57	17
30%	3:7	2, 20	119 ± 7	240 ± 11	77 ± 6	2.5 ± 0.5	50	25
10%	1:9	3, 30	118 ± 8	242 ± 9	76 ± 6	2.2 ± 0.4	57	7
0%	0:10	1, 9	118 ± 6	246 ± 8	76 ± 5	2.1 ± 0.5	78	11
ANOVA								
Mixed rearing			NS	*	NS	***	-	
Sex			NS	***	*	***	***	
Mixed rearing × Sex			NS	NS	NS	NS	NS	

Note: Values are expressed as mean ± standard deviation except for "Grades."

Abbreviation: NS, not significant.

^aGroup % indicates the barrows present in mixed rearing.

* $p < 0.05$. *** $p < 0.001$.

TABLE 3 Effect of mixed rearing on carcass traits of barrows or gilts of Kagoshima-Kurobuta (Berkshire) pigs

Group ^a	Carcass traits of barrows			Carcass traits of gilts		
	Backfat thickness (cm)	Top grade (%)	Lower grade (%)	Backfat thickness (cm)	Top grade (%)	Lower grade (%)
100%	2.9 ± 0.5	20	70	—	—	—
90%	2.7 ± 0.5	37	37	2.2 ± 0.3	67	0
70%	2.5 ± 0.3	57	21	2.3 ± 0.2	83	0
50%	2.8 ± 0.5	27	27	2.1 ± 0.4	87	7
30%	2.8 ± 0.4	17	34	2.4 ± 0.5	64	21
10%	2.5 ± 0.3	0	67	2.2 ± 0.4	63	0
0%	—	—	—	2.1 ± 0.5	63	11

Note: Values of “Backfat thickness” are expressed as mean ± standard deviation. —, not applicable.

^aGroup % indicates the barrows present in mixed rearing.

These metabolites were divided into four categories, as shown in Table 4.

There were nine metabolites related to steroid and steroid acid metabolism. For metabolites related to sex steroid hormones, 11 β -hydroxyandrostenedione and estriol were detected only in barrows in the 100% group, and 19-hydroxyandrostenedione was detected only in gilts in the 50% group, whereas 5 α -dihydroprogesterone, 19-hydroxytestosterone, 17 α -estradiol, and 16-epiestriol were detected both in the barrows and gilts in the 50% group.

There were 11 metabolites related to lipid metabolism. Five metabolites related to fatty acid metabolism (sphingomyelin, eicosenoic carnitine, docosanoic acid, polyunsaturated fatty acids [PUFA, 24:2], and butanoic carnitine) were detected in barrows in the 100% group; these metabolites were not detected in barrows in the 50% group. In comparison, eight metabolites were detected in the gilts in the 50% group, two of which (PUFA [24:2] and butanoic carnitine) were also detected in barrows in the 100% group, and four (3-hydroxytetradecanoic acid, PUFA [14:3], PUFA [22:6], and glyceric acid) were also detected in barrows in the 50% group.

For the other eight metabolites, six were detected in the serum of gilts, many of which were metabolites related to amino acid metabolism. Ergothioneine and *N*-methylalanine, metabolites of amino acids, were detected both in the barrows and gilts in the 50% group.

4 | DISCUSSION

Several reports showed that the barrows of Kagoshima-Kurobuta pigs have thicker backfat than the gilts and that the thicker backfat leads to a reduced carcass evaluation score (Chitose et al., 1994; Ishihara et al., 2016; Kenzaki et al., 2005; Ohkoda et al., 2017). Reducing the nutritional value in feed (Chitose et al., 1994; Komura et al., 1998) and changing the feeding method (Itou, 2009) were found to be effective for suppressing excessive backfat thickness. Ishihara et al. (2016) recommended the separated rearing of barrows and gilts. However, Ohkoda et al. (2017) showed that mixed rearing of barrows and gilts was most effective in suppressing excessive backfat thickness of barrows in Kagoshima-Kurobuta pigs. This study demonstrated that the

mixed rearing of Kagoshima-Kurobuta barrows and gilts was effective in reducing the backfat thickness of barrows and the optimum mixing ratio of barrows and gilts was 7:3 (the 70% group). This was also supported by the fact that the barrows in the 70% group had the thinnest backfat, the highest percentage of top-grade carcass, and the lowest percentage of lower grade carcass, when the average data of barrows and gilts were summarized separately (Table 3). The backfat thickness was strongly influenced by mixed rearing and sex, but the effect of mixed rearing was considered to be more pronounced in the groups consisted of more than half barrows. On the other hand, the effect of sex was considered to be more pronounced in the groups consisted of more than half barrows. It can be also interpreted that in response to the mixed rearing, the backfat of barrow becomes thinner from the 100% group to the 70% group and conversely thicker from the 50% group to the 30% group. Contrary to the change in backfat thickness, the percentage of top-grade carcasses increases from the 100% group to the 70% group and then decreases. The results of this analysis indicate that the mixed rearing improves carcass evaluation score via suppression of backfat deposition in barrows, but the effect may show a quadratic response.

When the data of barrows and gilts were summarized separately, the age at final was shortened by mixed rearing in barrows (100%, 241 days; 90%, 237 days; 70%, 237 days; 50%, 238 days; 30%, 233 days; 10%, 230 days), whereas it was slightly prolonged in gilts (90%, 241 days; 70%, 243 days; 50%, 244 days; 30%, 243 days; 10%, 243 days; 0%, 246 days). The effect of mixed rearing on the age at final was different between barrow and gilt, and the sum of these differences is reflected in the mean values of the groups. From the above, it can also be considered that mixed rearing accelerated the growth of barrows and shortened the shipping date, resulting in improved backfat thickness, under certain mixing ratio.

This phenomenon may also occur in pig breeds other than Kagoshima-Kurobuta (Berkshire), but the effect of mixed rearing on backfat thickness may be more pronounced in Berkshire barrows, which tend to have thicker backfat.

The present study found that different types of circulating steroids were detected in barrows in the 100% group (i.e., no mixing with gilts) compared with the gilts in the mixed group. However, the serum

TABLE 4 List of serum metabolites differentially detected in barrows reared alone (100% group) and in barrows and gilts reared together (50% barrows; 50% gilts)

Category and metabolites	Classification	Groups		
		100% Barrow	50% mixed Barrow	Gilt
Metabolites related to steroid metabolism				
11 β -Hydroxyandrostenedione	Sex steroid	+	ND	ND
Estriol	Sex steroid	+	ND	ND
19-Hydroxyandrostenedione	Sex steroid	ND	ND	+
5 α -Dihydroprogesterone	Sex steroid	ND	+	+
19-Hydroxytestosterone	Sex steroid	ND	+	+
17 α -Estradiol	Sex steroid	ND	+	+
16-Epiestriol	Sex steroid	ND	+	+
Metabolites related to steroid acid metabolism				
Cholic acid	Bile acid	ND	+	+
Taurocholic acid	Steroid acid	+	ND	ND
Metabolites related to lipid metabolism				
Sphingomyelin (d18:1/16:0)	Sphingophospholipid	+	ND	ND
Eicosenoic carnitine (20:1)	Acylcarnitine ester	+	ND	ND
Docosanoic acid (22:0)	SFA	+	ND	ND
Fatty acid (24:2)	PUFA	+	ND	+
Butanoic carnitine (4:0)	Acylcarnitine ester	+	ND	+
Pentadecanoic acid (15:0)	SFA	ND	ND	+
Fatty acid (22:3)	PUFA	ND	ND	+
3-Hydroxytetradecanoic acid (14:0)	Hydroxy fatty acid	ND	+	+
Fatty acid (14:3)	PUFA	ND	+	+
<i>cis</i> -4,7,10,13,16,19-Docosahexaenoic acid (22:6)	PUFA	ND	+	+
Glyceric acid	Oxidized glycerol	ND	+	+
Other metabolites				
Glutaric acid	Linear dicarboxylic acid	+	ND	ND
Gly-Asp	Peptide	+	ND	ND
N-Hexanoylsphingosine	Short-chain ceramide	ND	ND	+
Glucosamine	Amino sugar	ND	ND	+
Putrescine	Polyamine	ND	ND	+
Glutathione disulfide	Peptide	ND	ND	+
Ergothioneine	His derivative	ND	+	+
N-Methylalanine	Ala derivative	ND	+	+

Note: +, detected.

Abbreviations: ND, not detected; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

steroid profile was almost the same in both barrows and gilts in the mixed group. Four sex steroids were detected in the mixed-reared barrows and gilts, two of which (17 α -estradiol and 16-epiestriol) are hormones in the estrone-estradiol biosynthetic pathway. The biosynthetic pathways of 17 α -estradiol are complex and not entirely known. 17 α -Estradiol is synthesized by aromatization of epitestosterone to 17 α -estradiol by the enzyme cytochrome P450 aromatase in sites that have not been fully characterized (Finkelstein et al., 1981; Toran-Allerand et al., 2005). 19-Hydroxytestosterone can also be

metabolized as a precursor to estradiol (Watanabe et al., 1994), and 5 α -dihydroprogesterone is synthesized from progesterone (Guidotti et al., 2001; Mellon, 2007). The above reports indicated that these four steroid metabolites can be synthesized in the adrenal cortex, brain, ovaries, and peripheral tissues. The results of our experiment suggest that the synthesis of these steroids occurs constantly in the adrenal cortex or peripheral tissue in gilts but not in castrated males (i.e., barrows). However, it is possible that the mixed rearing induced synthesis of these steroids in the adrenal cortex or peripheral tissue

of barrows, producing a steroid profile similar to that of gilts. Estrogens are also synthesized in tissues expressing aromatase, such as adipose tissue, using adrenal-derived androgen precursors (Schiffer et al., 2019; Toran-Allerand et al., 2005). Sex steroid hormones play an essential role in initiating sexual differentiation and reproduction; estrogens have been reported to affect lipid metabolism (Nakayama et al., 2013). Therefore, steroid-induced effects on lipid metabolism may affect metabolites related to lipid metabolism in the mixed-reared barrows. In the current study, two estrogens (17 α -estradiol and 16-epiestriol) were induced in mixed-reared barrows. 17 α -estradiol is a minor and weak endogenous estrogen that is related to 17 β -estradiol. Although the biological activity of 17 α -estradiol is much weaker than that of 17 β -estradiol, it has been found to bind and activate the brain-expressed estrogen receptor X with a greater potency than that of 17 β -estradiol, suggesting that it may be the predominant endogenous ligand for this receptor (Toran-Allerand et al., 2005). 16-Epiestriol is also a minor and weak endogenous estrogen, and it is synthesized via estradiol, estrone, and 16-oxoestradiol (Adlercreutz et al., 1976). 11 β -Hydroxyandrostenedione, an androgen produced primarily in the adrenal glands (Pretorius et al., 2017), was detected only in barrows of the 100% group. This finding indicates that surgical castration promoted the synthesis of this androgen in the adrenal gland. Estriol is a metabolite from estradiol, but it is unclear why it was only detected in barrows in the 100% group. It remains unclear what specific gilt-induced stimuli produced the estrogen synthesis in barrows, but the endocrine status of sex steroid hormones in barrows may be similar to that present in gilts.

Nakajima et al. (2019) reported that there was no significant difference in lipid metabolism between two lines of pig with genetically low backfat (Landrace) and high backfat (Meishan), but backfat thickness was thicker in Meishan pigs. One possible explanation for this difference in backfat thickness is that differences in protein synthesis capacity, rather than changes in lipid metabolism, may affect backfat thickness. As a next step, it would be useful to investigate the mRNA expression levels of lipid and protein metabolism-related factors in the muscle tissues of Kagoshima-Kurobuta barrow under the mixed rearing by microarray analysis.

In addition to the above-mentioned endogenous steroids, genistein, which is derived from feed grains (soybean meal), was detected both in barrows and gilts in the 50% group but not in barrows in the 100% group (data not shown). This finding suggests that there is a metabolic or catabolic pathway in common for exogenous estrogen-like substances in the barrows and gilts in the 50% group. Steroids such as 17 β -estradiol and testosterone conjugate with glucuronic acid or sulfuric acid in the liver. Conjugated steroids are water soluble and do not bind to transported proteins and are excreted in bile, feces, and urine in small amounts (Auer et al., 2020; Kumar et al., 2013; Yanai et al., 2007). Therefore, it is possible that these steroids were transferred from gilts to barrows by the later eating feces and urine.

In summary, we propose that estrogens were induced in barrows when the barrows and gilts were mixed-raised, and as a result, the accumulation of subcutaneous adipose tissue was suppressed through alterations of lipid or protein metabolism. Further studies are needed

to elucidate the biological mechanism of estrogen induction in castrated males during exposure to females in the same environment.

4.1 | Conclusion

These results indicated that mixed rearing of barrows and gilts was effective for reducing the backfat thickness of Kagoshima-Kurobuta barrows and the optimal mixing ratio of barrows and gilts was 7:3. Sex steroid hormones induced by mixed rearing may influence the backfat thickness of barrows in mixed groups.

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CONFLICT OF INTEREST

No conflict of interest.

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