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Article

Effect of different dietary lysine level on the functioning of genes participating in buildup of intramuscular fat in pork

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ABSTRACT – The eating quality of pork, e.g. its taste and tenderness are favourably affected by inter- and intramuscular fat (IMF) content. Many genes are involved in forming the fat deposited in the meat and especially in between the muscle fibres, among others, the genes of the FABP family (FABP3, FABP4), the LEPR gene, the SDC and the FASN. The objective of this trial was to study whether a moderate change in dietary Lys content (approximately 6%) through the growing and fattening period results in a shift in the expression of genes involved in fat metabolism (FABP3, FABP4, LEPR, SDC and FASN). In the study, Danbred pigs were assigned from a fattening study that involved 96 pigs (50-50% barrows and gilts) from about 25 kg to 125kg live weight. The animals received three-phase feeding and the feeds were formulated according to their requirements. The dietary treatments were set by diets containing different Lys levels in all phases: 10.9, 9.1 g/kg, and 8.3 g/kg vs 11.5, 9.6 and 9.0 g/kg Lys, respectively. The pigs were slaughtered in 125 kg live weight and carcass classification through lean meat % was performed. Meat samples from the carcass were taken within 30 minutes post-mortem from the longissimus dorsi muscle. Gene expression levels were quantified using RT-qPCR analysis. The results indicated that variations in dietary lysine (Lys) content did not significantly influence slaughter quality or the expression levels of genes associated with fat metabolism. Consequently, it can be inferred that a 10% difference from the recommended Lys content does not alter lipid synthesis in the longissimus dorsi muscle of pigs.

Keywords: pork quality, intramuscular fat, pig, lysine, gene expression

INTRODUCTION

Pork contributes to the meat consumption of the world by 40 %, and its quality has a high impact on the preference of consumers. It is widely accepted that a high level of intramuscular fat content (IMF) influences positively the culinary quality of pork (*Laack* et al., 2001). In recent decades the selection of swine primarily focused on growth, feed efficiency and the ratio of lean meat. Due to

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the negative correlation between the ratio of lean meat and IMF content, current commercial swine strains contain less fat and have lower backfat thickness and fat content in the muscle than indigenous breeds. Because of this, the swine industry is highly interested in the increase of IMF content to satisfy consumer needs. IMF content shows a significant difference between breeds and between different lines within a breed (*Pena* et al, 2016). It is also influenced by sex, age and feeding strategy.

IMF incorporates all of the lipids which are found in the muscles and can be functional (cholesterol and phospholipids) or reserve lipids (triglycerides). Recently multiple genes have been identified that participate in the development of IMF and which play a key role in fat metabolism and the appearance of fat in muscle tissue (reviewed by *Malgwi* et al, 2022).

Nutrigenomic research has provided evidence that the bioactive components of food have an impact on the animal genome and the expression profile of genes. Nutrigenomic studies explain the effect of dietary components on transcriptomics and proteomics influencing the physiological state of the organism (*Fenech* et al, 2011).

It has been repeatedly reported that low lysine (Lys) content feed promotes the accumulation of IMF in the muscle of swine. There is evidence that Lys-deficient feed changes gene expression involved in fat synthesis (*Katsumata* et al., 2018), however, it is not clear whether moderately reduced Lys feed could result in a shift in gene expression responsible for fat metabolism. A dose-response relationship between dietary lysine (Lys) levels and intramuscular fat (IMF) content in pork has been observed. This relationship provides the basis for determining the optimal Lys level that maximizes both IMF content and growth performance in pigs (reference). Specifically, lower dietary Lys levels can enhance IMF content; however, they are associated with reductions in growth performance parameters such as body weight gain and daily feed intake (*Katsumata*, 2011).

Additionally, multiple studies have shown that most genes with relation to fat metabolism has indirect effects on the IMF content of pork. The effect of these genes, however, has been observed to be diverse in different muscles (*Malgwi* et al. 2022). Fatty acid binding proteins (FABPs) are intracellular proteins which take part in fatty acid transportation from the plasma membrane to the place of β-oxidation or to the synthesis of triacylglycerol and phospholipids. This group of proteins is built up by different types, including liver, heart, myelin, epidermis, brain, adipocytes and the bowel system (*Becker* et al, 1994; *Chmurzynka* et al 2006). The intracellular long-chain fatty acids in muscle cells primarily originate from substances transported by H-FABP (*Glatz* et

al. 2003). *Damon* et al. (2006) reported that the level of FABP4 (A-FABP) in the longissimus dorsi muscle has doubled in swine with higher IMF content than in the ones with lower IMF content, and positive correlation have been found between the level of FABP4 (A-FABP) and adipocyte number and lipid content. Stearoyl-CoA desaturase enzyme is encoded by the SCD gene which converts saturated fatty acids into mono-unsaturated (MUFA) ones. The SCD gene is an important regulator of lipid deposition and fatty acid synthesis in swine (*Malgwi* et al, 2022). The LEPR gene is known in context with the leptin receptor. It takes part in the take-up of nutrients and in regulation of energy metabolism. LEPR is a functional genetic marker which affects the growth and fat deposition of swine. An enhanced expression of this gene can be anticipated in animals with a higher level of fat deposition. The fatty acid synthase (FAS) enzyme is encoded by the FASN genes. This gene regulates the *de novo* synthesis of fatty acids from acetyl-CoA and malonyl-CoA in the presence of NADPH. The FASN gene initiates the synthesis of fatty acids and mono-unsaturated fatty acids during the early stages of lipid metabolism (*Malgwi* et al., 2022).

Therefore, the aim of this study was to investigate whether a moderate alteration in dietary lysine (Lys) content (approximately 6%) during the growing and fattening phases influences the expression of genes associated with fat metabolism.

MATERIALS AND METHODS

Experimental Design

The muscle samples were taken from experimental pigs fed different dietary Lys during the growing and fattening period. In the experiment, a total of 96 Danbred pigs (50-50% barrow and gilt) with an initial body weight of 25 kg ± 5 kg (SD) were involved. The animals were kept in groups, 8 pigs in each pen. Pigs were allocated to two treatment groups (n=48) that differed in dietary Lys content. Three-phase-feeding was applied, the compound feeds in the growing and fattening phase contained 10.9, 9.1, and 8.3 g/kg or 11.5, 9.6, and 9.0 g/kg Lys in treatment A and B, respectively. Methionine and Cystine, Threonine and Tryptophan to Lysine ratio was formulated according to the ideal protein concept. The feed was pelleted and offered *ad libitum* and water was freely available from drinkers.

Feeding

The feeds were formulated isonitrogenous and isocaloric on net energy bases. The crude protein and amino acid content of the experimental feeds except for

Lys were formulated according to the breeder's nutritional guidelines (*SEGES*, 2020). The composition and nutrient content of experimental feeds are shown in Table 1, 2, 3.

The pigs were slaughtered in 126 kg ± 4 kg (SD) live weight and carcass qualifications were performed in the slaughter house of the *MSC Vágóhíd* in Mohács.

Table 1.
The experimental feeds for 25-40 kg pigs

Components g/kg	**Treatments** A	B
Corn	0	0
Soybean meal	183	178
Wheat	401.5	399.7
Barley	350	355
Plant oil	24	24
Lyine-HCL	0.6	1.5
DL-Methionine	0.4	0.8
L-Threonine	0.4	0.8
L-Tryptophan	0.1	0.2
Premix 0,3%	40	40
Sum	**1000**	**1000**
Nutrients g/kg		
Dry matter	889.9	890.1
Net energy MJ/kg	10	10
Crude protein	159.9	159.8
Crude fat	42.6	42.5
Crude fiber	34.2	34.1
Crude ash	53.4	53.2
Lysine	10.9	11.5
Methionine+Cystine	6.1	6.5
Threonine	7.1	7.5
Tryptophan	2.2	2.3
Ca	8	8
P	5.3	5.3
AID* Lysine	9.9	10.5
AID M+C	5.5	5.9
AID Threonine	6.2	6.6
AID Tryptophan	2.0	2.1

* AID = apparent ileal digestible

Table 2.
The experimental feeds for 40-80 kg pigs

	Treatments	
Components g/kg	A	B
Corn	10	15
Soybean meal	163	158
Wheat	399.1	397.5
Barley	380	380
Plant oil	17	17
Lysine-HCL	0,6	1,4
DL-Methionine	0	0,3
L-Threonine	0.2	0.6
L-Tryptophan	0.1	0.2
Premix 0,3%	30	30
Sum	**1000**	**1000**
Nutrients g/kg		
Dry matter	888.2	888.2
Net energy MJ/kg	9.9	9.9
Crude protein	150.4	149.8
Crude fat	35.7	35.7
Crude fiber	34.4	34.2
Crude ash	50.9	50.7
Lysine	9.1	9.6
Methionine+Cystine	5.1	5.4
Threonine	6	6.3
Tryptophan	1.9	2
Ca	7.5	7.5
P	5.2	5.2
AID* Lysine	8.1	8.6
AID M+C	4.5	4.8
AID Threonine	5.1	5.4
AID Tryptophan	1.6	1.7

* AID = apparent ileal digestible

Table 3.
The experimental feeds for 80-120 kg pigs

Components g/kg	**Treatments** A	B
Corn	50	50
Soybean meal	131	128
Wheat	400.4	401.7
Barley	380	380
Plant oil	8	8
Lysine-HCL	0.5	1.4
DL-Methionine	0	0.2
L-Threonine	0	0.5
L-Tryptophan	0.1	0.2
Premix 0,3%	30	30
Sum	**1000**	**1000**
Nutrients g/kg		
Dry matter	886.4	886.6
Net energy MJ/kg	9.9	9.9
Crude protein	139.9	140.2
Crude fat	27.3	27.2
Crude fiber	34	33.9
Crude ash	48	47.9
Lysine	8.3	9
Methionine+Cystine	4.9	5
Threonine	5.5	5.9
Tryptophan	1.7	1.8
Ca	7	7
P	0.5	0.5
AID* Lysine	7.4	8
AID M+C	4.1	4.5
AID Threonine	4.7	5
AID Tryptophan	1.5	1.6

* AID = apparent ileal digestible

Assessment in the abattoir

At the slaughterhouse the swine were first lowered in a shaft where the concentration of carbon-dioxide is about 90-95%. The animals spent 1-1.5 minutes down in this shaft. The stunned swine were hanged by their feet with their head hanging down. Then they are transported on a conveyor belt where they are stabbed once and they are exsanguinated. Post mortem they are

scalded and their hair is removed, followed by their processing and dismemberment.

At this point carcass qualification and trade classification is performed. During the estimation of lean meat content (%) ultrasonic measuring equipment is used (UltraFom 300). About 7 cm from the plane of cut, between the second and third ribs the loin muscle diameter and backfat thickness is measured (in mm). The lean meat % is calculated automatically from loin area (converted from longissimus muscle diameter) and backfat thickness.

Gene expression studies

Sample collection and storage conditions

Meat samples were collected from randomly assigned 10 carcasses from each treatment group. Samples were taken from the left carcass in less than 45 minutes following the slaughter, from the *Musculus longissumus dorsi* between the 13th and 14th vertebra. The samples were placed in 2 mL DNase/RNase free Eppendorf tubes containing RNA stabilizing liquid (RNAlater™, Thermo Fisher Scientific. 168 Third Avenue. Waltham, MA USA 02451) with a 1:10 sample:liquid volume ratio to stabilize and protect the RNA content of the tissues from degradation with immediate RNase inactivation. After keeping the samples on 4 °C overnight, they were moved to a freezer and kept at -80 °C until further processing.

Sample Preparation, and RNA extraction

Prior to initiating RNA extraction from muscle tissue, ribonucleases (RNases), which are responsible for RNA degradation, are inactivated as an additional precaution to protect the integrity of the RNA. To achieve this, β-mercaptoethanol was used as the deactivating agent, ensuring the RNA remains intact and suitable for the subsequent downstream analyses. The process of RNA extraction started with homogenisation of the tissues in the lysis buffer of RNeasy Mini Kit (Qiagen, 19300 Germantown Road, Germantown, MD 20874, USA) in a Tissuelyser II high-throughput sample disruptor (Qiagen, 19300 Germantown Road, Germantown, MD 20874, USA). To avoid RNA degradation, the adapter of the homogenizer was cooled down to -20 ℃ and all the tools for sample preparation as well. RNA extraction was carried out according to the instructions of the manufacturer. To extract the RNA 30 mg of muscle tissue was used. For the homogenisation, RTL buffer (This is a lysis buffer which is used for the lysis of cells and tissues) was used, provided by the manufacturer. This was followed by a precipitation with alcohol. After which the resulting

solution was pipetted on the "RNeasy Mini spin column", which was centrifuged and flow-through was discarded. Centrifugation steps followed with the use of multiple washing buffers (RW1 buffer and RPE buffer). At last the RNA attached to the column was dissolved with RNA-ase free water with RNase free water.

All the tools for sample preparation and manipulation were pre-sterilised by heat-air steriliser (Celsius 2000 SLE 600, MEMMERT, Germany) on 180 °C for the elimination of microbes and the inactivation of RNases. The quantity and quality of the extracted RNA were measured by Thermo Scientific™ NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific™ 840274200, 168 3rd Ave, Waltham, MA 02451, Massachusetts, USA).

cDNA preparation

The cDNA preparation procedure was applied according to (*Korenková* et al., 2015) starting with the same initial and highest possible concentration of the extracted RNA (140 ng/μL), for which every sample was adjusted to, followed by cDNA transcription.

As the first step of cDNA transcription genomic DNA has to be eliminated. For this gDNA wipe-out buffer, the template RNA and RNase free water was brought into reaction mixture which was incubated for 2 minutes at 42 °C (as determined by the manufacturer and provided in the QuantiTect Reverse Transcription Kit). In the next step the mater mix needed for reverse-transcription is assembled for which the necessary reverse-transcriptase enzyme, the RT buffer and the RT primer mix was provided by the manufacturer. The RNA resulting from the first step was used as template (which does not contain genomic DNA). The resulting mixture was first incubated at 42 °C for 15 minutes, then at 95 °C for 3 minutes (QuantiTect Reverse Transcription Kit /Qiagen, 19300 Germantown Road, Germantown, MD 20874, USA/) and used for downstream application without dilution, since inhibitory effect using undiluted cDNA was not experienced (*Whelan* et al., 2003).

Primer information

The genes analyzed in this study included *LEPR* (Leptin Receptor), *FABP3* (Fatty Acid Binding Protein-3), *FABP4* (Fatty Acid Binding Protein-4), *SCD* (Stearoyl-CoA Desaturase), and *FASN* (Fatty Acid Synthase). The *β-ACTIN* gene was used as a reference housekeeping gene for normalization. The primer sequences for the analyzed genes are detailed in Table 4.

Table 4.
Primer sequences of the genes used for the gene expression study

Gene name	Forward- and reverse primer sequence	Reference
H-FABP (FABP3)	F: 5'AGTTTGATGAGACAACAGCAGATGA 3' R: 5'CAAGTTTGCCTCCATCCAGTGT 3'	*Tyra et al, 2011*
A-FABP (FAPB4)	F: 5' CTGGTACAGGTGCAGAAGTGG 3' R: 5' TTCTGGTAGCCGTGACACCT 3'	*Meng et al., 2018*
LEPR	F: 5' ACATTGCAGGGAAGGCATTT 3' R: 5'CAGTTTGCACCTGTTTGTTGAAA 3'	*Tyra et al, 2011*
SCD	F: 5' TTGCTCTGGGCGTTTGC 3' R: 5' CGAGCTTTGTAAGTTCGGTGACT 3'	*Meng et al., 2018*
FASN	F: 5'-CGTCCTGCTGAAGCCTAACTC-3' R: 5'-GCTCCTTGGAACCGTCTGTGT-3'	*Zhu et al, 2019*
β-ACTIN	F: 5'-ACTGCCGCATCCTCTTCCTC-3' R: 5'-CTCCTGCTTGCTGATCCACATC-3'	*Zhao et al, 2009*

qRTPCR conditions

Real-time PCR was performed using SYBR Premix and Mx3000P Real-Time PCR System (Agilent Technologies). Amplifications were performed in 10 µL reaction volume containing 5 µL of SYBR Premix 0.2 µL (stock concentration:10µM) of each primer,1 µL of diluted cDNA, and sterile water. The PCR amplification was carried out as follows: 95 °C for 15 min, then 40 cycles of 95 °C for 1s and 60 °C for 30 sec in normal two steps settings. PCR tests were performed with three parallels in case of every sample.

Calculations and statistical analysis

The C_t (threshold cycle) values given by qRT-PCR were evaluated according to the following conditions. The applied method for the determination of relative amount was the $2^{-\Delta\Delta Ct}$ method.

Change of relative gene expression= $2^{-\Delta\Delta Ct}$
Where:
$\Delta\Delta C_t = \Delta C_{t\ treated} - \Delta C_{t\ untreated}$
$\Delta C_t = (C_{t\ target\ gene} - C_{t\ reference\ gene})$

To better visualize the results, the log10 of $2^{-\Delta\Delta Ct}$ values were taken into account.

The gene expression and meat quality values were evaluated with a Student's t-test considering significant differences at the $p<0.05$ level. The data

showed normal distribution. The statistics were performed using R-program (R 4.1.2 for Windows, Rcmdr package).

RESULTS AND DISCUSSION

Pigs were slaughtered over 120 kg body weight in a commercial abattoir. There was a 3.5 kg difference in live weight at slaughter and a 2.7 kg difference in carcass weight (p=0.052). The higher Lys feed tended to increase the live weight and the carcass weight, however, there was no difference in backfat thickness, loin diameter and lean meat percentage between pigs of each treatment (Table 5). Our data suggest that Lys recommendation may be slightly overestimated since an approximately 6 % reduction in dietary Lys did not compromise the body weight and the lean meat content of the body. In line with our results, others have also found that a slight reduction in dietary Lys does not change the pig growth. Kumar et al. (2016) reported that 10 and 15% reduction in dietary Lys had no impact on growth performance and carcass parameters in crossbred Landrace pigs either. Results of *Jin* et al. (2010) indicated that finishing pigs fed a diet with 15% lysine restriction had no detrimental effects on growth performance and N utilization but could achieve substantial increases in marbling and longissimus fat content of pork.

Table 5.
Slaughter weight and carcass quality traits measured in the abattoir

	A treatment		B treatment		
	mean	SD	mean	SD	P-value
Live weight (kg)	124.4	2.4	127.9	4.5	0.052
Carcass weight (kg)	98.8	1.9	101.5	3.6	0.052
Backfat thickness (mm)	14.6	2.1	15.1	3.9	0.73
Loin diameter (mm)	63.5	2.9	63.0	4.9	0.80
Lean meat %	59.8	1.4	59.4	2.8	0.72

Treatment A: feed was formulated to contain 10.9 g/kg, 9.1 g/kg, 8.3 g/kg digestible Lys in grower 1, 2, and finisher phases respectively; Treatment B: feed was formulated to contain 11.5 g/kg, 9.6 g/kg, 9.0 g/kg in grower 1, 2, and finisher phases respectively

In nutrigenomics studies, the general objective is to evaluate whether expression of genes is affected by dietary treatments. In this research, the expression of genes determining IMF was measured in meat samples that originated

from pigs fed by different Lys content feed. The dietary contrast in Lys was 5.5% in phases of grower 1 and 2, and 8.4 % in the finishing phase.

The results of the gene expression in different dietary treatments are shown in Figure 1. Dietary Lys content had no significant impact on the expression of measured genes.

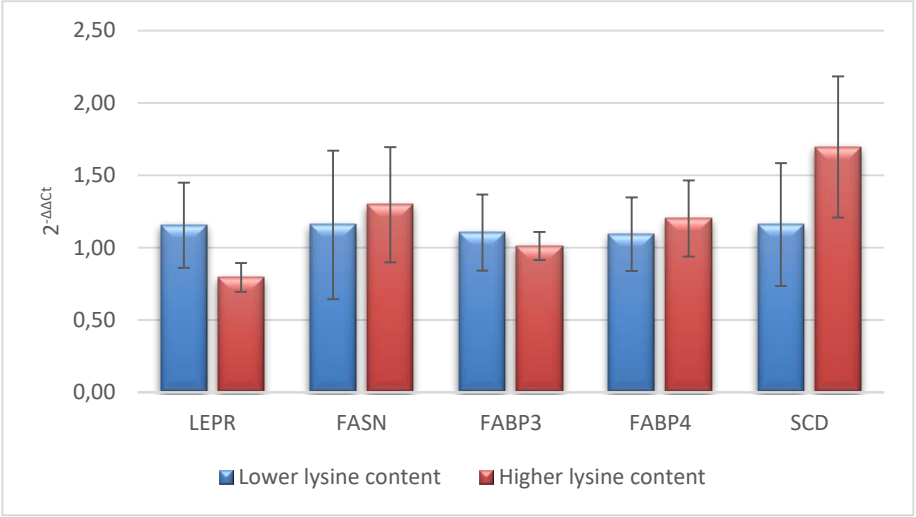

Figure 1. Level of gene expression in *m. longissimus dorsi* samples of pork when pigs were feed diets differed in digestible Lys content between 25-125 kg live weight.

Table 6.
Result of two-sample Student's t-test

	LEPR	***FASN***	***FABP3***	***FABP4***	***SCD***
two-sample t-test	0.21	0.67	0.99	0.91	0.30

The blue columns show the results of gene expression for feeding lower Lys content feeds (10.9 g/kg, 9.1 g/kg, 8.3 g/kg in grower 1, 2, and finisher phases respectively), the orange columns show the gene expression results for feeding high Lys content feeds (11.5 g/kg, 9.6 g/kg, 9.0 g/kg in grower 1, 2, and finisher phases respectively). In the case of *SCD* and *LEPR* genes, the means differ, but the individual variance within the group was high, and thus no reliable statistical difference could be found between the treatment groups with different dietary Lys content. The treatment did not affect the activity of *FABP3, FABP4* and *FASN* genes either. In general, the individual variances within the groups were relatively high with exception of *LEPR* and *FABR3* in high Lys group.

In this study, the experimental contrast was set to achieve a moderate difference in dietary Lys content and this may have been insufficient, or the longissimus dorsi muscle (*M. longissimus dorsi*) may not have been the most suitable tissue for detecting differences in the expression of the target genes with the levels of Lys supplied in the diets. *Katsumata* (2011) reported that IMF content of longissimus dorsi muscle was twice as high in pigs fed lysine-depleted diet, compared to control pigs (0.70% vs. 1.15% lysine; respectively). In a further study of Katsumata et al. (2018) when pigs were fed with significantly reduced Lys content diet (1.37% vs 0.78%) the level of Fatty Acid Synthase mRNA in the liver increased. The mean level of *FAS* mRNA in *Longissimus dorsi* muscle was also higher for those swine that received a diet with low Lys content (*Katsumata* et al, 2018). In line with that *Palma-Granados* et al. (2019) found that the effect of lysine deficiency on lipid metabolism was tissue-specific, with an activation of lipogenesis in longissimus and biceps femoris muscle but no apparent stimulation in backfat adipose tissue. However, it has to be stressed that the experimental contrast was really huge in both trials, the reduction in dietary Lys was more than 40% and 50% in the referred studies (*Katsumata* et al, 2018 and *Palma-Granados* et al., 2019, respectively).

The excessively low intramuscular fat (IMF) content in lean pig genotypes negatively impacts pork eating quality, necessitating precision feeding strategies informed by nutrigenomic insights. *Palma-Granados* et al. (2019) propose that lysine (Lys)-deficient diets could effectively increase IMF content in both lean and fat pig genotypes. However, it is critical that such strategies do not compromise growth performance, as reduced growth rates carry adverse economic and environmental consequences. Precision feeding approaches tailored to individual genotypes and production goals could include carefully timed and targeted Lys restrictions during the fattening phase, optimizing IMF content while maintaining efficient growth and minimizing resource use.

CONCLUSIONS

In conclusion, a 6-8% variation in dietary lysine (Lys) content relative to the recommended level did not significantly impact slaughter quality or fat synthesis in the longissimus dorsi muscle of lean pigs. Moreover, the expression of genes involved in fat metabolism, particularly those encoding fatty acid synthase (FASN) and stearoyl-CoA desaturase (SCD), exhibited considerable individual variability, even within a genotype subjected to intensive genetic selection. This suggests that genetic factors may play a substantial role in regulating fat metabolism, and minor dietary adjustments in Lys may not be sufficient to induce notable changes in fat deposition at the molecular level.

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