Insulin-Like Growth Factor I and II mRNA Levels in Rumen Wall of Calves Fed with Different Physical Forms of Diets

S. Akbari¹, S. Ansari Mahyari¹*, A. H. Mahdavi¹, A. Zahmatkesh², and E. Dehnavi¹

ABSTRACT

This study was designed to investigate the effects of physical forms and hay contents of diets on gene expression of insulin-like growth factor (IGF) I and II in rumen epithelium of Holstein calves. Twelve male calves were assigned to 4 treatments: ground (GR), texturized (TX), pellet (PL), and ground+10% forage (GF). Calves were weaned on day 50 of age and then slaughtered on day 70 after birth. Rumen epithelial tissue samples were immediately collected for quantification of mRNA abundance. Results indicated that only IGF I expression was influenced by the dietary treatments. A significant (p<0.05) correlation between IGF I expression and each of histological parameters denoted as length of rumen villi and diameter of keratinocyte layer was observed. No significant correlation between IGF II expression and rumen histological parameters was found (p>0.05). Regarding the results, higher IGF I expression in PL and TX treatments despite the low growth rate might be due to the challenging condition of developing rumen in calves. In fact, the rumen tissue attempted to maintain rumen pH at least by induction of a higher IGF I expression.

Keywords: IGF I; IGF II; Keratinocyte layer; Rumen villi, Holstein calves.

INTRODUCTION

Newborn dairy calves do not have any effective rumen function at early ages. Feeding with solid foods can increase relative weight of rumen compared to that of whole body weight (BW). Fermentation of the starter diets and feedstuff in rumen enhances the amounts of volatile fatty acids (VFA), especially propionic and butyric acids (Dai et al., 2010). These critical materials stimulate development of the ruminal mucosa in calves.

The main goal of using starter diets is to accelerate the growth of rumen papillae and finally gastrointestinal tract (GIT) and increase the ability of calves to be weaned sooner and consume milk replacers using other sources of foods as a ruminant (Malmuthuge et al., 2013). The GIT grows rapidly and changes notably its function during the initial postnatal period. Moreover, it is a major factor and affected by the somatotropic axis in endocrine, autocrine, and paracrine processes (Ontsouka et al., 2004b).

Apart from the significant effect of serum IGFs and IGF expression in the liver, studies have shown that local expression of IGF mRNAs occurs in ovine and bovine rumen and intestine (Georgieva et al., 2003a, b; Flaga et al., 2011; Cheng et al., 2012). Higher levels of IGF I mRNA in intestine, but not in liver, of pre-term pregnancy than in full-term pregnancy calves at birth suggests autocrine-paracrine roles of IGF I for regulation of intestine growth and indicates that IGF I is an essential factor in
pre-term intestinal activities. The IGF I mediates growth and differentiation of tissues during postnatal period (Hammon and Blum, 2002). It influences enterocyte proliferation and maturation and GIT morphology and functions in calves (Georgiev et al., 2003a; Roffler et al., 2003). In turn, IGF II expression, has been found to be involved in the mechanisms affecting the intestinal epithelium differentiation in calves, and is mostly important during prenatal period and for fetal growth and development (Georgieva et al., 2003a; Ontsouka et al., 2004b). Besides, it has been shown that age-dependent decrease in IGFs transcripts is related to the reduction in rumen papillae length and tunica muscularis thickness in calves (Flaga et al., 2011).

During immediate postnatal period, GIT changes morphologically and functionally in calves (Ontsouka et al., 2004b). Investigation of the factors involved in functional development of GIT improves rate of compatibility during postnatal period, and it would be possible to help reduce digestive defects and increase the efficiency of digestive system, health status, and growth performance (Georgiev et al., 2003a, b). Maturation of GIT and its associated organs in calves are influenced by dietary strategies and weaning-feeding managements in early life (Khan et al., 2011). Although there have been some studies considering effect of physical forms of dietary treatments on feed intakes and histological parameters, it is still questioned if different physical forms of diets can affect gene expression of growth factors in rumen tissue. Therefore, this study was designed to investigate the expression level of IGF I and IGF II genes in rumen epithelium of Holstein calves (Bos taurus) fed with different physical forms of starter diets and hay contents to achieve more information about GIT changes. In addition, the relation between IGFs gene expression and histological parameters of the rumen was evaluated.

MATERIALS AND METHODS

Research was performed in compliance with the Federation of Animal Science Societies in the Guide for the Care and Use of Agricultural Animals in Research and Teaching. Also, the killing procedure was according to Humane Methods of Slaughter Act (Federation of Animal Science Societies, 2010).

Animals, Feeding, and Experimental Design

This study was managed at FKA (Agro-Animal Husbandry Corporation, Isfahan, Iran) between February and May 2015. For comparison of gene expression profile in different treatments, 12 Holstein calves (Bos taurus) (41.3 ± 0.4 kg BW) were selected and randomly assigned to 4 treatments. Each treatment consisted of 3 male calves in a completely randomized design, according to previous researches. (Connor et al., 2008; Connor et al., 2014). All neonatal calves were housed in individual pens (1.2 m × 2.5 m) while separated from their dams at birth. The bed was filled with fresh sawdust, which was renewed daily. All calves received colostrum for 3 days (4 L/day). They were then fed by (4 L/day) milk in steel buckets twice daily from days 3 to 45. Feeding by milk reduced to half (i.e. 2 L/day and by morning feeding) from days 46 to 49 of age. All calves were weaned on day 50. From day 7 onward, all calves received the experimental diets. They had ad libitum access to starter diet and water. The total study duration was 70 d.

Dietary treatments were: 1) ground (GR), 2) texturized (steam flake corn and steam flake barley), (TX), 3) pellet (commercial pelleted starter mixture) (PL), and 4) ground + 10% forage (GF). The GF treatment was a mixture of GR starter feed supplemented by 10% alfalfa hay. Diets were formulated to be iso-energetic and iso-nitrogenous and with similar nutrient compositions, but the treatments were different in particle size.
Different Diet Forms Affect Growth Factor

(Table 1). Geometric mean particle size of starter diets was calculated using the method described by the American Society of Agricultural Engineers (ASAE, 1983). Starter diets were separately analyzed for dry matter, ash, crude protein, crude fat, Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and metabolizable energy using standard procedures (AOAC, 2000) (Table 2).

**Tissue Sampling and Processing**

On day 70 of age, the calves were slaughtered at the neck area by captive bolt stunning and cutting of the veins. The gastrointestinal tract was removed immediately and washed. The tissue samples were collected from cranio-ventral sac of rumen. Muscular tissues were separated from the lamina propria layer and washed by phosphate-buffered saline solution. Then, epithelial layer was inserted into 1.5 mL tubes containing DEPC (Sigma Aldrich Company, Germany) water and finally frozen in liquid nitrogen, and stored at −80 °C until analysis for *IGF I* and *IGF II* mRNA expression.

A previous data of villi length, villi width, and thickness of keratinocyte layer, epithelium, circular muscle, longitudinal muscle, total muscle, and rumen wall for the same animals (Pazoiki et al., 2017) were used to calculate the correlations between rumen histological parameters and expression levels of *IGF I* and *IGF II* mRNAs.

**RNA Extraction, RT-PCR and Real-Time-PCR**

Frozen tissue samples were grounded in liquid nitrogen in a mortar. Total RNA was extracted using TRIzol® Reagent (Thermo Fisher Scientific Inc. Invitrogen, USA). Quantity and quality of extracted RNA was verified using electrophoresis and NanoDrop spectrophotometer (Thermo Scientific, USA). An amount of 2000 ng RNA was

### Table 1. Particle size of different diets.

<table>
<thead>
<tr>
<th>Dietary Treatments</th>
<th>Particle Size (mm)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>0.72</td>
<td>3.34</td>
</tr>
<tr>
<td>TX</td>
<td>3.61</td>
<td>2.07</td>
</tr>
<tr>
<td>PL</td>
<td>4.53</td>
<td>1.58</td>
</tr>
<tr>
<td>GF</td>
<td>1.02</td>
<td>3.04</td>
</tr>
</tbody>
</table>

*a Dietary treatments were 1: GR (ground), 2: TX (texturized: steam flake corn and steam flake barley), 3: PL (commercial pelleted starter mixture) and 4: GF (ground + 10% forage). b Geometric mean particle size was calculated as described by ASAE, 1983.

### Table 2. Chemical composition of different diets.

<table>
<thead>
<tr>
<th>Chemical Composition (% of DM of total food)</th>
<th>Dietary Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>GR</td>
</tr>
<tr>
<td>98.30</td>
<td>98.02</td>
</tr>
<tr>
<td>CP</td>
<td>23.88</td>
</tr>
<tr>
<td>Crude fat</td>
<td>2.94</td>
</tr>
<tr>
<td>NDF</td>
<td>15.2</td>
</tr>
<tr>
<td>ADF</td>
<td>8.85</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.82</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.68</td>
</tr>
<tr>
<td>Ash</td>
<td>6.9</td>
</tr>
<tr>
<td>ME (Mcal/kg)</td>
<td>2.75</td>
</tr>
</tbody>
</table>

*a Dietary treatments symbols are defined under Table 1. b Chemical composition was explained in % dry matter. c ME was calculated from NRC (2001) and was shown in Mcal/kg.
used to construct cDNA by Fermentas RevertAid First Strand cDNA Synthesis kit (Hanover, Maryland, USA). The temperature condition of the thermal cycler (B960, Nison Instrument Limited, Shanghai, China) was practiced as following: 25 °C for 10 min at first, 42 °C for 60 min, and finally 90 °C for 10 min. All cDNA samples were stored at −20 °C until next analyses. Primers used for the amplification of IGF I, IGF II and β-actin genes were selected from previous studies (Armstrong et al., 2000; Kasimanickam and Kasimanickam, 2011; Nasiri et al., 2012). Nonetheless, in order to have the best choice, bioinformatic analysis was performed by considering melting temperature (Tm), GC %, AG, dimer and hairpin formation using Oligo (7.0) software and Primer-BLAST order of NCBI database. The sequences and length of utilized primers, Tm, GC %, and length of PCR products are shown in Table 3. The PCR reaction components were 4.5 μL PCR-master-mix (Viragene Akam Company, Tehran, Iran) (2.5 mM), 7.5 μL ddH2O, 2 μL cDNA, and 0.5 μL of each forward and reverse primers (10 pmol/μL) for a 15 μL final reaction solution. PCR program included a preliminary denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58, 53, and 63 °C for β-actin, IGF I, and IGF II, respectively for 30 sec, extension at 72 °C for 30 sec, and 5 min at 72 °C as final extension. Polymerase chain reaction was carried out to amplify 200, 196, and 200 bp fragments for β-actin, IGF I (parts of exons 2 and 3), and IGF II (parts of exons 2 and 3) genes, respectively. A negative control (water instead of cDNA) was considered in each run to check the accuracy of the reaction. PCR products were visualized in 1% agarose-TBE gel, after electrophoresis and staining.

In order to determine the changes in IGF gene expression in response to different treatments, Real-Time PCR was performed including a negative control, and cDNA of 3 calves of each treatment for each gene. β-actin was used as an internal control to normalize the expression of IGF mRNAs, as it was used in previous studies (Dervishi et al., 2010; Muscher et al., 2010; Tao et al., 2014) and it is considered as an stable reference gene in rumen samples in different rations (Die et al., 2017). Reaction components for Real-Time-PCR consisted of 5 μL SYBR Green kit (2X), 3 μL ddH2O, 1 μL cDNA, and 0.5 μL of each forward and reverse primers (5 pmol/μL) for a 10 μL final reaction solution. The Real-Time PCR (Applied Biosystems StepOne Plus) program was as explained before. Also, in order to ensure the absence of primer-dimer formation and unspecific amplification, a melting curve program (60-95 °C) was included.

### Statistical Analyses

The abundance of IGF I and II mRNAs were calculated using relative quantification by 2−ΔΔCT formula. The effect of different feeding types on IGFs expression was evaluated using the GLM procedure of SAS.
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software version 9.2 (SAS Institute, Cary, NC, USA), as a completely randomized design, using the following model (1):

\[ Y_{ijkl} = \mu + T_j + IBW_k + e_{ijkl} \quad (1) \]

Where, \( Y_{ijkl} \) was the gene expression ratio, \( \mu \) was the overall mean, \( T_j \) was the fixed effect of the treatments (dietary groups), \( IBW_k \) was the covariate effect of the initial body weight of calf, and \( e_{ijkl} \) was the random error.

Tukey-Kramer test was used for comparison of the treatment means. Pearson method was used to clarify the correlations of rumen histological parameters with expression levels of IGF I and II mRNAs (Georgiev et al., 2003a, b; Kravchenko et al., 2008). The significance level was declared at \( p \leq 0.05 \) for all the tests.

RESULTS

RNA electrophoresis and NanoDrop spectrophotometer verified the extracted RNA integrity and purity. The optical density ratio of 260 and 280 nm was between 1.8 and 2 for all samples. PCR results showed the amplification of 196, 200 and 200 fragments for IGF I, IGF II, and \( \beta \)-actin, respectively (Figure 1).

Quantitative PCR showed that abundance of IGF I mRNA differed significantly \( (p < 0.001) \) among different dietary treatments, but there was no significant difference in IGF II mRNA levels \( (p > 0.05) \). The average expression levels of IGF I and II mRNAs are shown in Table 4. The results showed that the physical form of starter diet can significantly affect the expression of IGF I, which increased in PL and TX \( (p < 0.05) \) (order of treatments: PL and TX > GR > GF).

Investigated traits and their correlation coefficients are presented in Table 5. Results showed that correlation between IGF I and IGF II was not significant. The correlation between IGF I gene expression and 2 of the histological parameters of the rumen was significant [-0.72 and 0.73] for length of villi and diameter of keratinocyte layer, respectively. The correlation between the expression of IGF II and histological parameters of rumen was not statistically significant.

DISCUSSION

Several reports have revealed that consumption of different foods and their

![Figure 1. PCR amplification results of IGF I, IGF II and \( \beta \)-actin (ACTB) in 1% agarose-TBE gel, L: 100 bp DNA ladder.](image)
Table 4. The expression levels of IGF I and II mRNAs normalized to β-actin, in rumen wall in different dietary treatments.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Dietary Treatments</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GR</td>
<td>TX</td>
</tr>
<tr>
<td>IGF I</td>
<td>2.91a</td>
<td>4.24a</td>
</tr>
<tr>
<td>IGF II</td>
<td>1.39</td>
<td>2.49</td>
</tr>
</tbody>
</table>

* Dietary treatments symbols are defined under Table 1. ab Values with different superscript letters are significantly different (p < 0.05).

Table 5. The coefficients of correlation between the expression of IGF I and II mRNAs and histological parameters of Holstein calves rumen.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Villi length</th>
<th>Villi width</th>
<th>Keratinocyte layer thickness</th>
<th>Epithelium thickness</th>
<th>Circular muscle thickness</th>
<th>Longitudinal muscle thickness</th>
<th>Total muscle thickness</th>
<th>Wall thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF I</td>
<td>-0.727</td>
<td>0.261</td>
<td>0.729</td>
<td>0.111</td>
<td>-0.66</td>
<td>-0.50</td>
<td>-0.59</td>
<td>-0.63</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>(0.03)</td>
<td>(0.03)</td>
<td>(0.78)</td>
<td>(0.11)</td>
<td>(0.32)</td>
<td>(0.22)</td>
<td>(0.18)</td>
</tr>
<tr>
<td>IGF II</td>
<td>-0.526</td>
<td>0.087</td>
<td>0.362</td>
<td>0.022</td>
<td>-0.153</td>
<td>0.625</td>
<td>0.451</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>(0.15)</td>
<td>(0.34)</td>
<td>(0.95)</td>
<td>(0.75)</td>
<td>(0.19)</td>
<td>(0.37)</td>
<td>(0.37)</td>
<td>(0.42)</td>
</tr>
</tbody>
</table>

* P < 0.05.

Physical form can increase the mRNA expression of IGFs and then modulate the amplification of epithelial cells and development of muscles of digestive tract (Shen, 2005; Flaga et al., 2009; Flaga et al., 2011). Ontsouka et al. (2004b) showed that adding dexamethasone (DEXA; 30 µg/kg BW per day) into an isoenergetic milk-based formula increased mRNA levels of IGF I in esophagus, rumen, jejunum, and colon, while it reduced IGF I levels in esophagus, fundus, duodenum, and ileum in colostrum-fed calves. They concluded that different feedings affected gene expression of the components of somatotropic axis in GIT of neonatal calves.

Moeini et al. (2016) studied the effects of physical forms of diet and hay content on blood metabolites, liver composition and intestinal morphology. They showed that feed efficiency in the texturized treatment was better and the ground treatment had the least efficiency among the treatments. As physical forms of PL and TX diets in starter mixture in the current study caused the highest expression level of IGF I mRNA among the experimental treatments, it seems that more nutrients in these treatments got the chance (through processing) to have an effect on expression of growth factor genes. Available nutrients in PL and TX treatments (through processing) may stimulate the expression of IGF I at rumen wall. However, it is notable that the expression of IGF I is affected by various stimuli controlled by different factors such as hormonal and nutritional status, specific characteristics of each tissue, and the mechanical pressure (Dai et al., 2010).

Calves in PL treatment showed the lowest ADG, DMI and feed efficiency (Moeini et al., 2016). Interestingly, the expression level of IGF I was the highest in PL treatment and then the order was followed by TX (numerally), GR and GF (statistically) treatments, respectively. The PL treatment, which had shown a weak performance in the study of Moeini et al. (2016), had the highest expression level of IGF I mRNA in the current study.

It has been demonstrated that using forage in starter diets stimulated the physical abrasion of feed on rumen papilla (Beiranvand et al., 2014; Omidi-Mirzaei et al., 2015) and improved the rumen wall (Suarez et al., 2007). Furthermore, GF
treatment, which was reported to have the highest ruminal pH, ADG and DMI among the treatments (Moeini et al., 2016), showed the lowest IGF I expression level in the present study. Therefore, we concluded that there might be a relation between ruminal pH, ADG, DMI and the expression of IGF I.

Yang et al. (1996) introduced IGF IEc, an isoform of IGF I, as a factor related to mechanical stimulation, and named it as mechano growth factor (MGF). It has been further demonstrated that acidic status and increased temperatures enhance the expression of MGF isoform in mice myoblasts (Kravchenko et al., 2008). It has been reported that the rumen pH was reduced in treatments lacking forage, because of decrease in rumination time span, amount of salivary secretion, and the ability of rumen to absorb VFA (Moeini et al., 2016). Hence, we concluded that the acidic status of the rumen injured the rumen wall and then, in response to this challenging situation, the rumen increased the expression of IGF I in PL and TX treatments compared with GF treatment. The higher expression of IGF I mRNA may be due to the damage resulting from a reduction in the rumen pH which makes a challenging condition for rumen at these treatments. However, the increased expression could not affect the tissue growth. It has been previously reported that diets consisting of more concentrate showed lower-developed rumen mucosa than those including concentrate and 10% hay (Beiranvand et al., 2014). Another assumption is that IGF I receptors might be damaged due to the acidic condition of rumen, or their expression might be hypothetically down-regulated as a result of low pH and, therefore, no cellular response would happen after IGF I expression. Consequently, the cells might have tried to express more IGF I transcripts to overcome the deficiency.

This study indicated a considerable, but not significant, difference in IGF II mRNA levels and, numerically, the order of treatments was as TX > PL > GR > GF. Flaga et al. (2011) studied the age-dependent changes (days 5, 12, 19 and 26 of calves’ life) in IGF I and IGF II mRNA levels in relation to GIT development in newborn calves. They detected that both IGF I and IGF II mRNAs were at the highest level on 5th day of life, and significantly decreased on days 12 to 19 in most of analyzed GIT parts, and started to increase slightly thereafter. They concluded that mRNA expression level depended on age and feed type.

Georgieva et al. (2003a, b) demonstrated that the expression of IGF II mRNA in liver and intestine of preterm calves (born after 277 days of pregnancy) was higher in comparison to full-term calves (born after 290 days of pregnancy), but not significant between preterm calves on 8th day of life after being fed for 7 day and full-term calves. These results confirm that IGF II is more functional during prenatal life and its function decreases in postnatal period. It was reported that IGF II expression level was affected by feeding type and dexamethasone treatment. Abundance of IGF II mRNA in rumen and jejunum was higher (p < 0.05) in formula-fed than in colostrum-fed calves and in pylorus was higher (p < 0.05) in DEXA-treated than in non-treated calves (calves on 5th day of life) (Ontsouka et al., 2004b). Considering previous studies, this is the first time that the expression level of IGF I and IGF II mRNAs is investigated in different physical forms of starter mixture. Hence, more studies are needed in this field to get a comprehensive knowledge.

The effect of dietary energy-dependent alterations has been studied on the expression of growth factor genes and a positive correlation has been reported between IGFs expression and rumen papilla length (Shen et al., 2004; Shen, 2005; Flaga et al., 2011). However, our study showed a positive correlation between IGF I expression and the diameter of keratinocyte layer and a negative correlation between the expression of IGF I and villi length (p < 0.05). In a previous study, it was demonstrated that the diameter of keratinocyte layer in dairy calves increased.
in concentrate treatments compared with concentrate + 10% forage (Beiranvand et al., 2014). Feeding calves with concentrate rich with fast fermentable carbohydrates can quickly reduce ruminal pH (Beharka et al., 1998), and cause overgrowth and keratinization of ruminal papillae (Bull et al., 1965). Beharka et al. (1998) have shown that calves fed with ground starter diets had shorter villi and lower villi surface area in the rumen compared with calves fed with unground diets, which clearly demonstrates that physical forms of starter diets affect papillary development of rumen. According to the present study, the most IGF I expression was in PL and TX treatments. Hence, there would be an assumption that body in the high-IGF I expression group confronts a challenging acidic rumen environment and attempts to alleviate this problem by increasing IGF I expression. In fact, GF treatment which has been shown in different reports to have the highest growth rate (Franklin et al., 2003; Coverdale et al., 2004; Moeini et al., 2016), had some expression of IGF I gene, but it was not as high as PL treatment.

Reports indicate a negative correlation between the expression of IGF I and II mRNAs and villi length in small intestine (Ontsouka et al., 2004a). It has been shown that intestinal villi width was independent of IGF I and IGF II (Flaga et al., 2011). In an investigation upon 24 calves on 8th day of life, no correlation was detected between histological parameters and the expression levels of IGF I mRNA expression in intestine (Velayudhan et al., 2008). High expression of IGF I mRNA in intestine on 5th day of life was accompanied by decrease in villi length. The reduction in IGFs mRNA levels after 5th day of life in the duodenum (IGF I and IGF II) and jejunum (IGF I) was associated with reduction in villi length (duodenum and jejunum). In general, it may be concluded that IGF I has a fluctuating role in different parts of digestive system and is affected by diet (Flaga et al., 2009). However, more studies considering more repetitions per treatment may be needed to understand the exact role of growth factors in changes of rumen histological parameters.

A comprehensive conclusion is listed in the following:

IGF I and not IGF II expression was affected by diet and ruminal environmental status.

High level of IGF I expression is not always an indicator of the rumen development and this may happen due to the cellular-molecular compatibility of the rumen to the environmental status (e.g. acidic environment of rumen).

Given the lack of significant correlation between IGF I and IGF II, the expression of these genes may be regulated independently and IGF II may have a less important role compared to IGF I in the rumen development at birth time.

ACKNOWLEDGEMENT

We thank Mr. Jamshid Jalilnezhad and his staff at FKA Agro-Animal Husbandry Corporation (Isfahan, Iran) for putting the calves at our disposal.

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سطوح mRNA فاکتورهای رشد شبه انسولین ۱ و ۲ در دیوارهای شکم‌های گوساله - های تنظیم شده با شکل‌های فیزیکی متفاوت جیره

