Jejunal barrier dysfunction of Datong Yak with high proportion concentrate dietary during the cold season

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ABSTRACT

Objective: Ruminant intestine health played a vital role in nutrient absorption and metabolism.

Methods: In present study, twelve Datong male Yak were randomly selected and slaughtered to determine jejunal digestive enzyme activity, morphology and protein composition between forage-fed and concentrate-fed during cold season in Tibetan Plateau.

Results: The results showed that Yak jejunum digestive enzyme activity and morphology of forage-fed group were better than that in concentrate-fed group. A total of 96 differentially expressed proteins were identified by label-free MS, which could be concluded to two predominant themes: protein structure and inflammatory response. Nine differentially expression proteins were correlated in yak jejunum damage in high dietary concentrate.

Conclusion: This fact was confirmed long-term high dietary concentrate feeding could damage the jejunum epithelial morphology and function.

Keywords: Feeding pattern (forage-fed and concentrate-fed), Morphology, Physiological function, Protein composition, Yak (Bos grunniens), Jejunum

50 **INTRODUCTION**

51 Yak (Bos grunniens) mainly live above an altitude of 3000 meters in the Tibetan plateau, and develop
52 a dogged anti-adversity ability to exist, multiply and produce during the long period of natural selecting
53 and artificial domestication [1]. Yak feeding is an economic pillar of Tibet plateau area. Tibetan plateau
54 is known as extreme harsh conditions, characterized by high altitude, severe cold, low air oxygen, strong
55 ultraviolet radiation and short forage growing season. Herbage and nutrients are often insufficient to
56 support the livestock during the cold period, especially when raised under traditional grazing
57 management [2]. The saying 'alive in summer, strong in autumn, thin in winter, tired in spring' has been
58 passed down from generation to generation by the local people.

59 The modern intensive management pattern recently had gradually replaced to traditional grazing pattern
60 in Tibet plateau area [3]. The dietary concentrate was more than 75% under modern intensive reared
61 pattern, Yak bodyweight had a faster daily gain, and herdsman had a higher economic benefit. The
62 ruminant need intake 40%-70% dietary roughage to maintain normal rumen function and
63 gastrointestinal microflora environment [4]. However, long-term high dietary concentrate feeding can
64 eventually have a serious impact on animal health, which could lead to rumen microorganisms
65 metabolic disorder (such as VFAs accumulated and pH decreased), then fermentable carbohydrate
66 entered small intestine through rumen, caused intestinal acidosis, changed the microbial community
67 structure, and destroyed the integrity of intestinal epithelial morphology and structure [5].

68 The dietary nutrients were digested in intestine digestive enzymes, and absorbed into organism through
69 intestinal epithelium to provide energy for animals [6]. Jejunum was an important organ of nutrition
70 digestion, absorption and metabolism, also an important barrier against harmful substances, played an
71 important role in yak health. The research showed that jejunal congenital barriers was easy to be
72 damaged by the influence of external factors, which leaded to enteric flora disturbance, decrease the
73 production performance. Yak were grazing on the pasture without supplementary dietary all the year in
74 traditional grazing management, and yak body had formed a unique intestinal environment and
75 digestive function to adapt to plateau grazing conditions. However, the modern intensive management
76 pattern changed yak diet habit, high proportion concentrate dietary reduced feedstuff residence time in

77 yak rumen, improved the amount of jejunum carbohydrate and jejunum microbial fermentation level,
78 which had a negative effect on the jejunum epithelial cells monolayer structure and damage the
79 intestinal barrier [7,8]. This may be an important physiological mechanism that high proportion
80 concentrate dietary destroy yak jejunum structure and barrier. Here, twelve Datong male Yak with
81 different feeding patterns (forage-fed and concentrate-fed) during cold season were randomly selected
82 and slaughtered to determine Yak jejunal digestive enzyme activity, morphology and protein
83 composition. Collectively, our results shed new light on the damage of long-term high dietary
84 concentrate feeding on Yak jejunum. Meanwhile, this study laid a technical foundation in yak organism
85 health associated with dietary structure.

86 **MATERIALS AND METHODS**

87 All procedures involving the use of animals were approved by the Animal Care Committee of Lanzhou
88 Institute of Animal Science & Veterinary Pharmaceutics, Chinese Academy of Agricultural Sciences.
89 Animal slaughtering was approved by the National Administration of Cattle Slaughtering and
90 Quarantine regulations (Qinghai, China).

91 **Study Site**

92 The study was performed at Haibei Tibetan Autonomous Prefecture, Qinghai Province of China,
93 situated at northeastern Qinghai-Tibetan Plateau. This area is over 3000m above sea level and has a dry
94 cold climate.

95 **Animals and Diets**

96 Twelve Datong male Yaks (4±0.5 years old) were selected under grazing conditions on natural
97 grasslands in Haibei Tibetan Autonomous Prefecture. All the Yak were equally distributed into 2
98 groups. The experimental group Yak were feed in a roofed shed via intensively reared pattern, and the
99 control group Yak were feed in grazing pasture during the experimental period. The experiment lasted
100 180 days from 16th November, 2018 to 4th May, 2019, and the first 10d was an adjustment period of
101 experimental group Yak and the following 170d was for data collection period.
102 Based on the determination of the feed intake of the grazing Datong Yak, we prepared the concentrated
103 feed based on the recommendations for Chinese Beef cattle Raising Standard (NY/T 815-2004). The

104 ingredients composition and nutritional level of experimental group Yak feed was presented in Table
105 1. The roughage was mixed ration of maize silage and oat hay in 1:1. The daily dietary of each yak
106 contained with 4kg concentrate and 2kg roughage (DM) during the experimental period. The mixed diet
107 nutrient composition was determined in Qinghai University laboratory by 'Feed Analysis and Quality
108 Test Technology' [9].

109 **Measurement of Samples**

110 Datong male yaks were stunned by captive bolt pistol and their blood was drained at the end of
111 experiment. The jejunum was immediately isolated after slaughtering, and approximately 10g of jejunal
112 contents were aliquoted into 2mL sterile tubes and then conserved at -80°C ultracold storage freezer for
113 digestive enzyme activity analysis. The activity of Amylases (AMS), Trypsinases (TRS), Lipases (LPS)
114 and Carboxymethyl cellulase (CLS) were determined According to Fan's method used commercially
115 TaKaRa kits [10].

116 The separated jejunum tissue was divided two parts. One part for histological examination were fixed
117 in 4% buffered formaldehyde for 72 hours, then the samples were dehydrated under different
118 concentration of glucose, embedded in frozen embedding medium, and serial 5-7 μm transverse sections
119 were cut and stained with hematoxylin and eosin (HE), which displayed villous height, villous width,
120 crypt depth, mucosa thickness and muscular thickness, respectively. Images were acquired using the
121 DP70 software (Olympus, Nagano, Japan) per transverse section of jejunum tissue. The slides were
122 observed using BX51 microscopy (Olympus, Nagano, Japan). Measurements were made using Image
123 Proplus5.1 software. 10 well-oriented and intact crypt-villus units of each slide were measured in
124 triplicate.

125 Other part was aliquoted into 1.5mL centrifugal tube, when the jejunum surface contaminants were
126 removed by washing with phosphate buffered saline solution, and then conserved at-80℃ ultracold
127 storage freezer for proteomics and qPCR analysis. proteomics analysis was conducted by Novogene
128 (China, Beijing). There were 3 replicates each group. Jejunum proteins were extracted by lysis buffer
129 method (7M Urea, 2M Thiourea, 4% CHAPS(w/v), 100 mM DTT, 60 mM Tris-HCl, 0.5% pH 3-10
130 Ampholine, 20μL proteinase inhibiors) [11]. Digestion of the protein (250 μg for each sample) was

131 performed according to the FASP procedure described. Label-free Mass Spectrometry (MS)
132 experiments were performed on a Q Exactive mass spectrometer that was coupled to an Easy nLC
133 system (Thermo Fisher Scientific, MA, USA). The MS data were analyzed using MaxQuant software
134 and compared with the UniProt Bos taurus database. Relative quantitative real-time PCR (qPCR) was
135 performed to determine the copy number of target genes. Shanghai Biological Engineering Ltd.
136 synthesized the primers. The qPCR reaction was carried out in a Light Cycler 480 fluorescence
137 quantitative meter (Fritz Hoffmann-La Roche Co. Limited, Basel, Switzerland).

138 **Statistical Analysis**

139 Functional annotation and classification of all identified proteins were determined by using the
140 Blast2GO program against the uniport database, including cell component, biological process, and
141 molecular function. Pathway analyses were extracted using the search pathway tool of the KEGG
142 mapper platform (http://www.genome.jp/kegg/mapper.html). Pathway enrichment statistics were
143 conducted by Fisher's exact test, and the pathways with a corrected p value < 0.05 were defined as the
144 most significant pathways. The STRING program (http://string-db.org/) for the retrieval of interacting
145 genes/proteins database for the prediction of the physical and functional interactions was used to
146 analyze the PPi. The graphical visualization and analysis of interaction network was performed in
147 Cytoscape software.

148 The results of the analysis were given as mean ± SD. Duncan's post hoc test was used to determine any
149 significant differences between groups. Differences were considered as significant at $P < 0.05$ and as a
150 trend at $P<0.1$.

151 **RESULTS**

152 **Digestive enzyme activity analysis of jejunum**

153 The activity of AMS, LPS, TRS as well as CLS in different feeding patterns were presented in Table 2.
154 The data indicated that the AMS and CLS activity of forage-fed group was higher than that in
155 concentrate-fed group, but no significant difference between those two groups ($P>0.05$); The LPS
156 activity of concentrate-fed group was significantly greater than that in forage-fed group ($P<0.05$); The
157 TRS activity of concentrate-fed group was significantly lower than that in forage-fed group ($P<0.05$).

158 In contrast, the Yak jejunum digestive enzyme activity of forage-fed pattern was better than concentrate-
159 fed pattern.

160 **Histological analysis of jejunum morphology**

161 The villous height, villous width, crypt depth, mucosa thickness and muscular thickness during forage-
162 fed group and concentrate-fed group were summarized in Table 3. The villous height, villous width,
163 mucosa thickness and muscular thickness of jejunum were significantly greater in forage-fed group
164 compared to that in concentrate-fed group ($p<0.05$). however, the crypt depth of control group was
165 lower than that in concentrate-fed group, but no significant difference between those two groups
166 ($P>0.05$).

167 **Changes in proteome profiles during different feeding pattern**

168 The changes proteins of yak jejunum during different feeding pattern were investigated using label-free
169 MS. A total of 96 differentially expressed proteins were identified between concentrate-fed group and
170 forage-fed group as determined using $P < 0.05$ and a quantitative ratio of > 2 or < 0.5. There were 17
171 up-regulated and 79 down-regulated differentially proteins in forage-fed group compared to that in
172 concentrate-fed group (Fig.1).

173 **Bioinformatics analyses**

174 To explore biological functions associated with differentially expressed proteins of yak jejunum during
175 different feeding pattern, enrichment analysis in the Gene Ontology (GO) contained cellular
176 components (CC), molecular function (MF) and biological process (BP) was performed (Fig. 2). The
177 major enriched terms of differentially expressed proteins were membrane (GO:0016020), cytoplasm
178 (GO:0005737) and mitochondrion (GO:0005739, GO:0005740) for cellular components. Differentially
179 expressed proteins were enriched of molecular function in oxidoreductase activity (GO:0016491), cell-
180 cell adherens junction (GO:0005525), adenylate kinase activity (GO:0004017), protein binding
181 (GO:0019904) and structural molecule activity (GO:0003954). Apoptosis (GO:0048468),
182 Inflammatory Response (GO:0016020), Cellular response to fatty acid (GO:0008152), Actin
183 cytoskeleton reorganization (GO:0055114) and Adenylate cyclase-inhibiting G protein-coupled
184 receptor (GO:0046034) were enriched in biological processes.

185 The major pathways associated with differentially expressed proteins of yak jejunum during
186 concentrate-fed group and forage-fed group were identified using KEGG Pathway Analysis via KAAS
187 software. 96 differentially expressed proteins across 20 pathways were significantly enriched ($p < 0.05$).
188 The significantly enriched pathways were Tight junction signaling pathway (ko04530), NF-kappa B
189 signaling pathway (ko04064), MAPK signaling pathway (ko04010), Cytokine-cytokine receptor
190 interaction (ko04060), Calcium signaling pathway (ko04020), and Signaling pathways regulating
191 pluripotency of stem cells (ko04550, Fig. 3). The results of KEGG pathway analysis matched the results
192 of GO analysis.
193 To explore the information of differentially expressed proteins of yak jejunum during concentrate-fed
194 group and forage-fed group network, connected proteins among the 96 proteins were analyzed via
195 STRING program and Cytoscape software. As showed, the results identified two predominant themes:
196 protein structure (Occluding, Claudin, Zo-1, ATP1B3 and HKDC1) and inflammatory response
197 (GPR41, IL-6, TNF-α and CCL5) constituted a complex and strong PPi network (Fig. 4).Therefore,
198 these target proteins could be regarded as important for jejunal structure, physiological function and
199 protein composition.

200 **Validation of differentially expressed proteins coding genes by qPCR**

201 As shown in Fig.5, the gene expression of OCLN, CLDN1, TJP1 and HKDC1 were higher in forage-
202 fed group than that in concentrate-fed group ($P<0.05$) via RT-PCR, and the ATP1B3 expression was
203 no significant difference between two groups, however, the gene expression of IL6, GPR41, CCL5 and
204 TNF were higher in concentrate-fed group than that in forage-fed group ($P<0.05$). In summary, the
205 results of selected differentially expressed proteins coding genes by RT-PCR were the same expression
206 tendency of the label-free analysis. The RT-PCR assay illustrated that the label-free results were reliable
207 for further analyses.

208 **DISCUSSION**

209 Jejunum was the main organ for dietary nutrition digestion, absorption and metabolism, and played an
210 important role in yak health. The dietary concentrate was more than 75% under modern intensive
211 management pattern to enhance daily gain and performance, However, long-term high dietary

212 concentrate feeding can eventually have a serious impact on animal health, such as jejunal acidosis [12].
213 The level of dietary nutrition digestion, absorption and metabolism could be reflected through digestive
214 enzyme activity. Starch was the main energy source for ruminants, and AMS could hydrolyze starch to
215 glucose, maltose and oligosaccharides, which acted on the 4-c-D-glycosidic bonds of starch molecule
216 [13]. Fat was the necessary materials of energy storage and nutrition in animal body, and the animal
217 essential fatty acids were mainly provided by dietary fat. LPS could digest dietary fat to absorbable
218 nutrition, such as free fatty acid, glycerin and monoglyceride. TRS could hydrolyze dietary proteins to
219 small molecular amino acids, those amino acids were involved in the pathway of intracellular signaling
220 transmission and apoptosis, and played an important role in animal immune response [14]. The ruminant
221 cellulose degrading mainly depended on the cellulase secreted by microorganism, and CLS activity was
222 an important reflection of ability to degrade cellulose [15]. In our research, yak jejunum digestive enzyme
223 activity would be in a weakened state with long-term high dietary concentrate feeding, we believed that
224 the digestion and absorption of forage-fed pattern was better than concentrate-fed group.
225 The morphophysiological variations in jejunal epithelium of ruminants measured the ability of dietary
226 nutrients digestion and absorption [16]. Changes in the structure of villous absorptive surface (height,
227 width and crypt) in jejunal epithelial was closely related with extent of enterocytes development, which
228 were direct representation of the quality of intestinal environment, and often be used as the indicator of
229 intestinal health [17]. The present research showed that the ability of dietary nutrients digestion and
230 absorption increased with increasing villous absorptive surface, and submucosa thickness and muscular
231 thickness of jejunum were decreased with long-term high dietary concentrate, decreasing the absorption
232 of nutrients in Yak jejunum. When the high concentrate dietary was be fed for a long-term, a large
233 number of nutrients were degraded into short-chain fatty acids (SCFAs) by microorganisms in rumen,
234 which lead to increase the jejunum volatile fatty acids (VFAs), and decrease the jejunum pH value.
235 VFAs had a strongly lipid solubility, when the pH value dropped below the critical value, VFAs would
236 infiltrate into jejunum mucosa cells to acidify cells, which damaged the intestinal barrier and caused
237 the inflammatory response [18]. In our study, we found that the significantly decreased results in
238 concentrate-fed group of yak jejunum villous height, villous width, mucosa thickness and muscular

239 thickness. these results agreed with that reported in the ruminant intestine when fed with higher
240 concentrate feedstuff.
241 When SCFAs in rumen permeated into Yak jejunum, which would destroy the structural integrity of
242 morphophysiological and damage epithelial barrier function [19]. Jejunal mechanical barrier played a
243 mechanical protective role in jejunum via the normal morphological structure of jejunal mucosa [20-22].
244 Tight junction proteins were the important part of jejunum mechanical barrier, composed of transporters
245 protein family, including Occludin and Claudin, which were mainly involved in enterocytes
246 proliferation differentiation, apoptosis and cell bypass permeability regulation. The damaged
247 mechanical barrier induced jejunal dysfunction, caused pathogens to infect enterocytes, it resulted in
248 tight junction structural damage, permeability increasing and inflammatory response [18]. According to
249 recent reports and our study results presented target proteins can be concluded to two predominant
250 themes: protein structure (Tight junction signaling pathway, Calcium signaling pathway and Signaling
251 pathways regulating pluripotency of stem cells) and inflammatory response (MAPK signaling pathway,
252 Chemokine signaling pathway and Cytokine-cytokine receptor interaction). GPR 41, a key activator of
253 jejunal mechanical barrier, participated in the regulation of body inflammatory response, and affect the
254 tight junction signaling pathway as a nutrient energy regulatory receptor [23]. The large concentrate
255 intake could start-up enterocytes gluconeogenesis process, activated the tight junction signaling
256 pathway, then regulated downstream related cell apoptosis pathways, which decreased the tight junction
257 proteins expressions, such as Occluding, Claudin and Zo-1. Meanwhile, the proliferation of Regulatory
258 T Cells to secrete many anti-inflammatory cytokines (IL-6, TNF-α, CCL5), promoted the inflammatory
259 response pathway (MAPK) related genes expression [24,25]. GPR41 was detected in the current study,
260 and presented significantly up-regulated in concentrate-fed group, it showed that long-term high dietary
261 concentrate feeding changed the normal jejunal metabolic pathways. Furthermore, several identified
262 candidate proteins were identified by label-free MS, followed by RT-PCR: Occluding, Claudin, Zo-1,
263 ATP1B3, HKDC1, IL-6, TNF-α and CCL5. Compared with forage-fed group, the expressing quantity
264 of Occluding, Claudin, Zo-1, ATP1B3 and HKDC1 in Yak jejunum presented significantly down-
265 regulated in concentrate-fed group, however, IL-6, TNF-α and CCL5 showed a contrary tendency, it

266 indicated that a large number of SCFAs in jejunum of the high-concentrate dietary could damage the
267 mechanical barrier, reduce the differentially expression proteins. At the same time, GPR41 was
268 activated to up-regulate the expression, and promoted the proinflammatory factors expression. In this
269 research, we found that feeding pattern result in Yak different jejunum physiological function,
270 morphology and protein composition. This fact was confirmed long-term high dietary concentrate
271 feeding can damage the jejunum epithelial morphology and structure. Therefore, we hold that the high
272 proportion concentrate dietary was only suitable for short-term fattening pattern, not cultivation of
273 reserved yak.

274 **CONFLICT OF INTEREST**

275 We certify that there is no conflict of interest with any financial organization regarding the material
276 discussed in the manuscript.

277 **ACKNOWLEDGMENTS**

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366

367

368 **Table 1** Ingredients and proximate nutrition content of concentrate

Composition	Content (%)	Nutritional composition	Content
Maize	67.0	ME b	3.01 Mcal/kg
Soybean meal	5.0	CP	14.88 %DM
Cottonseed meal	10.0	Ca	0.66 %DM
Wheat bran	14.0	P	0.43 %DM
Salt	0.5		
Sodium Bicarbonate	1.5		
Stone Powder	1.5		
Premix Feed a	0.5		

369 a The premix provided the following per kg of diets：VA 12000IU, VD 20 00IU, VE 30IU, Cu 12mg, Fe 64mg,

370 Mn 56mg, Zn 60mg, I 1.2mg, Se 0.4mg, Co 0.4mg;

371 b ME was calculation value (NRC,2007), others were measured value.

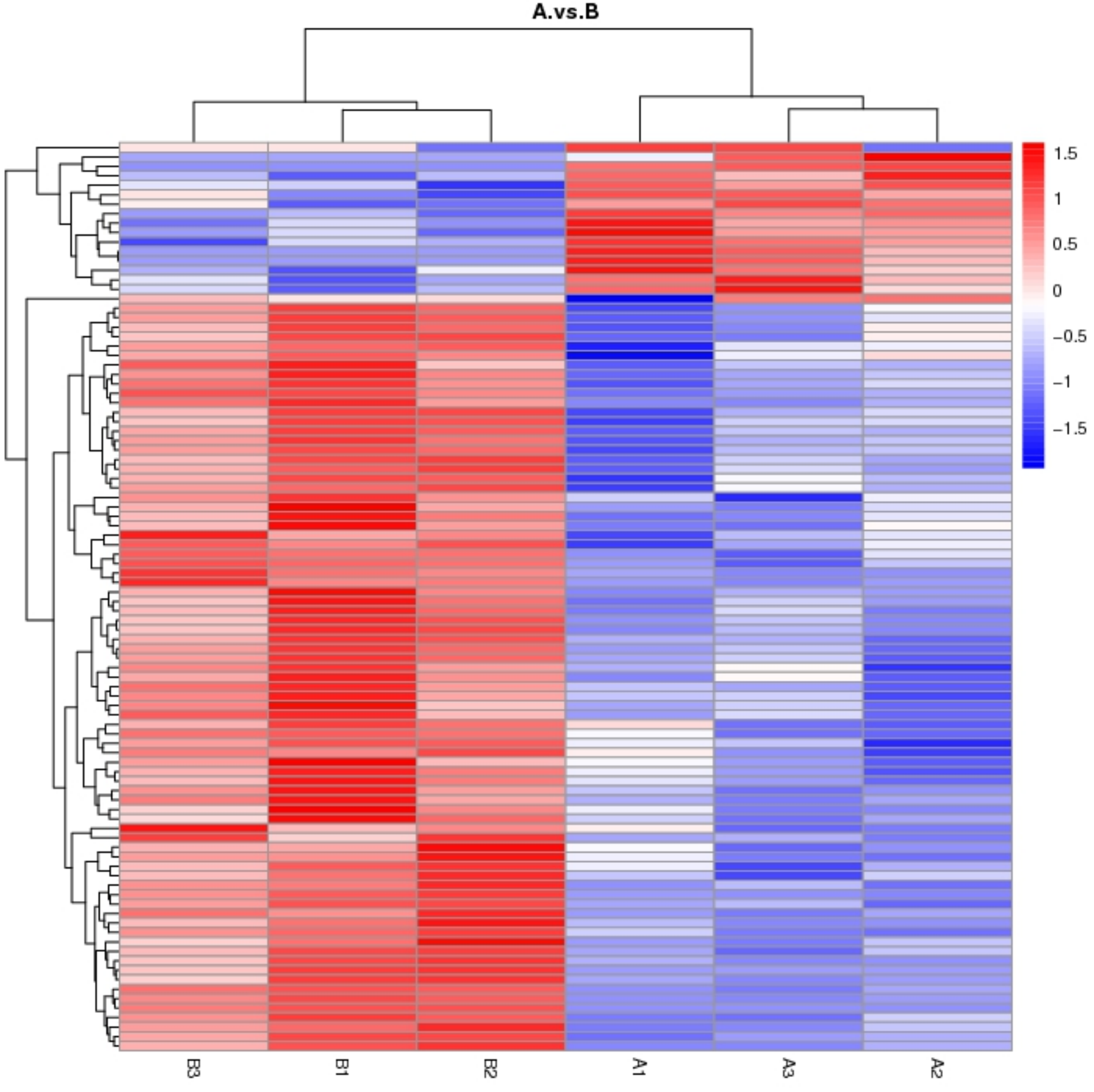
372 **Table 2** The alkaline phosphatase activity of yak jejunum in different feeding pattern

	AMS	LPS	CLS	TRS
forage-fed	0.176±0.043	0.218±0.072a	4.102±0.835	70.642±7.206a
concentrate-fed	0.121±0.071	0.542±0.053b	2.145±0.332	36.109±3.058b

373

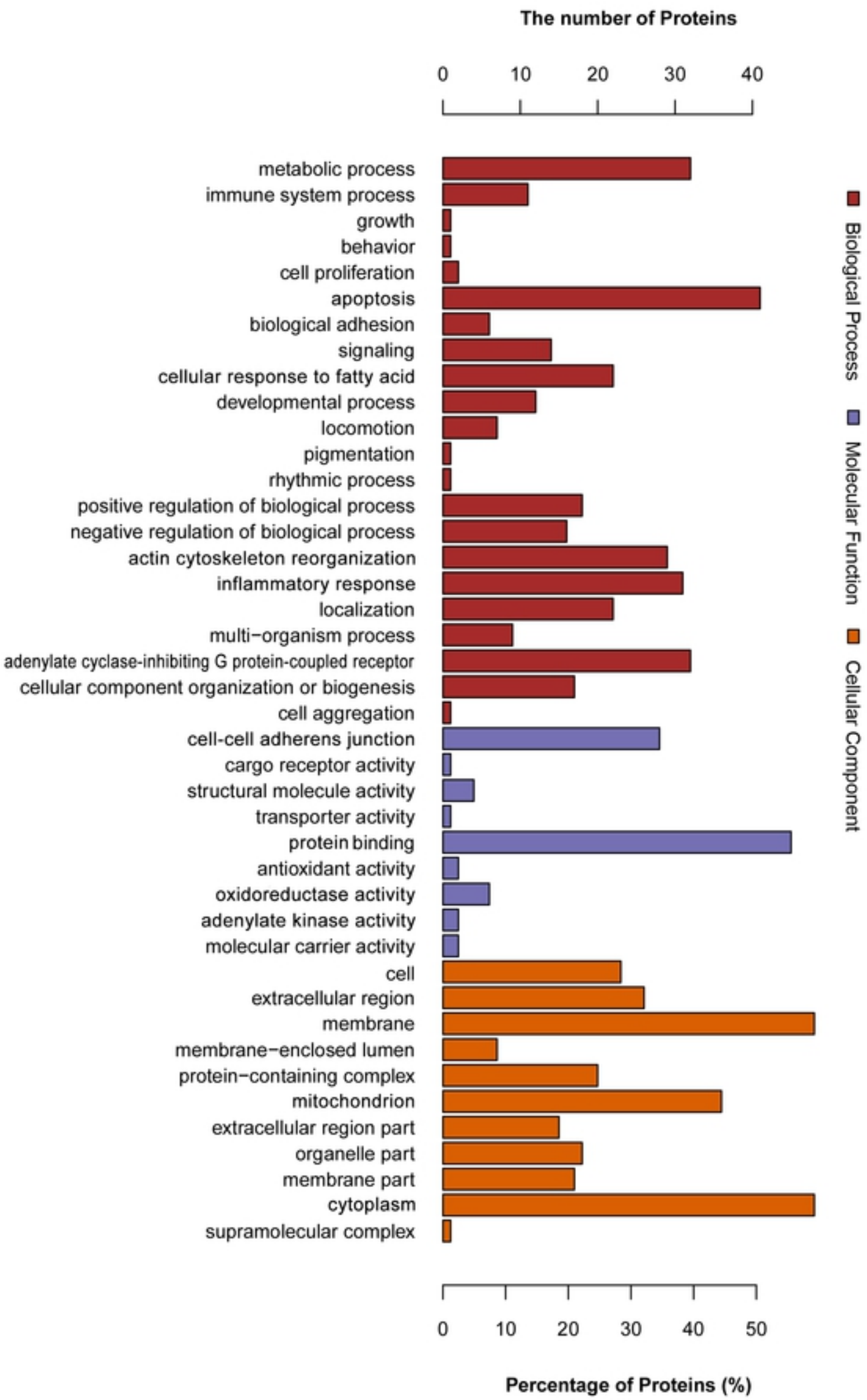
Table 3 The villous height, villous width, crypt depth, mucosa thickness and muscular thickness of yak jejunum in different feeding pattern

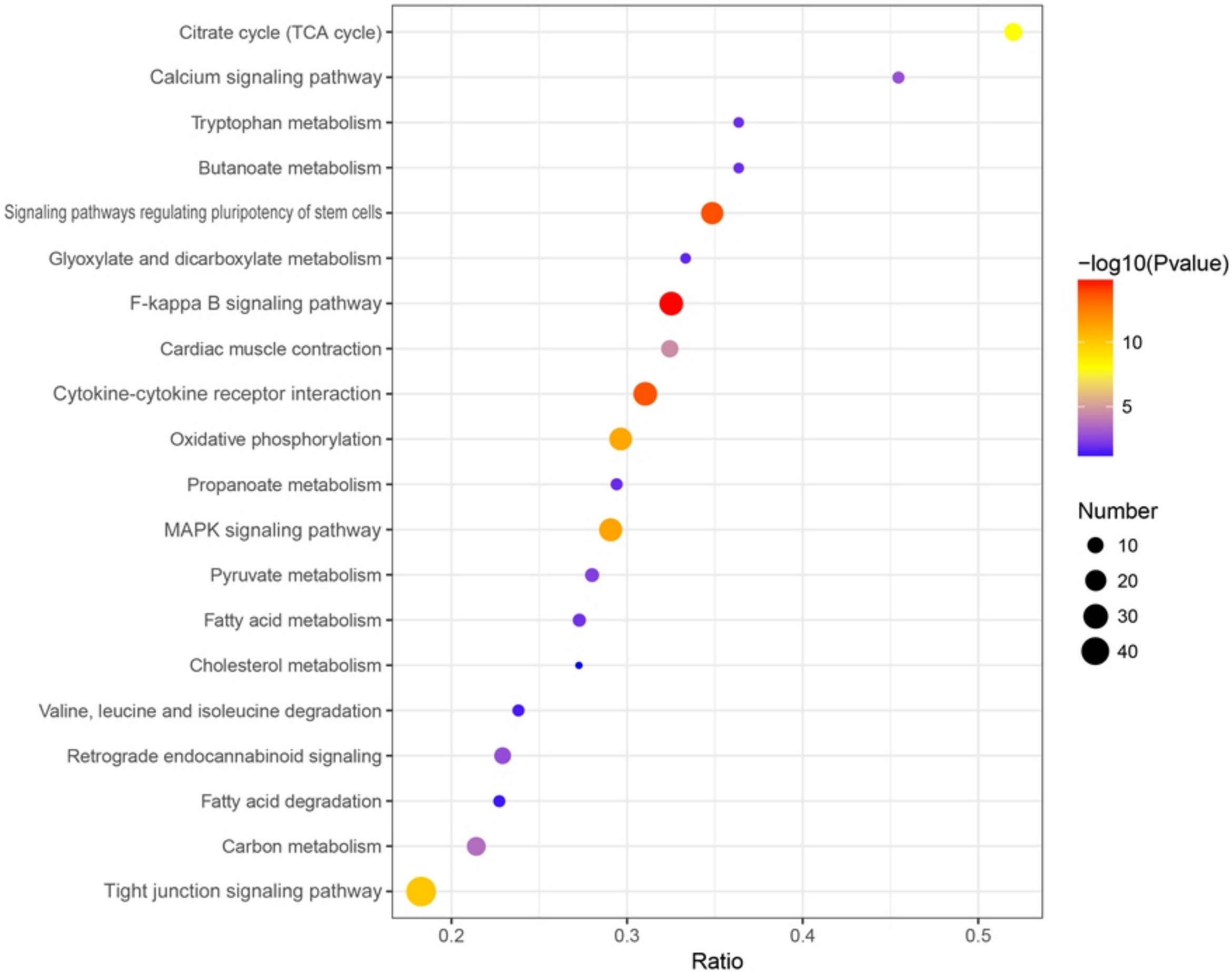
	villous height	villous width	crypt depth	mucosa thickness	muscular thickness
forage-fed	563.33±11.48a	149.29±2.67a	178.84±6.59	808.38±15.52a	528.91±20.93a
concentrate-fed	408.31±10.46b	82.88±5.45b	194.61±8.37	939.18±14.14b	751.51±15.14b



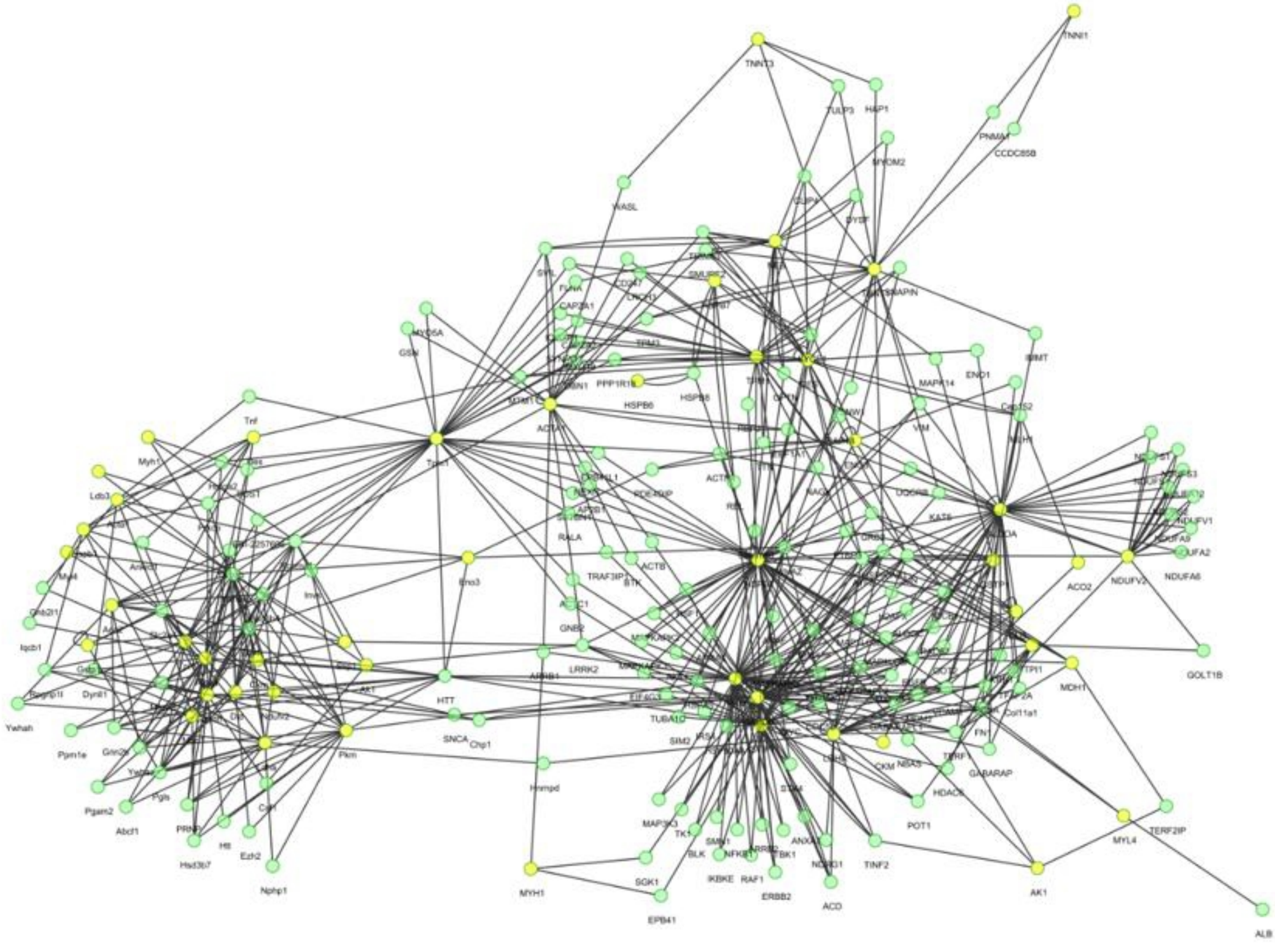
Figure

Figure

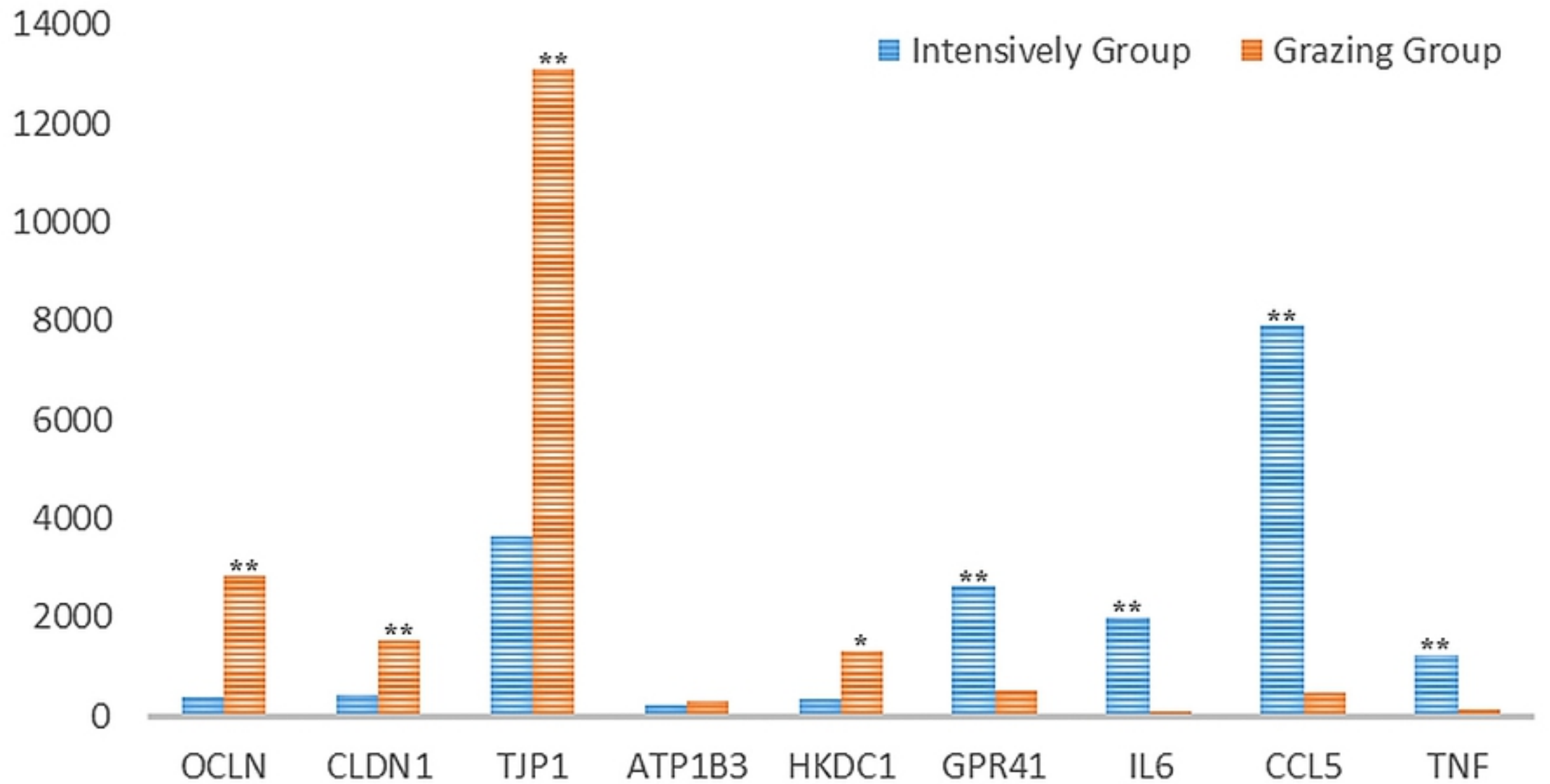

The number of Proteins
0
10
20
30
40
metabolic process
immune system process
growth
behavior
cell proliferation
apoptosis
biological adhesion
signaling
cellular response to fatty acid
developmental process
locomotion
pigmentation
rhythmic process
positive regulation of biological process
negative regulation of biological process
actin cytoskeleton reorganization
inflammatory response
localization
multi−organism process
adenylate cyclase-inhibiting G protein-coupled receptor
cellular component organization or biogenesis
cell aggregation
cell-cell adherens junction
cargo receptor activity
structural molecule activity
transporter activity
protein binding
antioxidant activity
oxidoreductase activity
adenylate kinase activity
molecular carrier activity
cell
extracellular region
membrane
membrane−enclosed lumen
protein−containing complex
mitochondrion
extracellular region part
organelle part
membrane part
cytoplasm
supramolecular complex
Biological Process
Molecular Function
Cellular Component
0
10
20
30
40
50
Percentage of Proteins (%)


Citrate cycle (TCA cycle)
Calcium signaling pathway
Tryptophan metabolism
Butanoate metabolism
Signaling pathways regulating pluripotency of stem cells
Glyoxylate and dicarboxylate metabolism
F-kappa B signaling pathway
Cardiac muscle contraction
Cytokine-cytokine receptor interaction
Oxidative phosphorylation
Propanoate metabolism
MAPK signaling pathway
Pyruvate metabolism
Fatty acid metabolism
Cholesterol metabolism
Valine, leucine and isoleucine degradation
Retrograde endocannabinoid signaling
Fatty acid degradation
Carbon metabolism
Tight junction signaling pathway
0.2
0.3
0.4
0.5
Ratio
−log10(Pvalue)
10
5
Number
10
20
30
40

Figure


TNNI1
TNNT3
TULP3
HAP1
PNMA1
CCDC85B
MYOM2
WASL
DYSF
SMURF2
SNAPIN
MYO5A
GSN
IBMT
ENO1
MAPK14
PPP1R18
HSPB6
HSPB8
Tnf
Myh1
Ldb3
VIM
KAT6
RALA
ACTB
TRAF3IP2
BTK
Eno3
ACO2
NDUFV2
NDUFA6
NDUFV1
GNB2
LRRK2
ARRB1
HTT
SNCA
Chp1
Pkm
Hnmpd
Iqcb1
Dynll1
Ywhah
Pgm1e
Pgam2
Abcf1
Hsd3b7
Ezh2
Nphp1
MYH1
SGK1
EPB41
IKBKE
RAF1
ERBB2
ACD
TINF2
POT1
HDAC6
GABARAP
FN1
Col11a1
MDH1
GOLT1B
MYL4
TERF2IP
AK1
ALB

Figure


14000
12000
10000
8000
6000
4000
2000
0
Intensively Group
Grazing Group
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*
**
**
**
**
OCLN
CLDN1
TJP1
ATP1B3
HKDC1
GPR41
IL6
CCL5
TNF

Figure