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**Effects of Dietary β -Carotene Supplementation on Vitamin A and β -Carotene Levels,
Antioxidant Responses, and Milk Composition in Holstein Cows**

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ABSTRACT

Background: The transition from late gestation to early lactation challenges antioxidant
defense in dairy cows. This study evaluated the effects of dietary β -carotene supplementation
from 60 d prepartum to 90 d postpartum on milk composition, vitamin A and β -carotene
status, antioxidant responses, and β -carotene oxygenase 1 (BCO1) and β -carotene oxygenase
2 (BCO2) mRNA abundance in Holstein cows.

Results: Twenty-four multiparous Holstein cows were assigned to 0, 600, 1,200, or 1,800
mg/d β -carotene (n = 6 per treatment). Supplementation had limited effects on milk yield and

most milk components, but decreased somatic cell count in mature milk. Dietary β -carotene increased serum and hepatic β -carotene concentrations and promoted hepatic vitamin A accumulation, whereas serum vitamin A and mature milk vitamin A and β -carotene concentrations were not markedly affected. Supplementation also enhanced serum antioxidant capacity by increasing total antioxidant capacity, superoxide dismutase, and glutathione peroxidase activities, and by reducing reactive oxygen species, total oxidant status, and inducible nitric oxide synthase. Tissue responses were tissue dependent, but antioxidant enzyme activities generally improved and malondialdehyde concentrations decreased in liver and intestinal tissues. BCO1 and BCO2 expression patterns suggested that the duodenum and jejunum were the main intestinal sites associated with β -carotene metabolism.

Conclusions: Dietary β -carotene supplementation from late gestation to early lactation supported β -carotene and vitamin A status and improved redox balance in Holstein cows. The most consistent responses were observed at 1,200 to 1,800 mg/d.

Keywords: β -carotene; Holstein cows; vitamin A; oxidative stress; antioxidant status; milk composition

Background

The transition from late gestation to early lactation is a metabolically demanding period for dairy cows. During this stage, rapid physiological changes associated with colostrum synthesis and increasing milk production may increase reactive oxygen species production and challenge antioxidant defense systems. Impaired redox balance during early lactation has been associated with metabolic stress and health-related disorders in dairy cows [1–3].

Therefore, nutritional strategies that support antioxidant defense and immune function may help improve cow resilience during the transition period [4, 5].

β -Carotene is an important dietary carotenoid and a precursor of vitamin A in cattle [6, 7]. However, its bioavailability can be affected by ruminal degradation, intestinal absorption, and conversion to retinol, and responses to supplemental β -carotene may vary with basal β -carotene intake and vitamin A status [7]. In addition to its role in vitamin A metabolism, β -carotene has antioxidant and immune-regulatory properties [8]. Dairy cows cannot synthesize β -carotene and must obtain it from dietary sources. However, plasma β -carotene concentrations may decrease around calving, and β -carotene status is closely related to dietary intake [9]. Thus, β -carotene supplementation may be beneficial during the transition period and early lactation. Previous studies have shown that β -carotene supplementation can increase serum β -carotene and vitamin A concentrations in dairy cows, although its effects on milk yield and milk composition are inconsistent [10]. Similarly, different supplies of vitamin A during the transition period have been reported to affect milk retinol concentration and somatic cell count (SCC) responses without consistently improving milk yield or milk components [11]. β -Carotene supplementation has also been reported to affect colostrum quality and β -carotene transfer to calves [12, 13].

Although β -carotene has been studied in relation to serum status, colostrum quality, and production performance, most previous studies have focused mainly on blood, milk, or colostrum responses. Less information is available on whether graded β -carotene supplementation from late gestation to early lactation is accompanied by coordinated changes in hepatic and intestinal β -carotene/vitamin A status, tissue antioxidant responses, and the

expression of β -carotene oxygenase genes across different intestinal segments. The liver is the major storage site for vitamin A, whereas the small intestine is a key site for carotenoid absorption and cleavage. Therefore, evaluating liver and intestinal tissues together may provide a more complete understanding of the biological effects of β -carotene supplementation in dairy cows. The objective of this study was to evaluate the effects of dietary β -carotene supplementation from 60 d prepartum to 90 d postpartum on milk yield and composition, vitamin A and β -carotene status, serum and tissue antioxidant responses, and β -carotene oxygenase 1 (BCO1) and β -carotene oxygenase 2 (BCO2) mRNA abundance in liver and intestinal tissues of Holstein cows. We hypothesized that β -carotene supplementation would increase circulating and hepatic β -carotene availability, promote hepatic vitamin A accumulation, improve serum and tissue antioxidant status, and alter the relative mRNA abundance of BCO1 and BCO2 in a tissue-dependent manner.

Methods

All animal procedures were reviewed and approved by the Ethics Committee for Animal Care and Use of Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences, China (Approval No. 2025-0088). The study was reported in accordance with the Animal Research Reporting of In Vivo Experiments (ARRIVE) 2.0 guidelines where applicable.

Animals and Experimental Design

The study was conducted at an experimental dairy farm in Toketuo County, Inner Mongolia Autonomous Region, China. Twenty-four multiparous Holstein cows with similar body weight (550 ± 32.71 kg) and parity (2.5 ± 0.48) were used in this study. Cows were dried off 60 d before the expected calving date and were randomly assigned to 1 of 4 treatment groups

according to body weight, body condition score (BCS), parity, and expected calving date, 89
with 6 cows per treatment. Cows were eligible for enrollment if they were clinically healthy 90
multiparous Holstein cows, had no apparent metabolic or infectious disease before the start of 91
the experiment, and had similar body weight, body condition score, parity, and expected 92
calving date. These inclusion criteria were established before treatment allocation. No cows 93
were excluded from the experiment or from the statistical analysis, and all allocated cows 94
were included in the final data set ($n = 6$ cows per treatment). After balancing cows according 95
to body weight, body condition score, parity, and expected calving date, treatment allocation 96
was performed using a random number table. 97

The β -carotene supplement used in this experiment was a commercial product containing 98
10% β -carotene (DSM Co., Ltd., China). Cows were supplemented with 0, 600, 1,200, or 99
1,800 mg/d active β -carotene from 60 d before expected calving to 90 d postpartum. 100
Accordingly, cows in the supplemented groups received 6, 12, or 18 g/d of the commercial 101
product to provide 600, 1,200, or 1,800 mg/d active β -carotene, respectively. The daily 102
amount of supplement was weighed individually, mixed with 200 g of concentrate, and 103
offered before the morning total mixed ration (TMR) feeding to ensure complete intake. 104
Cows in the control group received the same amount of concentrate without supplemental β - 105
carotene. 106

Throughout the experiment, cows were housed in naturally ventilated freestall barns and had 107
ad libitum access to feed and fresh water. Total mixed rations were offered twice daily at 108
0630 and 1600 h. Diets were formulated to meet the nutrient requirements of dairy cows 109
according to the National Research Council [14]. Three diets were used during the 110

experiment: a dry-period diet, a periparturient diet, and a lactation diet. The dry-period diet was fed from 60 to 22 d before expected calving, the periparturient diet was fed from 21 d before expected calving to calving, and the lactation diet was fed from calving to 90 d postpartum. The ingredients and nutrient composition of the diets are shown in Table 1. Cows were observed daily by farm personnel throughout the experiment for general health, feed intake, behavior, and signs of discomfort or disease. Standard farm management and veterinary care were provided as needed to reduce pain, suffering, or distress. No unexpected adverse events, deaths, or removals occurred during the study.

At calving, cows were moved to individual maternity pens bedded with clean, dry straw and monitored by farm personnel. Colostrum was collected from each cow within 1 h after calving at the first milking and before calves were allowed to suckle. After calving, cows were moved to a lactating cow freestall barn and milked twice daily at 0800 and 1800 h. Milk yield was recorded at each milking from d 1 to 90 postpartum.

Table 1. Dietary ingredients and nutrient composition of the dry-period, periparturient, and lactation diets (DM basis)

Items	Dry period diet	Periparturient diet	Lactation diet
Ingredient, % of DM			
Corn	10.67	17.61	23.32
Whole-crop corn silage	28.54	29.63	29.65
Sugar cane molasses	0.00	0.49	0.00
Soybean meal	1.71	4.00	7.28

Cotton seed meal	1.23	3.33	4.66
Rape seed meal	0.86	0.00	0.00
Corn gluten meal	0.00	0.00	1.16
Wheat bran	2.85	6.67	3.79
Distillers dried grains with solubles	5.14	3.73	4.85
Limestone	0.55	0.45	1.13
Corn germ meal	5.14	7.39	0.00
Leymus chinensis	42.81	25.93	3.64
Alfalfa hay	0.00	0.00	18.19
Anti-mould agent	0.02	0.02	0.03
Premix (Mineral salts and vitamins) ¹	0.32	0.49	0.54
NaHCO ₃	0.00	0.00	0.81
MgO	0.00	0.00	0.38
Total	100	100	100

Nutrient composition, % of DM unless otherwise noted

NEL, MJ/kg of DM	5.30	5.61	6.02
CP	10.76	12.05	14.41
Ca	0.40	0.31	0.72
P	0.29	0.28	0.33
ADF	29.83	23.44	21.84
NDF	37.91	35.62	34.17
β-Carotene, µg/100 g of DM	86.50	77.20	91.20

1 Premix supplied the following per kg of diet dry matter: Se, 0.30–0.75 mg; Fe, ≥20 mg; Zn, 126

≥40 mg; Mn, ≥40 mg; Cu, 10–35 mg; vitamin A, 8,000 IU; vitamin D3, ≥2,400 IU; vitamin 127

E, ≥ 40 mg.	128
2 Nutrient contents were measured values.	129
3 NEL = net energy for lactation, estimated according to NRC [14].	130
Dry Matter Intake	131
Feed offered and refusals were recorded daily for each cow throughout the postpartum	132
period. The dry matter content of the diets was determined regularly, and individual dry	133
matter intake (DMI) was calculated as the difference between dry matter offered and refused.	134
Daily DMI was summarized for each cow at d 1, 7, 14, 21, 35, 60, and 90 postpartum.	135
Vitamin A and β-Carotene Analysis	136
Vitamin A and β -carotene concentrations in milk, serum, liver, intestinal tissues, and diets	137
were determined by high-performance liquid chromatography (HPLC). All sample	138
preparation procedures were performed under dim light to minimize degradation of retinoids	139
and carotenoids. Vitamin A standard and β -carotene standard (C4582-10MG; purity $\geq 95\%$)	140
were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol, ethanol,	141
petroleum ether, and dichloromethane were used for sample extraction and chromatographic	142
analysis.	143
For serum analysis, 400 μ L of serum was mixed with 400 μ L of anhydrous ethanol and 200	144
μ L of ascorbic acid ethanol solution as an antioxidant. After vortexing, samples were	145
extracted with ethanol–petroleum ether mixture, centrifuged, and the upper organic phase was	146
collected. The extraction was repeated 3 times, and the combined extracts were evaporated	147
under nitrogen and reconstituted in methanol before HPLC analysis. Milk samples were	148
thoroughly mixed before analysis and extracted using the same organic solvent extraction	149

procedure. 150

For liver and intestinal tissue analysis, approximately 2 g of tissue sample was homogenized 151
and mixed with anhydrous ethanol and ascorbic acid. Samples were saponified with 50% 152
NaOH, extracted with petroleum ether, and centrifuged. The upper organic phase was 153
collected, washed with distilled water until neutral, passed through anhydrous sodium sulfate, 154
evaporated to dryness, and reconstituted in methanol before injection. For diet analysis, 155
approximately 0.5 g of ground TMR sample was mixed with ascorbic acid, anhydrous 156
ethanol, and petroleum ether, followed by extraction in a 30°C water bath with ultrasonic 157
treatment. After centrifugation, the supernatant was collected, evaporated to dryness, and 158
reconstituted in methanol before HPLC analysis. 159

Chromatographic separation was performed using a PerkinElmer Altus A10 HPLC system 160
(PerkinElmer Inc., Waltham, MA, USA) equipped with a C18 reversed-phase column (250 × 161
4.6 mm). The column temperature was maintained at 30°C, the flow rate was 1.0 mL/min, 162
and the injection volume was 10 µL. Vitamin A was detected at 325 nm, and β-carotene was 163
detected at 455 nm using a ultraviolet (UV) detector. Quantification was performed using 164
external standard curves prepared from serial dilutions of vitamin A and β-carotene standards. 165
Concentrations were calculated according to sample volume or weight and expressed as 166
µg/mL for milk and serum, µg/g of tissue for liver and intestinal tissues. Method recovery 167
was evaluated using representative milk and serum samples spiked with known amounts of 168
vitamin A and β-carotene. In milk samples, recovery rates ranged from 82.1 to 85.3% for 169
vitamin A and from 80.7 to 85.8% for β-carotene. In serum samples, recovery rates ranged 170
from 82.1 to 105.6% for vitamin A and from 80.6 to 98.3% for β-carotene. Standard curves 171

were prepared for each analytical batch, and sample concentrations were calculated only 172
when the correlation coefficient of the standard curve was greater than 0.99. These recovery 173
results were used to verify the reliability of the extraction and HPLC quantification 174
procedures for vitamin A and β -carotene in milk and serum samples. 175

Milk Sampling and Analysis 176

Cows were milked twice daily at 0800 and 1800 h throughout lactation. Daily milk yield was 177
recorded for each cow from d 1 to 90 postpartum. Colostrum samples were collected within 1 178
h after calving at the first milking and before calves were allowed to suckle. Mature milk 179
samples were collected during the last 3 d of the experimental period. On each mature milk 180
sampling day, morning and afternoon milk samples were composited at a ratio of 60:40. The 181
composite samples collected during the last 3 d were further pooled for each cow to represent 182
the mature milk sample. Additional milk samples were collected on d 1, 2, 3, 7, 21, 35, 60, 183
and 90 postpartum for analysis of milk vitamin A and β -carotene concentrations. On each 184
mature milk sampling day, morning and afternoon milk samples were composited at a ratio of 185
60:40 according to the approximate daily distribution of milk yield between the 2 milkings, 186
except for colostrum collected at the first milking after calving. All milk samples were stored 187
at -20°C until subsequent analysis. Milk fat, protein, lactose, and total solids were 188
determined using a milk composition analyzer (Master Classic, LM2, Europe), and SCC was 189
measured using a Fossomatic 5000 Basic instrument (FOSS Electric, Hillerød, Denmark). 190
Feed offered and refusals were recorded daily for each cow, and dry matter intake (DMI) was 191
calculated as the difference between dry matter offered and refused. Milk yield was recorded 192
at each milking from d 1 to 90 postpartum. Fat-corrected milk (FCM) was calculated as 4% 193

FCM = $0.4 \times \text{milk yield} + 15 \times \text{milk fat yield}$. Milk production efficiency was calculated as 194
FCM divided by DMI. 195

Blood Sampling and Serum Analysis 196

Blood samples were collected on the last day of the experimental period via the 197
coccygeal vein into 10-mL evacuated tubes. The samples were kept on ice and subsequently 198
centrifuged at $2,500 \times g$ for 15 min at 4°C to separate serum. The serum samples were 199
immediately collected and stored at -20°C until further analysis. 200

Serum antioxidant and oxidative stress-related indices were determined using 201
commercial assay kits according to the manufacturers' instructions. Total antioxidant 202
capacity (T-AOC; A015-1-1) was measured using a colorimetric assay, superoxide dismutase 203
(SOD; A001-3-2) activity was determined using the water-soluble tetrazolium salt-1 (WST-1) 204
method, glutathione peroxidase (GSH-Px; A005-1-2) activity was measured using the 5,5'- 205
dithiobis-(2-nitrobenzoic acid) (DTNB) colorimetric method, reactive oxygen species (ROS; 206
E004-1-1) levels were quantified using a dichlorofluorescein-based fluorescence probe assay, 207
and inducible nitric oxide synthase (iNOS; H372-1-2) was determined using a double- 208
antibody sandwich enzyme-linked immunosorbent assay (ELISA). These kits were purchased 209
from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Total oxidant status 210
(TOS) was measured using a commercial colorimetric assay kit (E-BC-K802-M; Elabscience 211
Biotechnology Co., Ltd., Wuhan, China), based on the oxidation of ferrous ions to ferric ions 212
and subsequent formation of a ferric ion–xylenol orange complex detected at 590 nm. 213

Tissue Sampling and Analysis 214

At the end of the experimental period, 6 cows from each treatment were humanely 215

slaughtered at an approved abattoir, and liver, duodenum, jejunum, and ileum tissues were 216
collected immediately after slaughter. Slaughter was performed at an approved abattoir 217
following standard humane procedures. Animals were monitored before transport and 218
slaughter, and no additional experimental procedures were performed. Intestinal samples were 219
collected from standardized locations, including the proximal duodenum, mid-jejunum, and 220
distal ileum. Tissue samples were rinsed with sterile saline, blotted dry, snap-frozen in liquid 221
nitrogen, and stored at -80°C until further analysis. Before analysis, tissue samples were 222
homogenized in ice-cold sterile saline and centrifuged to obtain the supernatant for 223
biochemical assays. T-AOC (A015-1-1) was measured using a colorimetric assay, SOD 224
(A001-3-2) activity was determined using the water-soluble WST-1 method, reduced 225
glutathione (GSH; A006-2-1) content was measured using the 5,5'-dithiobis-(2-nitrobenzoic 226
acid) colorimetric method, GSH-Px (A005-1-2) activity was determined using a colorimetric 227
assay based on the oxidation of reduced glutathione, catalase (CAT; A007-1-1) activity was 228
measured using the ammonium molybdate colorimetric method, and malondialdehyde 229
(MDA; A003-1-2) content was quantified using the thiobarbituric acid method. All assay kits 230
were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and all 231
procedures were performed according to the manufacturer's instructions. 232

Quantitative Real-Time PCR Analysis

 233

Total RNA was extracted from liver, duodenum, jejunum, and ileum tissues using RNAiso 234
Plus reagent (9109; Takara Bio Inc., Shiga, Japan) according to the manufacturer's 235
instructions. RNA concentration and purity were assessed by spectrophotometry, and samples 236
with A260/A280 ratios between 1.8 and 2.0 were used for cDNA synthesis. Reverse 237

transcription was performed using PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time; RR047A; Takara Bio Inc.) according to the manufacturer's protocol. Quantitative real-time PCR was performed using TB Green Premix Ex Taq II (Tli RNaseH Plus; RR820A; Takara Bio Inc.) on a real-time PCR system. The amplification program consisted of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Melting-curve analysis was performed to confirm amplification specificity. Relative mRNA abundance was calculated using the $2^{-\Delta C_t}$ method, with β -actin as the reference gene. For heatmap visualization, relative expression values were log10-transformed. Statistical analysis was performed using ΔC_t values.

Table 2. Primer sequences used for RT-qPCR

Target gene	Accession number	Primer sequence (5'to 3') F=forward, R=reverse	Amplicon length (bp)
BCO1	NM_001009465.2	F:TCAGGAGGTGCGGAGAAGACTTC R: TCCAGCAGCGTCGTAGTGAGG	155
BCO2	NM_001009313.3	F: AGGTTGACGTGGTGAACAAGACAC R: TTACTGGCTCCTGGTACCGGAAC	94
β -actin	NM_001009392.1	F: GCAAGGACCTCTACGCCAACAC R: CTTGACAGCCACCACGCCTATC	198

Statistical Analysis

Data were analyzed using Statistical Analysis System (SAS) software (version 9.4; SAS Institute Inc., Cary, NC). The individual cow was considered the experimental unit. Before statistical analysis, residuals were checked for normality and homogeneity of variance. SCC was log-transformed when necessary, whereas untransformed means are presented in the

figures. 253

Unless otherwise indicated, the number of experimental units for each treatment was 6 cows. 254

The exact number of cows included in each analysis was therefore $n = 6$ per treatment for 255

milk production, milk composition, serum variables, tissue antioxidant indices, and gene 256

expression analyses. 257

Treatment effects were estimated as least squares mean differences with 95% confidence 258

intervals (CI) from the corresponding statistical models when a significant treatment effect 259

was detected. 260

For variables measured repeatedly over time, including milk yield, milk production 261

efficiency, milk vitamin A concentration, and milk β -carotene concentration, data were 262

analyzed using the MIXED procedure of SAS. The model was: 263

$$Y_{ijk} = \mu + TRTi + T_j + (TRT \times T)_{ij} + COWk(TRTi) + e_{ijk} \quad 264$$

where Y_{ijk} is the dependent variable, μ is the overall mean, $TRTi$ is the fixed effect of β - 265

carotene treatment, T_j is the fixed effect of time, $(TRT \times T)_{ij}$ is the interaction between 266

treatment and time, $COWk(TRTi)$ is the random effect of cow nested within treatment, and 267

e_{ijk} is the residual error. Time was used as the repeated factor, and cow within treatment was 268

used as the subject. Several covariance structures were compared, and the final covariance 269

structure was selected according to the lowest Akaike information criterion. Degrees of 270

freedom were adjusted using the Kenward–Roger method. 271

Milk composition variables, including fat, protein, lactose, total solids, and SCC, were 272

measured once from pooled milk samples and were analyzed separately for colostrum and 273

mature milk. Serum variables and tissue antioxidant indices were analyzed as single- 274

measurement data using the following model:

$$Y_{ij} = \mu + TRTi + e_{ij}$$

where Y_{ij} is the dependent variable, μ is the overall mean, $TRTi$ is the fixed effect of β -carotene treatment, and e_{ij} is the residual error. When a significant treatment effect was detected, least squares means were compared using Tukey's multiple comparison test. Data are presented as least squares means with pooled standard error of the mean (SEM) unless otherwise stated. Statistical significance was declared at $P < 0.05$, and tendencies were discussed at $0.05 \leq P < 0.10$.

For BCO1 and BCO2 mRNA abundance, data were analyzed by one-way analysis of variance (ANOVA) using the GLM procedure of SAS. When gene expression was compared among tissues, tissue was included as the fixed effect. When gene expression was compared among β -carotene treatments within the same tissue, treatment was included as the fixed effect. Gene expression data were analyzed using ΔCt values, whereas $\log_{10}$ -transformed relative expression values were used only for graphical presentation. When a significant effect was detected, means were separated using Tukey's multiple comparison test.

Results

Dry Matter Intake

As shown in the figure, DMI increased progressively with advancing days postpartum in all treatment groups. Two-way ANOVA showed that dietary β -carotene supplementation had no significant effect on DMI ($P = 0.8276$), whereas time had a significant effect ($P < 0.0001$). No significant treatment $\times$ time interaction was observed ($P = 0.0627$), although a tendency was noted. Overall, DMI was relatively low on d 1 postpartum and increased gradually

thereafter, reaching higher levels on d 60 and d 90 postpartum. The differences among the 0, 600, 1,200, and 1,800 mg/d β -carotene groups were small, indicating that β -carotene supplementation did not markedly alter the postpartum pattern of DMI.

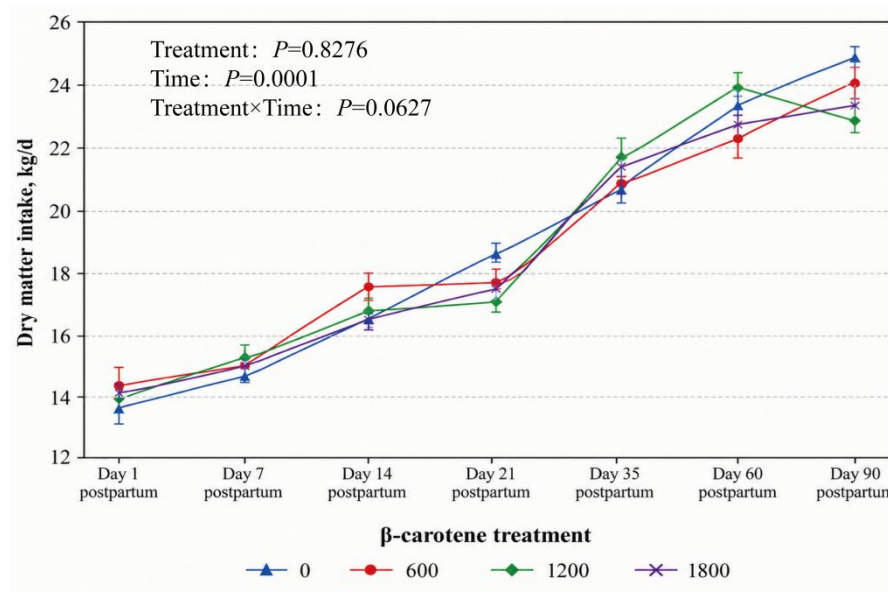


Fig. 1. Effects of dietary β -carotene supplementation on dry matter intake of dairy cows during the postpartum period. Cows were supplemented with 0, 600, 1,200, or 1,800 mg/d β -carotene. The effects of treatment, time, and treatment $\times$ time interaction were analyzed by two-way ANOVA.

Milk Production and Composition

To evaluate the effects of dietary β -carotene supplementation on lactational performance, milk yield was monitored from early lactation to d 90 postpartum, and milk composition was determined using colostrum and mature milk samples. The effects of dietary β -carotene supplementation on milk yield and composition are shown in Fig. 2A,B, and temporal changes in milk yield and milk production efficiency are presented in Fig. 2 C,D. Dietary β -carotene supplementation did not significantly affect milk yield, fat

percentage, total solids, protein percentage, or lactose percentage in colostrum ($P > 0.05$). 312

However, SCC in colostrum was affected by treatment ($P < 0.01$), with greater SCC observed 313

in cows supplemented with 1,200 and 1,800 mg/d β -carotene than in cows receiving 0 or 600 314

mg/d. In mature milk, milk yield, fat percentage, protein percentage, total solids, and lactose 315

percentage were not affected by β -carotene supplementation ($P > 0.05$). Notably, SCC in 316

mature milk was significantly affected by dietary β -carotene supplementation ($P < 0.01$). 317

Compared with the control group, cows supplemented with β -carotene had lower SCC, with 318

the lowest value observed in the 600 mg/d group. 319

As shown in Fig. 2C, milk yield changed over time during the postpartum period. Milk yield 320

increased from d 1 to d 60 postpartum and then declined slightly by d 90 postpartum. 321

Significant effects of treatment, time, and treatment $\times$ time interaction were observed for milk 322

yield ($P < 0.01$). Milk production efficiency also changed during the postpartum period (Fig. 323

2D). A significant treatment effect was observed for milk production efficiency ($P < 0.01$), 324

whereas the effects of time and treatment $\times$ time interaction were not significant ($P = 0.78$ 325

and $P > 0.99$, respectively). 326

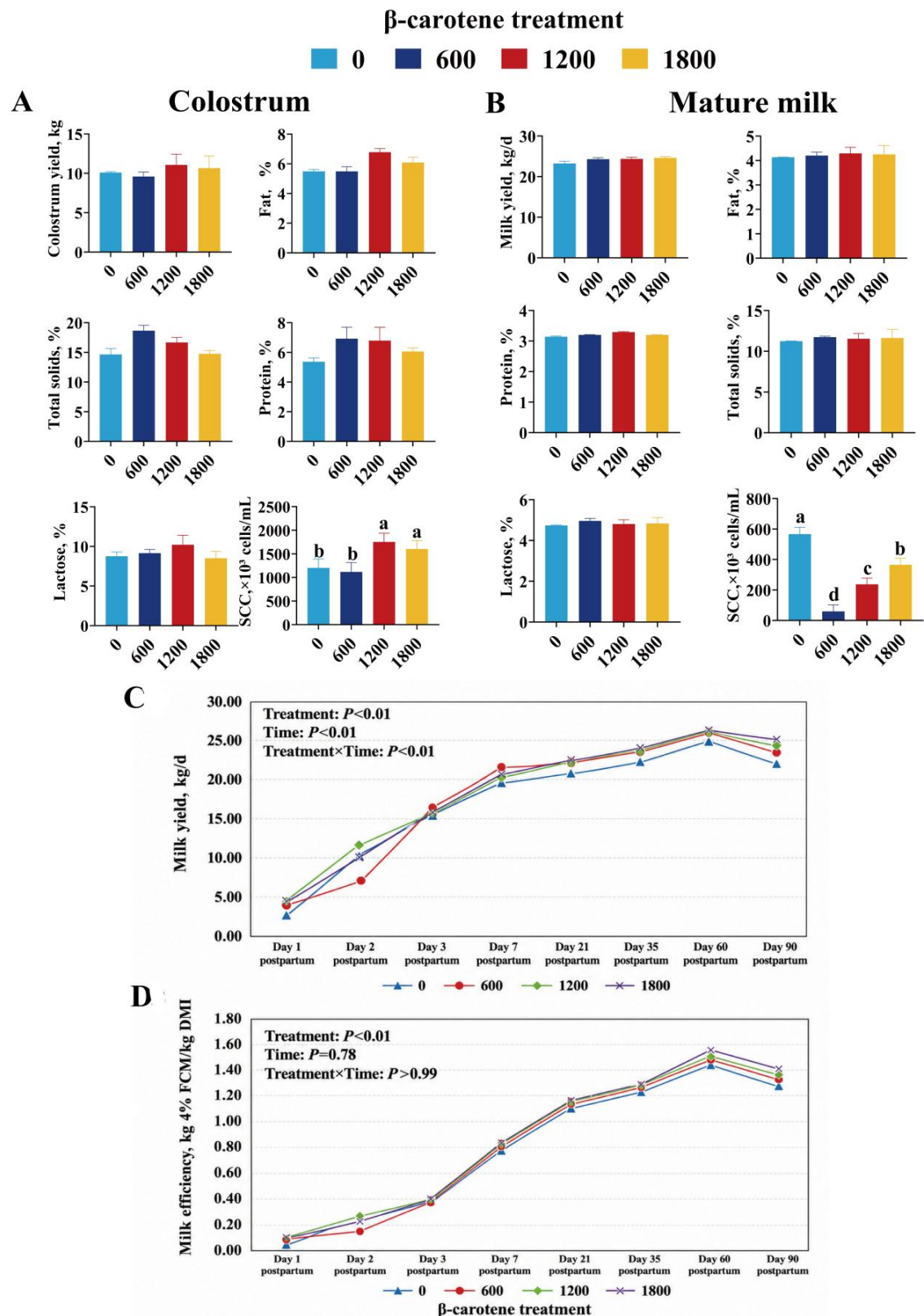


Fig. 2. Effects of dietary β -carotene supplementation on colostrum composition, mature milk composition, milk yield, and milk efficiency in Holstein cows. Treatments were 0, 600,

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1,200, and 1,800 mg/d β -carotene. Values are means $\pm$ SEM. Bars with different letters differ (P < 0.05). Treatment, time, and treatment $\times$ time effects are shown for repeated-measures data. SCC = somatic cell count; FCM = fat-corrected milk; DMI = dry matter intake.

Vitamin A and β -Carotene Concentrations in Milk, Serum, and Liver

To further evaluate whether dietary β -carotene supplementation altered vitamin A and β -carotene status in dairy cows, vitamin A and β -carotene concentrations were determined in colostrum, mature milk, serum, and liver. In addition, temporal changes in milk vitamin A and β -carotene concentrations from d 1 to 90 postpartum are shown in Fig. 3E, F. β -Carotene treatment affected vitamin A and β -carotene concentrations in colostrum, serum, and liver (Fig. 3A-D). In colostrum, vitamin A concentration was greatest in the 1,200 mg/d group and lowest in the 1,800 mg/d group (P < 0.05). Colostrum β -carotene concentration was also greatest in the 1,200 mg/d group, followed by the 600 and 1,800 mg/d groups, and was lowest in the control group (P < 0.05). In mature milk, vitamin A and β -carotene concentrations were not significantly different among treatments. Serum vitamin A concentration was not affected by β -carotene treatment, whereas serum β -carotene concentration was higher in the 1,200 and 1,800 mg/d groups than in the 0 and 600 mg/d groups (P < 0.05, Fig. 3C). In liver, both vitamin A and β -carotene concentrations increased with β -carotene supplementation, with the greatest values observed in the 1,800 mg/d group (P < 0.05, Fig. 3D).

Milk vitamin A and β -carotene concentrations changed over time and were affected by treatment and treatment $\times$ time interaction (P < 0.01; Fig. 3E,F). Both vitamin A and β -carotene concentrations were highest during the first days postpartum and declined sharply by d 7 postpartum, remaining at relatively low levels thereafter. The early postpartum increase in

milk β -carotene was most evident in the 1,200 mg/d group.

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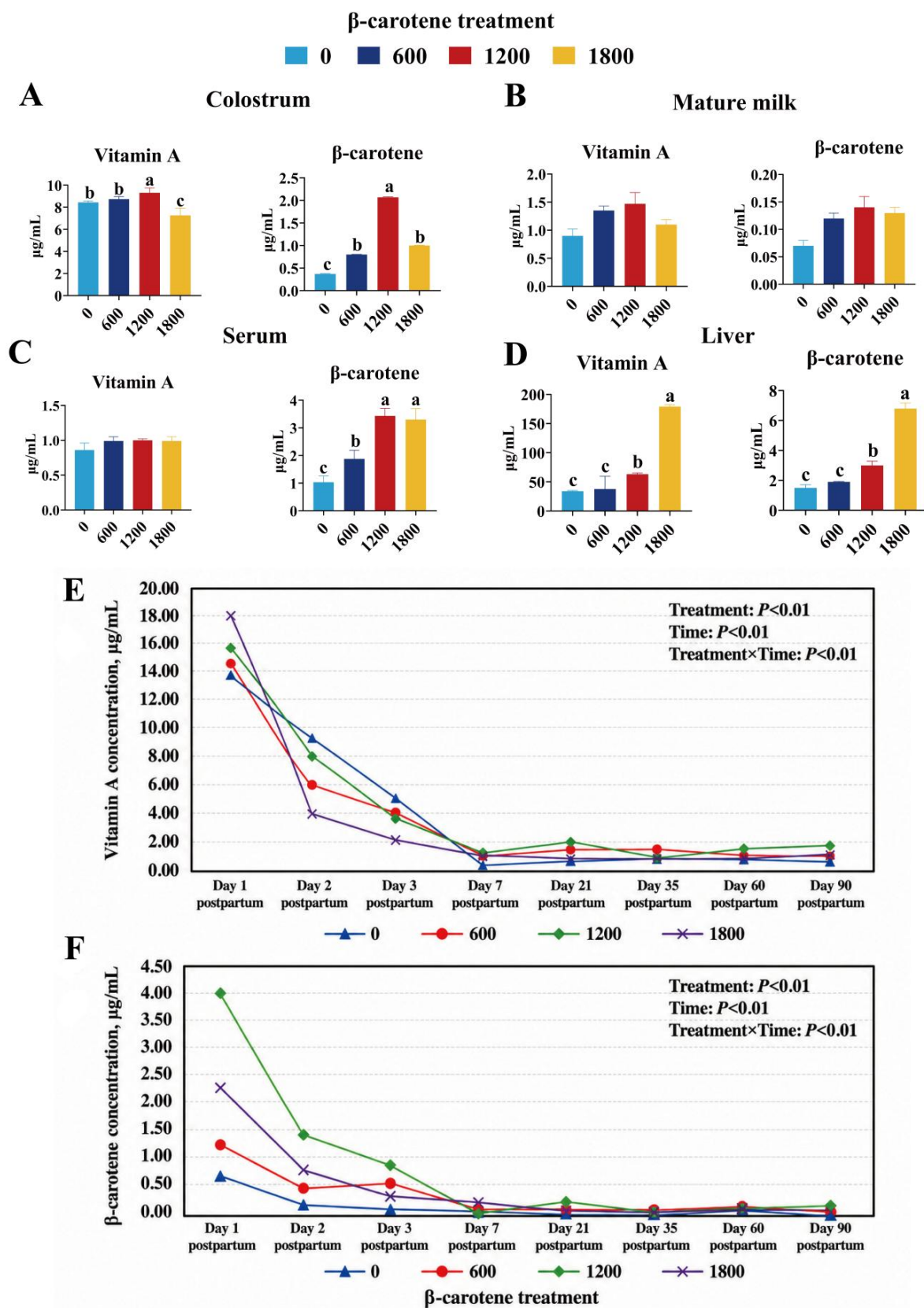


Fig. 3. Effects of dietary β -carotene supplementation on vitamin A and β -carotene

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concentrations in milk, serum, and liver of Holstein cows. A: Vitamin A and β -carotene 355
concentrations in colostrum.B: Vitamin A and β -carotene concentrations in mature milk. C: 356
Vitamin A and β -carotene concentrations in serum.D: Vitamin A and β -carotene 357
concentrations in liver.E: Milk vitamin A concentration from d 1 to d 90 postpartum.F: Milk 358
 β -carotene concentration from d 1 to d 90 postpartum. Values are least squares means $\pm$ SEM. 359
Bars with different lowercase letters differ significantly ($P < 0.05$). Treatment, time, and 360
treatment $\times$ time effects are shown for panels E and F. 361

Serum Oxidative Stress, Antioxidant, and Endocrine-Related Indices 362

As shown in Fig. 4, β -carotene treatment significantly affected antioxidant and oxidative 363
stress-related indices. Compared with the 0 and 600 mg/d groups, the 1,200 and 1,800 mg/d 364
groups had higher T-AOC concentrations ($P < 0.05$). GSH-Px activity was greatest in the 365
1,200 mg/d group, followed by the 1,800 mg/d group, whereas the control group showed the 366
lowest value ($P < 0.05$). SOD activity increased with β -carotene supplementation and was 367
higher in the 1,200 and 1,800 mg/d groups than in the 0 and 600 mg/d groups ($P < 0.05$). In 368
contrast, ROS concentration was reduced by β -carotene treatment, with the lowest value 369
observed in the 1,800 mg/d group ($P < 0.05$). TOS was lower in the 600 and 1,800 mg/d 370
groups than in the control group, while the 1,200 mg/d group showed an intermediate value. 371
Similarly, iNOS concentration was lower in all β -carotene-supplemented groups than in the 372
control group, with the lowest value observed in the 1,200 mg/d group ($P < 0.05$). 373

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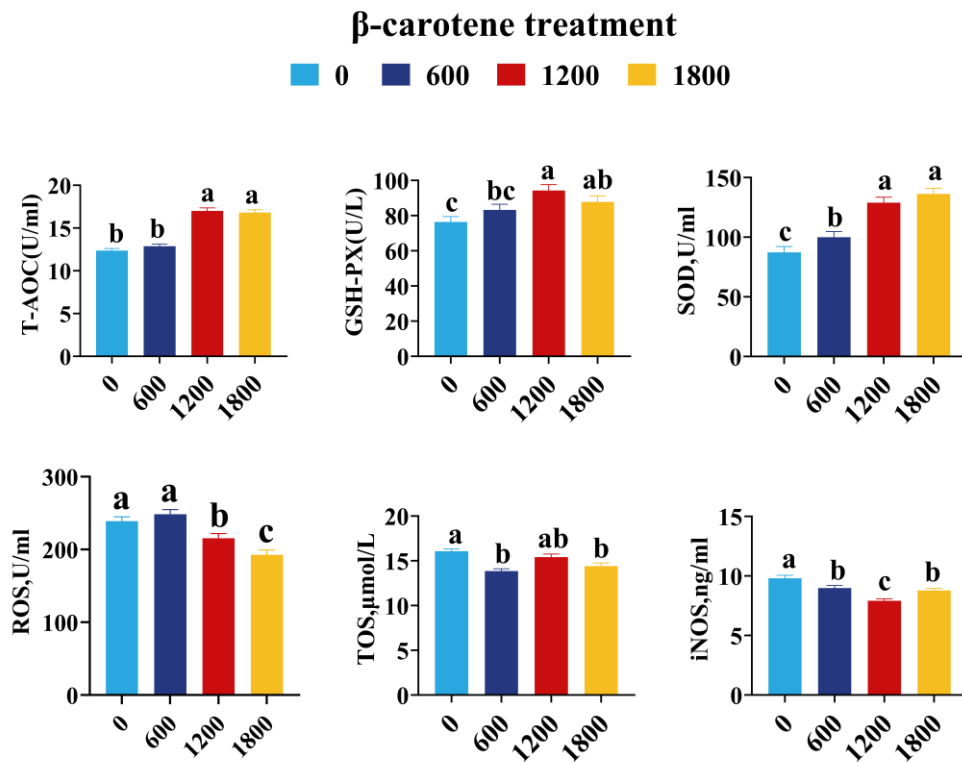


Fig. 4. Effects of dietary β -carotene supplementation on serum oxidative stress and antioxidant indices in Holstein cows. Data are presented as least squares means $\pm$ SEM. Different lowercase letters indicate significant differences among treatments within the same index ($P < 0.05$). T-AOC = total antioxidant capacity; GSH-Px = glutathione peroxidase; SOD = superoxide dismutase; ROS = reactive oxygen species; TOS = total oxidant status; iNOS = inducible nitric oxide synthase.

Antioxidant Responses in Liver and Intestinal Tissues

Dietary β -carotene supplementation altered antioxidant and oxidative stress-related indices in liver and intestinal tissues, and the responses differed among tissues and supplementation levels (Fig. 5).

Dietary β -carotene supplementation affected antioxidant indices in the liver and small intestine. Compared with the control group, β -carotene supplementation generally increased

T-AOC, SOD, GSH-Px, and GSH concentrations or activities, although the response differed among tissues. The improvement was most evident in the liver, duodenum, and ileum, particularly in the 1,200 and 1,800 mg/d groups for T-AOC and SOD. For lipid peroxidation, β -carotene supplementation reduced MDA concentration in most tissues. The 600 and 1,200 mg/d groups showed lower MDA concentrations in the liver, jejunum, and ileum compared with the control group, indicating reduced oxidative damage. Overall, dietary β -carotene improved antioxidant capacity and alleviated lipid peroxidation in hepatic and intestinal tissues, with tissue-specific responses among different supplementation doses.

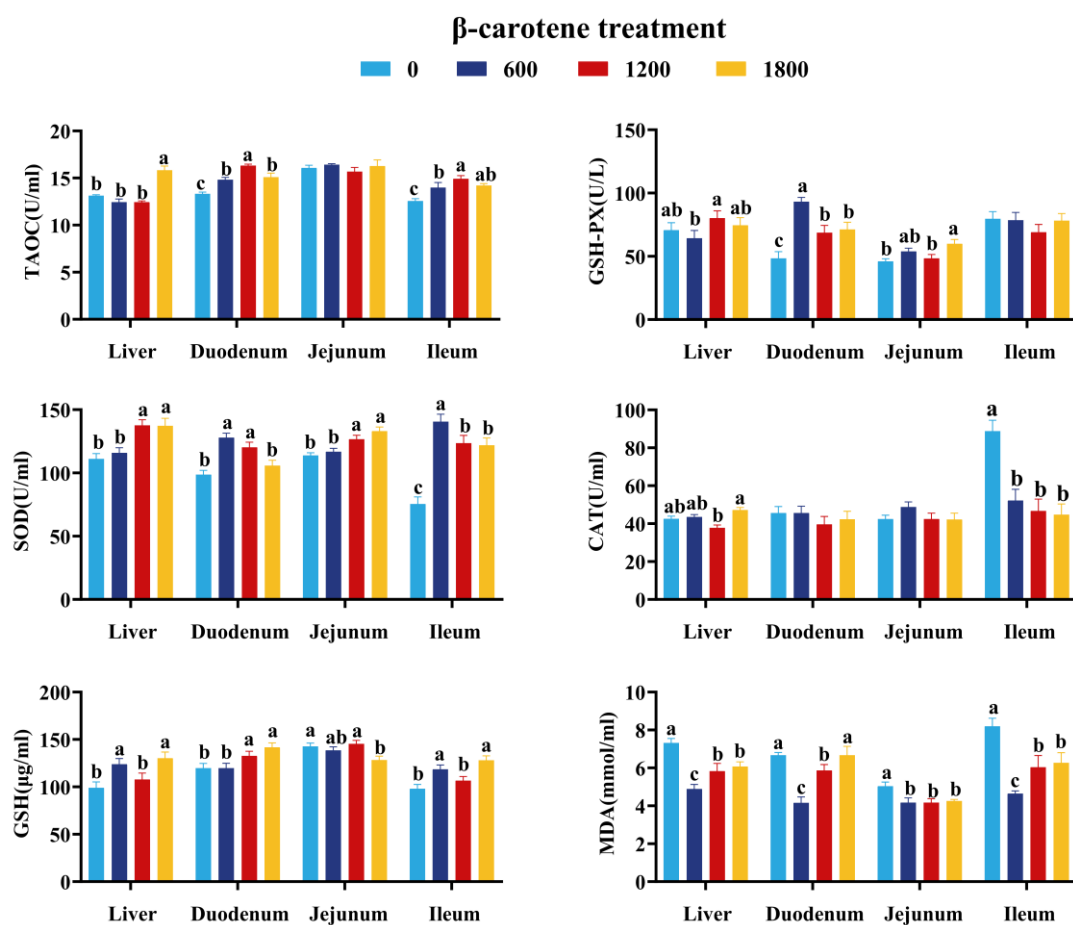


Fig. 5. Effects of dietary β -carotene supplementation on antioxidant responses in liver and

intestinal tissues of Holstein cows. Data are presented as least squares means $\pm$ SEM. 399
Different lowercase letters indicate significant differences among treatments within the same 400
tissue and index ($P < 0.05$). T-AOC = total antioxidant capacity; GSH-Px = glutathione 401
peroxidase; SOD = superoxide dismutase; CAT = catalase; GSH = reduced glutathione; MDA 402
= malondialdehyde. 403

Expression of β -Carotene Oxygenase Genes in Liver and Intestinal Tissues 404

The relative mRNA abundance of BCO1 and BCO2 in liver and intestinal tissues is shown in 405
Fig. 6. For graphical presentation, relative expression values were log10-transformed. BCO1 406
mRNA abundance differed among tissues, with greater expression observed in the duodenum 407
than in the liver, jejunum, and ileum. This result suggests that the duodenum was the major 408
site associated with central cleavage of β -carotene to vitamin A precursors. BCO2 mRNA 409
abundance was relatively greater in the duodenum and jejunum than in the liver and ileum, 410
indicating a broader distribution of BCO2 in the proximal small intestine. Within individual 411
tissues, β -carotene supplementation did not produce a consistent dose-dependent response in 412
BCO1 or BCO2 expression. Overall, these results indicate that BCO1 and BCO2 expression 413
patterns were mainly tissue dependent, with the proximal small intestine being the principal 414
site associated with β -carotene metabolism. 415

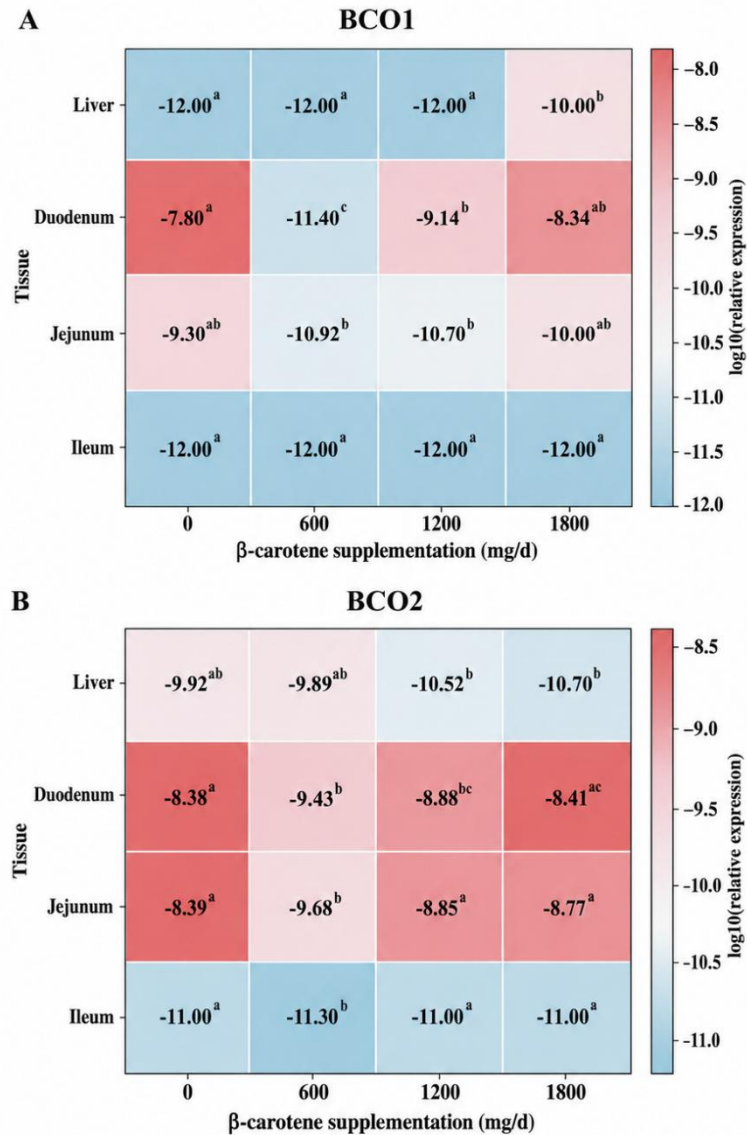


Fig. 6. Relative mRNA abundance of BCO1 and BCO2 in liver and intestinal tissues of Holstein cows supplemented with β -carotene. A: Heatmap of BCO1 relative mRNA abundance in liver, duodenum, jejunum, and ileum. B: Heatmap of BCO2 relative mRNA abundance in liver, duodenum, jejunum, and ileum. Cows received 0, 600, 1,200, or 1,800 mg/d β -carotene. Values are shown as $\log_{10}$ (relative mRNA abundance). Red indicates higher relative mRNA abundance, and blue indicates lower relative mRNA abundance.

Different lowercase letters within the same tissue indicate significant differences among β -carotene treatments ($P < 0.05$). 423
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Discussion 425

The transition from late pregnancy to early lactation is accompanied by marked changes in nutrient partitioning, endocrine regulation, immune function, and redox balance [15, 16]. 426
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During this period, dairy cows are susceptible to oxidative stress because the rapid increase in metabolic demand for colostrum and milk synthesis may exceed endogenous antioxidant defenses[3, 5]. β -Carotene is a provitamin A carotenoid and also functions as a lipid-soluble antioxidant. Therefore, dietary β -carotene supplementation during the prepartum and early lactation periods may influence both vitamin A/ β -carotene status and oxidative stress-related responses in dairy cows. In the present study, dietary β -carotene supplementation had limited effects on milk yield and most milk components, but altered SCC, increased serum and hepatic β -carotene status, promoted hepatic vitamin A accumulation, enhanced serum antioxidant capacity, and modulated antioxidant responses in liver and intestinal tissues. 428
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In the present study, β -carotene supplementation did not markedly affect milk yield or most milk components in either colostrum or mature milk. This finding is consistent with previous studies reporting that β -carotene supplementation increased circulating β -carotene concentrations but did not consistently improve milk yield or common milk components in dairy cows [10, 17]. Oliveira et al. [17] supplemented prepartum dairy cows with β -carotene and found increased plasma β -carotene concentration around calving, but no clear response in milk yield. Similarly, Prom et al. [10] reported that prepartum β -carotene supplementation 437
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increased serum β -carotene and vitamin A concentrations in Holstein cows but did not
improve overall production performance. These findings suggest that the productive response
to β -carotene supplementation may depend on basal β -carotene and vitamin A status, forage
carotenoid supply, and ruminal degradation [18]. In contrast, other studies have reported
changes in colostrum composition or milk component responses after β -carotene
supplementation [19]. Somatic cell count is widely used as an indicator of udder health and
mammary inflammatory status [20, 21]. In the present study, SCC in mature milk was
decreased by β -carotene supplementation, with the lowest value observed in cows receiving
600 mg/d. This result suggests that appropriate β -carotene supplementation may have a
beneficial effect on udder health-related traits. However, the SCC response differed between
colostrum and mature milk. In mature milk, β -carotene supplementation reduced SCC, which
may suggest a lower mammary cellular response during early lactation. In contrast, SCC in
colostrum was higher in cows receiving 1,200 and 1,800 mg/d β -carotene. Colostrum
collected at the first milking after calving differs markedly from mature milk in cellular
composition, immune activity, and mammary epithelial remodeling status. Therefore, a
higher SCC in colostrum may reflect physiological changes associated with colostrogenesis
and mammary remodeling, rather than a pathological inflammatory response. Previous
studies have also reported inconsistent relationships among vitamin A, β -carotene, SCC, and
udder health during the periparturient period [22, 23]. Therefore, the present SCC results
should be interpreted cautiously, and further studies including mastitis records, differential
somatic cell count, immunoglobulin G, lactoferrin, and mammary inflammatory markers are
needed to clarify whether β -carotene affects mammary immune status.

A clear response to β -carotene supplementation was observed in β -carotene status. Serum β -carotene concentration increased with dietary β -carotene, indicating that the supplemented β -carotene was absorbed and contributed to circulating β -carotene availability. This result agrees with previous reports showing that blood β -carotene concentration is closely related to dietary supply in dairy cows [9, 10]. The concomitant increase in hepatic β -carotene, together with the marked increase in hepatic vitamin A concentration, further indicates that absorbed β -carotene was either stored in tissues or converted to retinoids. This response is biologically plausible because cattle can accumulate β -carotene more readily than many other ruminant species, and β -carotene is an important provitamin A carotenoid in dairy cattle [6, 7]. In agreement with this interpretation, Jin et al. [24] reported that increasing β -carotene supplementation increased serum and liver β -carotene concentrations and also enhanced hepatic retinol storage in cattle. In the present study, the greatest hepatic vitamin A concentration in cows receiving 1,800 mg/d β -carotene likely reflects greater conversion of β -carotene to vitamin A and subsequent hepatic storage. However, serum vitamin A was not significantly affected, suggesting that circulating vitamin A was relatively stable despite changes in hepatic reserves. This may be related to the tight homeostatic regulation of plasma retinol when vitamin A status is adequate [6, 7]. Interestingly, mature milk vitamin A and β -carotene concentrations were not significantly altered by treatment, despite the clear increases in serum and hepatic β -carotene. This finding does not necessarily indicate limited absorption, but rather suggests that the transfer of these compounds into mature milk was limited under the conditions of this study.

Milk vitamin A and β -carotene concentrations were greatest during the first few days 487
postpartum and decreased rapidly thereafter. This pattern is biologically reasonable because 488
colostrum is enriched in fat-soluble vitamins, proteins, minerals, and immunologically active 489
compounds, whereas their concentrations generally decline as secretion transitions to mature 490
milk. [25–27]. Aragona et al. [12] reported that prepartum β -carotene supplementation altered 491
colostrum composition in Holstein cows, supporting the concept that carotenoid status can 492
influence colostrum constituents. In the present study, β -carotene concentration in colostrum 493
was greatest in the 1,200 mg/d group, whereas mature milk β -carotene concentration was 494
much lower and was not significantly affected by treatment. These results suggest that β - 495
carotene supplementation may influence carotenoid enrichment of colostrum more strongly 496
than mature milk. This response may be related to the high transfer of fat-soluble 497
micronutrients into colostrum and the rapid decline in colostrum-specific secretion after 498
calving. 499

β -Carotene is not only a precursor of vitamin A but also an important antioxidant nutrient in 500
dairy cows [28]. Therefore, its effects are usually reflected not only in vitamin status, but also 501
in oxidative balance, immune function, and metabolic adaptation [29, 30]. Previous studies 502
have shown that β -carotene status is associated with periparturient health and immune 503
function in dairy cows. For example, LeBlanc et al. [31] reported that serum β -carotene, 504
retinol, and α -tocopherol were associated with the risk of periparturient disorders, including 505
retained placenta and mastitis. Michal et al. [32] also found that dietary β -carotene modulated 506
blood and mammary leukocyte function in periparturient dairy cows, suggesting a role of β - 507
carotene in host defense. In the present study, β -carotene supplementation increased T-AOC, 508

SOD, and GSH-Px activities, while decreasing ROS, TOS, and iNOS concentrations. These 509
results indicate that β -carotene improved antioxidant capacity and reduced oxidative burden. 510
This finding is generally consistent with Vázquez-Añón et al. [33], who reported that 511
antioxidant supplementation improved antioxidant status in lactating dairy cows. However, in 512
our study, the improvement was more evident at the 1,200 and 1,800 mg/d levels, rather than 513
showing a completely linear dose response. This suggests that β -carotene may have an 514
effective range, and that increasing the dose does not necessarily produce a proportional 515
biological response. 516

BCO1 and BCO2 are key genes involved in β -carotene metabolism. BCO1 catalyzes the 517
central cleavage of β -carotene to retinal, which is the direct precursor of vitamin A, whereas 518
BCO2 participates in eccentric carotenoid cleavage and helps regulate carotenoid 519
homeostasis. Therefore, changes in these two genes may reflect the capacity of cows to 520
convert dietary β -carotene into vitamin A-related metabolites and to regulate tissue 521
carotenoid accumulation [34, 35]. The tissue distribution of BCO1 and BCO2 suggests that β - 522
carotene metabolism mainly occurs in the small intestine. BCO1 was predominantly 523
expressed in the duodenum, supporting the role of this tissue in converting β -carotene into 524
vitamin A precursors. This result is consistent with Morales et al. [36], who reported that 525
 β,β' -carotene-15,15'-oxygenase was expressed in the duodenum and liver of cattle, with the 526
duodenum being an important site for β -carotene cleavage. A similar tissue-dependent pattern 527
was also reported in yaks, where BCO1 mRNA and protein expression varied among 528
intestinal and metabolic tissues under different nutritional seasons [37]. Compared with 529
BCO1, BCO2 showed relatively higher expression in both the duodenum and jejunum, 530

indicating a broader role in intestinal carotenoid cleavage and metabolic regulation. This 531
agrees with previous findings that BCO2 is involved in carotenoid degradation and tissue 532
carotenoid accumulation. In cattle, a mutation in BCO2 was shown to affect milk and serum 533
 β -carotene concentrations and milk color [38]. In sheep, a nonsense mutation in BCO2 was 534
strongly associated with carotenoid accumulation in adipose tissue and the yellow-fat 535
phenotype [39]. Overall, the present results indicate that BCO1 may be more closely related 536
to duodenal conversion of β -carotene to vitamin A precursors, whereas BCO2 may contribute 537
more broadly to carotenoid degradation and homeostasis in the duodenum and jejunum. The 538
consistently low expression of both genes in the ileum suggests that the distal intestine 539
contributes little to β -carotene metabolism. The relatively low intestinal expression observed 540
in the 600 mg/d group also differs from a simple dose-dependent response, suggesting that β - 541
carotene-metabolizing enzymes may be regulated by tissue carotenoid status, vitamin A 542
feedback. 543

The present study has some limitations. Milk composition was determined using colostrum 544
samples collected within 1 h after calving and mature milk samples collected during the last 3 545
d of the experimental period, rather than continuous weekly milk composition data 546
throughout lactation. In summary, dietary β -carotene supplementation from the prepartum 547
period to early lactation had limited effects on milk yield and most milk components, but it 548
reduced SCC in mature milk, increased serum and hepatic β -carotene status, promoted 549
hepatic vitamin A accumulation, enhanced serum antioxidant capacity, and modulated 550
antioxidant responses in liver and intestinal tissues. The most consistent benefits were 551
observed for serum antioxidant status, serum and hepatic β -carotene concentrations, and 552

tissue redox-related indices. These findings suggest that β -carotene supplementation, 553
particularly at 1,200 to 1,800 mg/d, may improve antioxidant and health-related responses in 554
Holstein cows during the transition from late pregnancy to early lactation. 555

Conclusions 556

Dietary β -carotene supplementation from 60 d prepartum to 90 d postpartum had limited 557
effects on milk yield and most milk components in Holstein cows. However, supplementation 558
improved β -carotene status, increased hepatic vitamin A accumulation, enhanced serum 559
antioxidant capacity, and modulated antioxidant responses in liver and intestinal tissues. β - 560
Carotene supplementation also reduced SCC in mature milk, although the SCC response 561
differed between colostrum and mature milk. Overall, these findings indicate that β -carotene 562
supplementation can support β -carotene and vitamin A status and redox balance during the 563
transition from late gestation to early lactation. The responses were not uniformly dose 564
dependent, but 1,200 and 1,800 mg/d produced the most consistent improvements in systemic 565
 β -carotene status and antioxidant-related indices. 566

Abbreviations 567

ADF acid detergent fiber 568

ANOVA analysis of variance 569

ARRIVE Animal Research Reporting of In Vivo Experiments 570

BCO1 β -carotene 15,15'-oxygenase 1 571

BCO2 β -carotene oxygenase 2 572

BCS body condition score	CI	573
CAT: catalase		574
CI: confidence interval		575
CP: crude protein		576
Ct: cycle threshold		577
DM: dry matter		578
DMI: dry matter intake		579
DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid)		580
ELISA: enzyme-linked immunosorbent assay		581
FCM: fat-corrected milk		582
GLM: general linear model		583
GSH: reduced glutathione		584
GSH-Px: glutathione peroxidase		585
HPLC: high-performance liquid chromatography		586
iNOS: inducible nitric oxide synthase		587
MDA: malondialdehyde		588
NDF: neutral detergent fiber		589
NEL: net energy for lactation		590
NRC: National Research Council		591

ROS: reactive oxygen species	592
SAS: Statistical Analysis System	593
SCC: somatic cell count	594
SEM: standard error of the mean	595
SOD: superoxide dismutase	596
T-AOC: total antioxidant capacity	597
TMR: total mixed ration	598
TOS: total oxidant status	599
UV: ultraviolet	600
WST-1: water-soluble tetrazolium salt-1	601
Declarations	602
Ethics approval and consent to participate	603
All animal procedures were reviewed and approved by the Ethics Committee for Animal Care	604
and Use of Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences, China	605
(Approval No. 2025-0088). Written informed consent for the use of the animals and sample	606
collection was obtained from the owner or authorized representative of the experimental dairy	607
farm. The study was reported in accordance with the ARRIVE 2.0 guidelines where	608
applicable.	609
Consent for publication	610
Not applicable.	611

Availability of data and materials 612

All data generated or analysed during this study are included in this published article. 613

Competing interests 614

The authors declare that they have no competing interests. 615

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Authors' contributions 619

X.X. and K.Y. contributed equally to this work. Conceptualization, X.X. and K.Y.; 620

methodology, L.C. and C.L.; software, K.Y.; formal analysis, K.Y., M.Z., and L.S.; resources, 621

M.G.; writing—original draft preparation, L.C. and X.X.; writing—review and editing, H.H. 622

and M.G.; supervision, H.H.; project administration, H.H.; funding acquisition, H.H.; animal 623

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