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Effects of increasing n3:n6 ratio by replacing extruded soybeans with extruded flaxseed on dry matter intake, rumen fluid bacteria, and liver lipid metabolism in transition cows

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Abstract

Background The drop of dry matter intake (DMI) and rise of milk production in transitional dairy cows would mobilize reserved fat and disrupt lipid metabolism, eventually attributed to negative energy balance (NEB) and immune injury. The positive effect of n-3 polyunsaturated fatty acids (PUFA) on regulating energy metabolism and inflammation has been elucidated, however, the lack of regulatory mechanism of dairy cows deserves further investigation. In this study, 30 Holstein transition cows were divided into the control (CON) and HN3 groups based on the n-3: n-6 PUFA ratio in the diet.

Results The results showed that compared to the CON group, high n-3: n-6 PUFA ratio-supplemented cows in the prepartum phase reduced the relative abundance of gram-negative bacteria in the rumen, the concentration of lipopolysaccharide in the plasma and liver also significantly decreased ($P < 0.05$). Transcriptomic analysis of the liver showed that the NF-κB signaling pathway significantly down-regulated and the taste transduction pathway up-regulated ($P < 0.05$) in the HN3 group. In the postpartum phase, a high n-3/n-6 PUFA ratio in the diet increased the relative abundance of *Prevotella*, *Succinimonas* and *Treponema* in the rumen, at the same time, orexins in plasma were also changed ($P < 0.05$). Further, the insulin resistance pathway significantly down-regulated and the taste transduction pathway up-regulated ($P < 0.05$) in the liver.

Conclusions Overall, these results showed that a high n-3: n-6 PUFA ratio in the diet attenuates inflammatory responses in the prepartum phase and increases milk protein in the postpartum phase of transitional dairy cows. Appropriate increase in the proportion of n-3: n-6 PUFA ratio in the diet may be an effective measure to alleviate postpartum metabolic disease in dairy cows.

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Keywords N-3/n-6 PUFA ratio, Transition cows, Rumen fluid bacteria, Liver lipid metabolism

Background

The roles of polyunsaturated fatty acids (PUFA) in numerous biological processes, including the regulation of organizational functions, reduction of disease risk, and enhancement of immunity are well recognized [1, 2]. Despite their highly bioactive ingredient, n-3 and n-6 PUFAs compete in enzymatic systems crucial to metabolic processes [3, 4]. Studies have shown that n-3 PUFA inhibits the activity or levels of NF-κB signal transduction, and decreases the levels of pro-inflammatory factors [5, 6], but n-6 PUFA is considered as a pro-inflammatory substance [7].

In dairy cow diets, n-3 and n-6 PUFA are primarily provided by flaxseeds and soybeans, respectively. Our previous study showed that a high n-3/n-6 PUFA group in the cow diet attenuated inflammatory responses in transition cows [8, 9]. Transition cows often experience serious disturbances in the homeostasis of rumen fluid bacteria [10, 11], lipid metabolism disorders in the liver due to insufficient feed intake, and increased energy output from milk production, which further affect the energy balance [12], and induce serious diseases [13]. Therefore, attenuating inflammatory responses is important for transition cows. However, the specific mechanisms underlying the impact of high levels of n-3/n-6 PUFA in transition cows remain unknown.

Rumen contains a diverse community of anaerobic bacteria [14], which play an active role in protein synthesis and carbohydrate digestion [10, 15]. In addition to playing a crucial role in host metabolism, rumen fluid bacteria also regulate immune responses [13]. Suppose rumen function and feed intake cannot be adapted and recovered. In that case, a negative energy balance will inevitably occur, which will continue to affect cows' lactation and reproductive performance of cows. Research has shown that n-6 and n-3 PUFA exert contrasting effects on the gut microbiome. By increasing n-3/n-6 PUFA ratios in the diet, gram-negative *Enterobacteriaceae* growth can be reduced, *Bifidobacterium* and *Akkermansia* growth can be improved, and lipopolysaccharide (LPS) production can be reduced, thereby preventing gut barrier inflammation [16, 17].

Therefore, the balance in the dietary n-3: n-6 PUFA ratio is related to normal metabolism and immunity [18]. We speculated that elevated n-3/n-6 PUFA levels might ameliorate the imbalance in energy supply and reduce the inflammatory response by changing the rumen microbiota and liver metabolism. The objective of this study was to investigate the specific mechanisms through which high n-3/n-6 PUFA levels in the diet can attenuate immunosuppression and inflammatory responses, providing insights into feeding management in transition cows.

Materials and methods

Animals and sample collection

The animal experimental protocol was according to the ethical policies and procedures approved by the Committee of Animal Welfare and Animal Experimental Ethical Inspection of China Agricultural University, Beijing, China (protocol number: CAU20201024-2).

The animal experiment was conducted at Beijing Zhongdi Animal Husbandry Technology Co. Ltd. (39°30′ N, 116°33′ E). This experiment selected 30 multiparous Holstein cows with similar parity, body weight, body condition score, and milk yield. A completely randomized design was used, and at 240 days of pregnancy, the cows were randomly assigned to 2 isoenergetic and isoprotein treatments: one was fed a low n-3/n-6 PUFA diet (prepartum: 0.33; postpartum: 0.12) supplementing 8% dry matter of extruded soybeans (CON), and the other group was fed a high n-3/n-6 PUFA diet (prepartum: 1.56; postpartum: 0.63) supplementing 3.5% dry matter of extruded flaxseed (HN3). The feed ingredient composition and nutrient contents are listed in Table 1. The experimental period lasted 9 weeks, consisting of a 7-day adaptation period followed by a 56-day experimental period (prepartum: 28d; postpartum: 28d). Cows were fed twice daily at 0700 and 1500 h (5–10% orts on an as-fed basis), throughout the entire experiment, they had ad libitum access to both water and feed. Five cows were excluded because of mastitis, lameness, and left displaced abomasum at the end, and the remaining 25 animals were assigned to the trial, including 13 cows in the CON group and 12 cows in the HN3 group.

A Roughage Intake Control system (Insentec) monitored daily feed intake. Meanwhile, Samples of diet were collected weekly and stored at -20 °C until chemical composition analysis was conducted at the State Key Laboratory of Animal Nutrition in China. The dry matter (DM) content of the TMR and feeds was analyzed at 135 °C for 2 h following AOAC methods (1990). The crude protein (CP) content was determined using a Rapid N Exceed analyzer (Elementar) according to method 984.13 [19], while the ether extract (EE) and acid detergent fiber (ADF) were measured following method 920.39 and method 973.18c of the AOAC (1990), respectively. Finally, the neutral detergent fiber (NDF) is based on Van Soest et al. [20] with the ANKOM 2000i automatic fiber analyzer (Beijing Anke Borui Technology Co. Ltd.). Postpartum cows were milked four times daily at 0000,

Table 1 The ingredients and chemical composition of the close-up and milking cow TMR (DM basis)

Item	Prepartum		Postpartum	
	CON	HN3	CON	HN3
Ingredients (%)				
Oat hay	47.62	47.62		
Alfalfa hay			10.24	10.24
Corn silage	21.55	21.61	30.06	30.10
Alfalfa silage			7.71	7.76
Corn fine	2.99	2.74	10.35	9.37
Soybean Hulls	7.69	7.70	2.61	2.63
Corn steam flakes			10.15	10.21
Soybean meal	1.88	6.58	9.23	14.48
Corn gluten feed	4.07	4.06		
Urea	0.61	0.61		
Cottonseed fuzzy			5.30	5.33
Sugarcane molasses			2.55	2.56
Diamond V XP	0.26	0.26	0.16	0.16
Hydrogenated FA				
Extruded soybean	8.00		8.00	
Extrude flaxseed		3.50		3.50
Dry cow premix	4.32	4.31		
DCAD supplement	1.01	1.01		
Milking cow premix			3.64	3.66
Nutrition level				
CP(%)	15.47	15.52	17.31	17.39
NE_L(Mcal/kg)	1.28	1.28	1.69	1.71
NFC(%)	29.46	29.30	41.53	41.35
NDF(%)	49.82	49.67	32.65	31.64
EE(%)	3.72	3.82	5.35	5.49
C16:0 (g/d)	46.89	43.36	98.60	94.02
n-3: n-6	0.33	1.56	0.12	0.63

DM: Dry matter, CP: Crude protein, NE_L: Net energy of lactation, a calculated value according to NRC (2001), NFC: Non-fibrous carbohydrate, NDF: Neutral detergent fiber, EE: Ether extract, UFA: Unsaturated fat acid, n-6: C18:2n6 (Linoleic acid, LA), n-3: C18:3n3 (α- Linolenic acid, ALA). Dry cow premix: 1 kg of premix included vitamin A 918,750 IU, vitamin D3 253,125 IU, vitamin E 8,066 mg, niacin 6,650 mg, Ca 35 g, P 20 g, Cu 1146 mg, Mn 4248 mg, Zn 5362 mg, I 45 mg, Se 36 mg. Milking cow premix: 1 kg of premix included vitamin A 440,000 IU, vitamin D3 110,000 IU, vitamin E 4000 IU, niacin 400 mg, Ca 152 g, P 41 g, Cu 750 mg, Mn 1140 mg, Zn 2970 mg, I 30 mg, Se 24 mg. DCAD supplement: -107.43 (mEq/kg) = (Na%/0.023 + K%/0.039-Cl%/0.0355-S%/0.016). CON: Control treatment, HN3: High n-3 PUFA treatment

0600, 1200, and 1800 h, with milk production measured at each milking using the ALPROTM system (DeLaval). Milk samples were collected using milk sampling devices (ALPROTM). A 50-mL aliquot of milk was taken on the last day of every two weeks. On collection day, four aliquots were pooled into a single composite sample based on the proportion of milk produced at each milking. Bromopol (a milk preservative from D&F Control Systems) was then added, and the sample was stored at 4 °C for milk composition analysis. Milk samples were sent to the Beijing Dairy Cow Center for the analysis of milk composition, including fat, protein, and lactose. At 7 days before calving, and 28 days after calving, two hours after milking and feeding, blood samples were obtained into evacuated 10-ml test tubes (Vacutainer, Becton Dickinson, Rutherford, NJ, USA) containing ethylene diamine tetraacetic acid (EDTA) by venipuncture of the coccygeal vessels. The tubes with EDTA were centrifuged at 3,000 × g for 12 min in a refrigerated centrifuge at 4 °C, and the resulting plasma samples were stored at -80 °C for subsequent testing and analysis. Ruminal fluid samples were collected with a rumen sampling tube at the same time point as blood collection, then stored at – 80 °C for further 16S rRNA gene sequencing. The first 50 mL of ruminal fluid was discarded to prevent contamination with saliva.

Hepatic tissue was collected 7 d before calving and 28 d after calving as described in a previous study [21]. A stainless-steel percutaneous hepatic biopsy tool (31 cm long, 6 mm in diameter) was used. Before the puncture, a 20 cm × 20 cm area near the 10th and 11th intercostal spaces on the right side was shaved. The area was cleaned with povidone-iodine and disinfected with 75% alcohol, followed by a subcutaneous injection of 2% lidocaine hydrochloride (L7780; Solarbio) for local anesthesia. A 3 mm incision was made on the skin using a scalpel. The biopsy needle was inserted through the intercostal muscles into the liver. The liver tissue was carefully rinsed with sterile isotonic saline (IN9000; Bio) to remove blood from the sample. The biopsy tissue was immediately frozen in liquid nitrogen until further analysis.

Enzyme-Linked Immunosorbent Assay (ELISA) of relevant indicators in plasma

The levels of LPS, orexins, neuropeptide Y (NPY), and peptide tyrosine (PYY) in the plasma were measured according to the commercial ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China). The specific performance characteristics of this kit include the following: the linear regression of the standard curve shows a correlation coefficient (R-value) greater than or equal to 0.99 with the expected concentrations, and there is no cross-reactivity with other structurally similar soluble substances. Both the inter-assay and intra-assay CVs are less than 15%.

The absorbance values were measured, and the LPS, Orexins, NPY, and PYY were calculated using the corresponding standard curves. Specifically, the standard wells and test sample wells should be prepared. Then, add 50 μL of the standard solution to each standard well. For the test sample wells, add 10 μL of the testing sample followed by 40 μL of sample diluent. Leave the blank wells empty. Next, add 100 μL of HRP-conjugate reagent to each well, cover the plate with an adhesive strip, and incubate for 60 min at 37 °C. After incubation, aspirate each well and perform the washing step five times. After the final wash, remove any residual wash solution by aspirating or decanting, then invert the plate and blot it gently on clean paper towels. Add 50 μL of chromogen

solution A and 50 μL of chromogen solution B to each well. Mix gently and incubate the plate for 15 min at 37 °C under light-proof conditions. Finally, add 50 μL of stop solution to each well, measure the optical density at 450 nm within 15 min.

DNA extraction, PCR amplification, and 16S rRNA sequencing of rumen microbiota

DNA extraction was performed using the TIANamp Bacteria DNA kit (Tiangen, China) following the manufacturer's protocols. Amplification of the V3-V4 region of the bacterial 16S rRNA genes was performed using the primers 341 F (5′-CCTACGGGNGGCWGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT′) [22]. After determining the genomic DNA quality and concentration with NanoDrop 1000 spectrophotometer, the Illumina MiSeq PE 300 platform was used to sequence. Paired-end reads were produced, followed by bioinformatics analysis. Sequence quality control was performed using Trimmomatic (version 0.36) [23] and Pear v.0.9.6 [24] software to remove low quality by window. Additionally, the FLASH software combined pairs of reads into a single sequence by identifying overlapping regions between the paired-end reads [25]. Following the removal of low-quality data, sequences were clustered into operational taxonomic units (OTUs) using Vsearch software (version 2.7.1), with a criterion of at least 97% similarity [26]. The final results included 735,982 sequences and 52,178 OTUs.

Liver RNA extraction and transcriptomic analysis

The TRIzol Reagent was utilized to extract total RNA from the liver of cows. RNA integrity and purity were evaluated using the Bioanalyzer 2100 from Agilent Technologies, CA, USA. Subsequently, 24 RNA samples (six from each group) were randomly selected for cDNA library preparation. Sequencing libraries were constructed using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® from NEB, USA. The prepared libraries were then subjected to paired-end sequencing at Allwegene Technology (Beijing, China) using an Illumina NovaSeq 6000 platform. Following the removal of low-quality reads, adaptor sequences, and reads containing more than 5% unknown bases in raw reads, clean reads were obtained [27].

Targeted metabolomic analysis of liver

The metabolomic analysis was conducted using liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS). Accurately weigh approximately 50.0 mg of liver tissue into a 2 mL centrifuge tube. Add 250 μL of chloroform and methanol (2:1) solution. Add two steel beads into the tube, and grind at a frequency of 66 Hz for 4 min at 4 °C. Centrifuge the sample at 12,000 g for 15 min at 4 °C. Transfer the entire supernatant into a new 2 mL centrifuge tube and lyophilize the sample. After lyophilization, store the sample at -20 °C until further analysis. Before performing the analysis, reconstitute the lyophilized extract with 100 μL of 20% methanol aqueous solution. For quality control (QC) sample preparation, take an equal volume of supernatant from each group, mix thoroughly by vortexing. The liquid chromatography-mass spectrometry (LC-MS) platform utilized was built around the Thermo UHPLC-Q Exactive HF-X Vanquish Horizon system, which was equipped with an Accucore C30 column (100 mm × 2.1 mm i.d., 2.6 μm; Thermo, USA). The peak detection, alignment, and identification for raw data were processed in the lipidomic processing software LipidSearch (Thermo Fisher Scientific). The metaboAnalyst 5.0 (https://www.metaboanalyst.ca/) was used to analyze downstream data. The screening criteria of significantly different metabolites were set as variable influence on projection (VIP) > 1 and $P < 0.05$. MetaboAnalyst was employed to conduct Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.metaboanalyst.ca/) enrichment analysis of various metabolites [28].

Statistical analysis

A completely randomized design with repeated measures was used for the analysis of production performance and plasma parameter data. Group, time, and their interaction were considered as fixed effects, while cows within the group were treated as random effects. When significant interactions or differences due to dietary treatments were detected, significant differences in the table were denoted by the letters A and B. Statistical significance was considered at $P < 0.05$.

The α-diversity and β-diversity indices of rumen fluid bacteria were calculated using QIIME [29]. Linear discriminant analysis effect size (LEfSe) analysis was conducted to identify features with significant differential abundance. Subsequently, linear discriminant analysis (LDA) was employed to assess the effect size of each taxon that was distinctively abundant (LDA score > 2, $P < 0.05$) [30]. Afterward, the raw sequence data of liver transcriptome and 16S rRNA for rumen fluid bacteria were subjected to the Sequence Read Archive (SRA) of the NCBI under accession number PRJNA1111922 and PRJNA1083270, respectively.

Based on the length of the gene and the reads count mapped to this gene, we calculated the FPKM (fragments per kilobase of transcript per million fragments mapped) [31]. Differential expression analyses of the HN3 and CON groups were performed with a particular screening criterion (false discovery rate (FDR) < 0.05, fold change |log2 (FC)|>1) using edgeR [32]. Gene Ontology (GO) functional enrichment was performed using Goseq [33].

Table 2 Effect of n-3/n-6 PUFA ratio on the DMI, the concentration of PYY, Orexins, NPY, and LPS in the plasma

Items	Pre 7d		Post 28d		SEM	*P*-value		
	CON	HN3	CON	HN3		Time	Treatment	Interaction
DMI(kg/d)	10.837	12.843	19.747	20.347	0.602	< 0.001	0.433	0.198
PYY(pmol/L)	12.904	12.064	11.820	13.987	0.391	0.728	0.070	0.104
Orexins(ng/ml)	14.579^{B}	14.379^{AB}	12.257^{A}	15.070^{AB}	0.404	0.004	0.019	0.006
NPY(pg/ml)	3536.711	3157.215	3212.550	3377.645	94.320	0.227	0.551	0.180
LPS(ng/ml)	7.097^{A}	5.104^{B}	5.996^{B}	5.658^{AB}	0.204	0.009	0.469	0.003

$^{A-B}$Different superscript letters in each indicator represent a significant difference ($P < 0.05$)

DMI, dry matter intake; PYY, peptide-YY; LPS, Lipopolysaccharide; Pre7d, 7 days before calving; Pos28d, 28 days after calving; NPY, Neuropeptide Y. DMI data quoted from [8]

Table 3 Effect of n-3/n-6 PUFA ratio on milk yield and composition

Items	2wk		4wk		SEM	*P*-value		
	CON	HN3	CON	HN3		Time	Treatment	Interaction
Yield, kg/d	40.738	40.932	48.167	48.786	1.078	< 0.001	0.943	0.793
Fat, kg/d	1.760	1.788	1.917	1.937	0.020	< 0.001	0.485	0.777
Protein, kg/d	1.276^{A}	1.483^{B}	1.498^{A}	1.517^{A}	0.021	0.038	< 0.001	< 0.001
Lactose, kg/d	2.167	2.191	2.562	2.571	0.041	< 0.001	0.636	0.661

$^{A-B}$Different superscript letters in each indicator represent a significant difference ($P < 0.05$)

Finally, the pathway enrichment analysis was conducted by KEGG [34].

Results

The DMI, the content of LPS, PYY, NPY and orexins in plasma and milk yield and composition

Except for orexins and LPS, treatment and the interaction of treatment and time had no effects on DMI and other plasma indices (Table 2). Treatment by time interactions were observed for orexins in plasma during the entire transition period ($P < 0.05$). Specifically, The HN3 treatment decreased the orexins concentration in the plasma relative to the CON group before calving, but the opposite occurred after calving. In addition, treatment by time interactions were observed in the LPS concentrations in plasma between the CON and HN3 treatments during the whole trial period. HN3 significantly decreased the LPS concentration in the prepartum while had no effects on the postpartum period. Additionally, DMI was significantly affected ($P < 0.001$) by time.

In addition, milk yield and composition were significantly influenced by time ($P < 0.05$) throughout the postpartum period (Table 3). However, except for protein ($P < 0.05$), these parameters were not affected by treatment. We found that milk protein content increased significantly during the first two weeks after calving ($P < 0.05$), but no significant effect was observed by the fourth week.

Rumen fluid bacteria diversity and composition

Clean tags were used to generate OTUs by clustering under 97% similarity level conditions and subsequent bioinformatic statistical analyses. we obtained 2167, 2145, 1848, and 1674 OTUs in pre_HN3, pre_CON, post_HN3 and post_CON, respectively. Estimates of richness and diversity showed that the number of OTU, Chao 1 values, Shannon index values, and Simpson's index values in the rumen did not differ between the CON and HN3 groups ($P > 0.05$). The PCA analysis showed no significant separation between the CON and HN3 groups in the rumen during the prepartum and postpartum periods ($P > 0.05$) (Fig. S1). Additionally, at the phylum level, Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Patescibacteria dominated the rumen, accounting for more than 95% of the rumen fluid bacteria across all samples (Fig. 1A). Subsequently, when the LEfSe of rumen fluid bacteria from the phylum to genus level were analyzed in the prepartum and postpartum periods, as shown in Fig. 2B, C, which showed that the HN3 group increased the relative abundance of *Endomicrobium*, *Family_XIII_UCG_001*, and *Desulfobulbus*, while reducing the relative abundance of *Ruminobacter*, *Lachnospiraceae_ND3007_group* during the prepartum period; during the postpartum period, the HN3 group increased the relative abundance of *Prevotellaceae_Ga6A1_group*, *Treponema*, *Succinimonas*, and *Prevotella*, while reducing the relative abundance of *Succiniclasticum*. These data indicate that different n-3: n-6 PUFA ratios have different effects on ruminal bacterial composition during the pre-and postpartum periods. In addition, we analyzed the co-occurrence network at the genus level for the HN3 and CON groups, and both formed co-occurrence networks dominated by *Prevotella*, *Rikenellaceae_RC9_gut_group*, *NK4A214_group*, and *Lachnospiraceae NK3A20 group*; however, the network relationships in the CON group were more complex than those in the HN3 group (Fig. 1D and E). The random forest model at the genus level was used to identify rumen fluid bacterial biomarkers, and

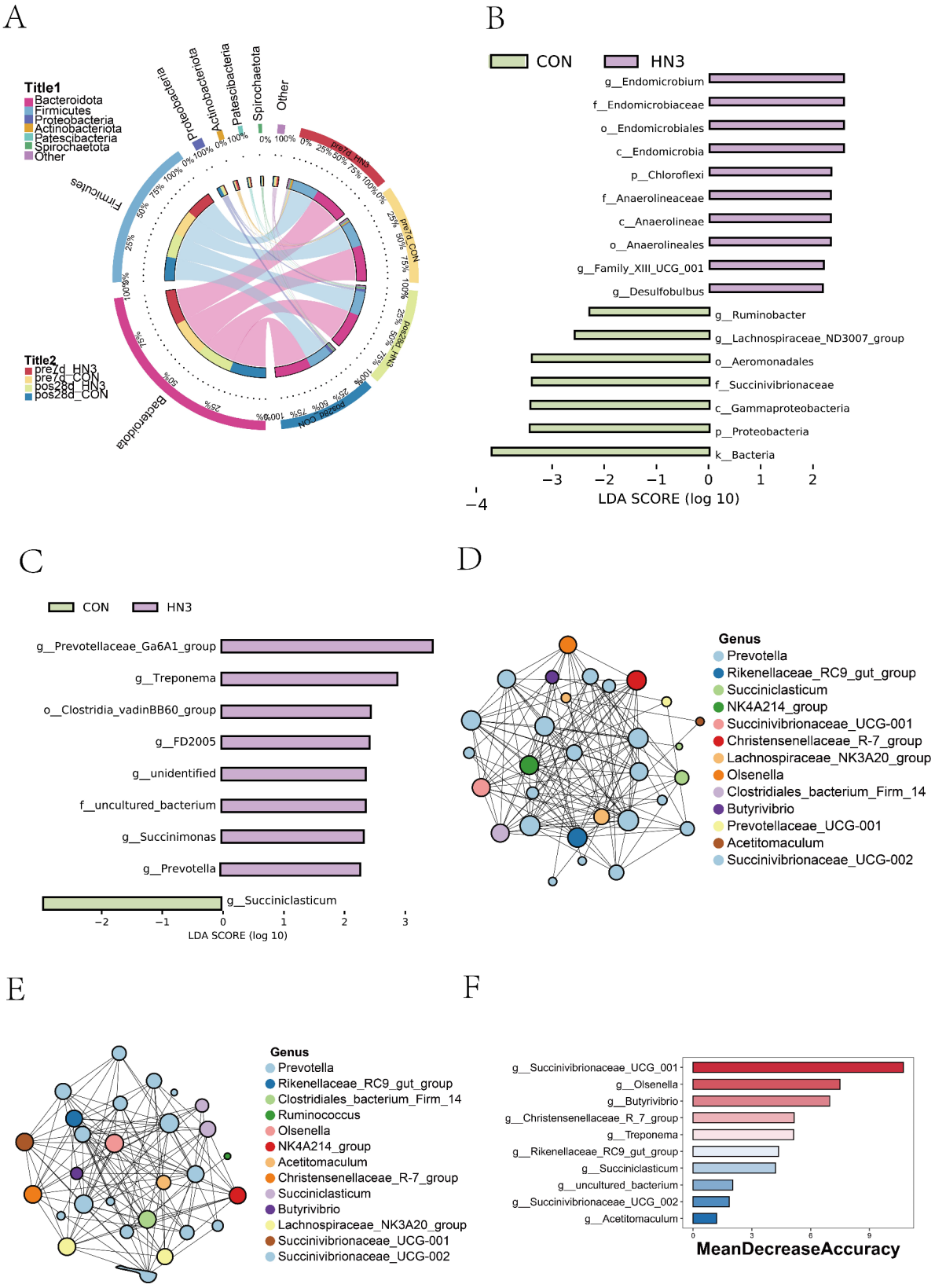

Fig. 1 (See legend on next page.)

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Fig. 1 Effect of n-3/n-6 PUFA ratio on the rumen fluid bacteria community. The chord diagram shows the contribution of different rumen fluid bacteria communities at the phylum level (**A**). Linear discriminant analysis (LDA) demonstrated the rumen fluid bacteria community between CON and HN3 groups from phylum to genus level in the prepartum (**B**) and the postpartum (**C**) period with *P* value < 0.05 and LDA score > 2. The interactions network among bacteria at the genus level for the CON (**D**) and the HN3 (**E**) group. (**F**) Random forest analysis of rumen fluid bacteria at the genus level, the greater the Mean Decrease Accuracy, the greater the contribution of the genus

the results showed that *Succinivibrionaceae_UCG_001* had the greatest contribution among all bacterial genera, followed by *Oisenella* and *Butyrivibrio* (Fig. 1F).

The transcriptional profile in the liver

Considering that the liver serves as the primary energy metabolizing and supplying organ of the host, we performed transcriptome sequencing of liver samples to study the effects of different n-3: n-6 PUFA ratios on substance metabolism and signal transduction in the liver. First, the plots of the principal component analysis of total RNA-sequenced genes showed no clear separation between the CON and HN3 groups (Fig. S2). Using the criteria of a false discovery rate (FDR) < 0.05 and a fold change (FC) > 2 or < 0.5, a total of 150 differentially expressed genes (DEGs) were identified in the liver during the prepartum period, including 89 up-regulated and 61 down-regulated genes (Fig. 2A); at the same time, a total of 162 DEGs in liver in the postpartum periods were identified, including 88 up-regulated and 74 down-regulated genes (Fig. 2B). The Vene diagram showed that there were two upregulated genes and four down-regulated genes common to both the prepartum and postpartum periods (Fig. 2C and D).

We further analyzed the DEGs, which were annotated into three gene ontology categories: biological processes, cellular components, and molecular functions. In the prepartum period, most of the DEGs were enriched in the category of biological processes, including organic hydroxy compound transport, regulation of cellular ketone metabolic processes, and regulation of cholesterol esterification, whereas in terms of molecular function, DEGs were categorized mainly into sterol transfer activity and cholesterol transfer activity (Fig. 2E). In the postpartum period, most DEGs were enriched in only one biological process pathway for the regulation of chemokine-mediated signaling. Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted. As shown in Fig. 2F and G, 11 and 12 differential metabolic pathways were enriched during the prepartum and postpartum periods, respectively. It is worth noting that both 7 days prior to calving and 28 days after calving, the results show that genes associated with the taste transduction pathway were enriched in all treatment groups and had the highest gene ratio in both the prepartum and postpartum periods.

Lipid metabolomic analysis in the liver

In total, 1,553 characteristic ion peaks were extracted in the positive spectral mode, and 925 in the negative spectral mode. These substances were identified as triacylglycerols (30.51%), glycerophosphocholines (20.96%), glycerophosphoethanolamines (11.40%), and glycerophosphoserine (8.64%) (Fig. 3A). Furthermore, to identify the significantly different metabolites between the HN3 and CON groups during the prepartum and postpartum periods, respectively, VIP derived from OPLS-DA analysis was used to select metabolites with potential biomarkers (Fig. 3B and D). Based on the criteria of $P < 0.05$ and VIP > 1, 98 and 126 differential metabolites were screened between the HN3 and CON groups during the prepartum and postpartum periods, respectively. During the prepartum period, the differential metabolites were mainly glycerophosphoethanolamines (29.41%), glycerophosphocholines (26.47%), glycerophosphoinositols (8.82%), and glycerophosphoserines (8.82%) (Fig. 3C). In contrast, in the postpartum period, the differential metabolites were mainly concentrated in triradyl glycerols (26.19%), glycerophosphocholines (23.81%), glycerophosphoethanolamines (23.81%), and ceramides (9.52%) (Fig. 3E). Additionally, from the Venn results, we identified 14 shared differential metabolites between the prepartum and postpartum periods, mainly PC and PE (Fig. 3F), which contributed significantly to the differences between the HN3 and CON groups (Fig. 3G). Specifically, the HN3 group showed reduced LPS content during the prepartum period, but it did not significantly affect the postpartum period (Fig. 3H).

To further explore the differences in the metabolic pathways between the HN3 and CON groups, KEGG enrichment analysis was performed. The results showed that 20 and 16 differential metabolic pathways were enriched during the prepartum and postpartum periods, respectively (Fig. 4A and C). The KEGG pathway differential abundance score plot showed a trend of down-regulation of differential metabolite expression in 16 pathways during the prepartum period, and no pathway was significantly upregulated ($P > 0.05$) (Fig. 4B), During the postpartum period, nine pathways showed a trend of downregulation of differential metabolite expression and seven pathways showed a trend of upregulation of differential metabolite expression, including glycerophospholipid metabolism, choline metabolism in cancer, and insulin resistance ($P < 0.05$) (Fig. 4D). These results suggest that different n-3: n-6 PUFA ratios have different

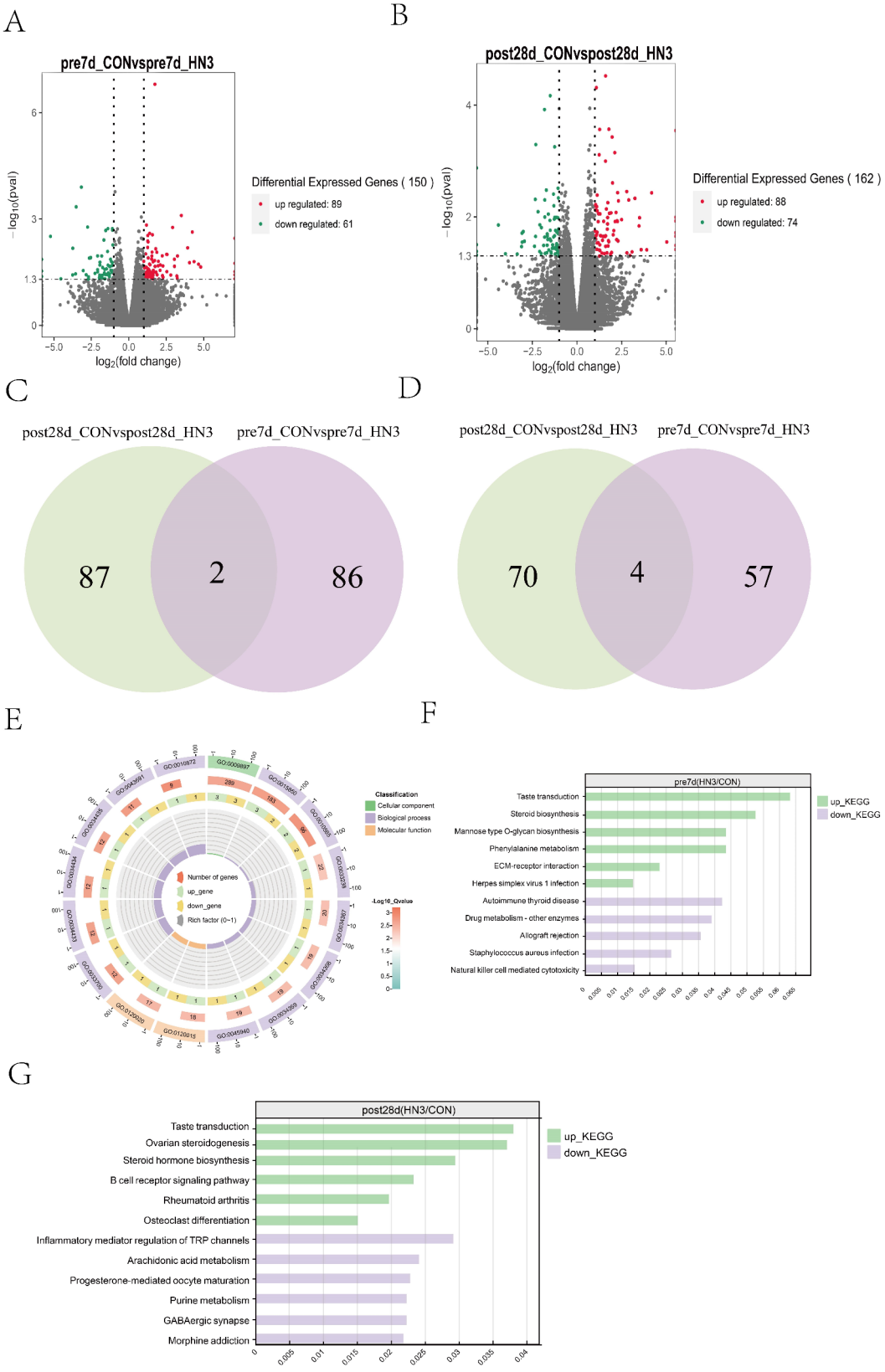

Fig. 2 (See legend on next page.)

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Fig. 2 Effect of n-3/n-6 PUFA ratio on the transcriptional profile in the liver. (**A**) Volcano plot of differentially expressed genes (DEGs) of the prepartum and the postpartum (**B**) period. The red pots and the green pots indicate upregulated and downregulated genes, respectively. (**C**) Venn diagram of up-regulated genes and down-regulated genes (**D**) shared prepartum and postpartum period. (**E**) GO circle chart of the differentially expressed genes based on gene ontology categories in the prepartum period. (**F**) Kyoto encyclopedia of genes and genomes enrichment analysis of the differentially expressed genes in the prepartum and the postpartum (**G**) period. The vertical axis represents the pathway name, and the horizontal axis represents the gene ratio of the pathway

effects on liver lipid metabolism pathways during the prepartum and postpartum periods.

Discussions

The transition period is a critical stage in the life of dairy cows during which reduced feed intake and increased energy requirements can lead to excessive lipolysis [35, 36]. Excessive lipolysis in transition cows is a multifactorial condition in which gut microbial dysbiosis, host metabolism, and immune function are altered [37]. We aimed to investigate whether a high n-3/n-6 PUFA ratio in diet could increase DMI by regulating neuropeptides that stimulate feed intake, such as NPY and orexins, which are important metabolic substances that influence the brain's neural circuits through peripheral signals and participate in the regulation of feed intake [38]. Therefore, we measured plasma levels of PYY, NPY, and orexins. Although the increase in DMI was not statistically significant, interestingly, we found that orexins levels were influenced by time, treatment, and the interaction between time and treatment. It is well known that the hypothalamus is a key brain region that helps maintain homeostasis, regulate the endocrine system, and adapt to changes in the external environment [39, 40]. Previous studies in dairy cows have shown that the hypothalamus can affect feeding behavior, energy metabolism, and peripheral inflammation through mechanisms such as the vagus nerve and humoral pathways [41, 42], and that there is a significant relationship between metabolic changes in the hypothalamus, blood, and milk after dietary changes [43]. Based on the above research, we hypothesize that a high n-3/n-6 PUFA ratio may influence the secretion of hypothalamic neuropeptides as a means of affecting energy metabolism. However, this hypothesis requires further experimental validation.

Accumulating evidence has demonstrated that the rumen, the main digestive organ of ruminants, contains microorganisms that substantially affect the physiological and metabolic health of organ function, including the liver [44–47]. Previous studies have revealed that n-3 PUFA has a positive effect on the gut microbiota of mice [48], humans [49, 50], pigeon squabs [51] pigs [52] and steers [53]. To date, the effect of n-3 PUFA on the rumen fluid microbiota of transition cows has been demonstrated in the current study. Interestingly, we also observed that changes in the n-3: n-6 PUFA ratio had different effects on rumen microbial structure during the prepartum and postpartum periods, which may be related to the physiological status of the host around calving.

More specifically, in the postpartum period, a high dietary n-3/n-6 PUFA ratio increases the relative abundance of *Prevotella* and *Succinimonas*, which play essential roles in protein and polysaccharide processing and starch degradation, respectively [54, 55], which may be an important reason for the significant increase in milk protein content during the postpartum period. In addition, a higher relative abundance of *Treponema* was found in the HN3 group, which has also been observed in high-milk-yield Holstein dairy cows [56]. At the same time, our present study also shows that the HN3 group reduced the relative abundance of *Ruminobacter*, *Lachnospiraceae_ND3007_group*, *Aeromonadales*, Succinivibrionaceae, Gammaproteobacteria, Proteobacteria in the prepartum period, it is noted that these are all gram-negative bacteria. Gram-negative bacteria have an outer membrane composed primarily of LPS, which enters the liver through the peripheral circulatory system and induces an inflammatory response in the host when the permeability of the intestine is altered [57]. The present study identified that a high n-3/n-6 PUFA ratio in the diet significantly reduced the content of LPS in the plasma and liver, which is consistent with previous findings that the gut microbiome is affected by n-3 PUFAs, mainly by affecting gut microbes' type and abundance as well as pro-inflammatory mediator levels [58, 59]. Further, the gut microbiota affects these reciprocal responses by producing neurotransmitters and microbial metabolites including secondary bile acids, tryptophan, and short-chain fatty acid, which all affect the host's CNS [60]. Taken together, we speculate that the n-3/n-6 PUFA ratio affects orexin secretion by influencing the abundance of related microbes. Therefore, further bacterial isolation and culture experiments will be included in our future research program to explore these specific mechanisms. Subsequently, we performed random forest and interaction analyses because the rumen microbiota plays a crucial role in energy metabolism and the inflammatory response. The results show that *Succinivibrionaceae_UCG_001* had the greatest contribution and close interactions with other genera, both in the CON and HN3 groups. However, the interactions among the CON groups were more complex, which might be because n-3 PUFAs can attenuate immune responses, thereby

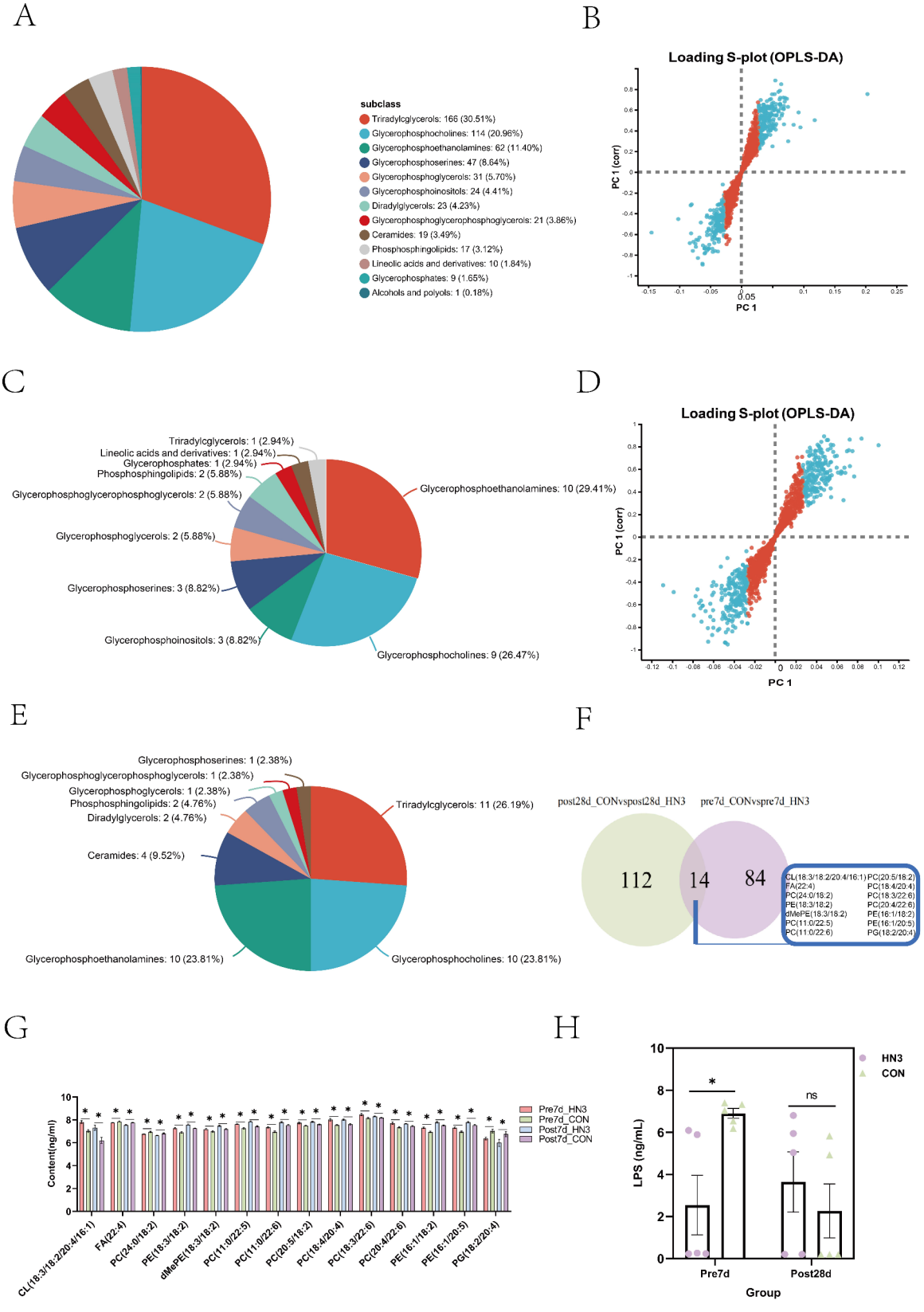

Fig. 3 (See legend on next page.)

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Fig. 3 Effect of n-3/n-6 PUFA ratio on the lipid metabolites in the liver. A pie chart (**A**) shows biochemical categories of identified metabolites. Significant differential metabolites screened by Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) (VIP > 1, and $P < 0.05$) are shown in an S-plot in the prepartum (**B**) and the postpartum (**D**) period, with the blue and red dots indicating VIP values greater than or equal to and less than 1, respectively. The biochemical categories of differential metabolites of prepartum and postpartum are demonstrated in (**C**) and (**E**), respectively. (**F**) Venn diagram of the shared differential metabolites in the prepartum and postpartum period. (**G**) The content of all shared differential metabolites is expressed as mean ± SEM. (**H**) The content of lipopolysaccharide (LPS) in the liver. *$P < 0.05$; ns, not significant

reducing unfavorable interactions between microbial communities, while a low n-3:n-6 PUFA ratio may make the response of the rumen microbial community more complex under stress.

The liver performs multiple functions in metabolism and growth [61]. Additionally, the liver is an organ regulating innate immunity and plays an important role in response to bacterial infection [62]. Based on the altered LPS content in the liver, and to further identify the alterations in related genes and pathways, we randomly selected six cows from each group for transcriptome analysis of the liver to identify critical differentially expressed genes. In this study, the results of DEGs enrichment and KEGG enrichment analysis in the prepartum period show that the liver also has immune-related genes upregulated including the B cell receptor signaling pathway, which indicates that the liver is immunologically active; this may be associated with the fact that a high n-3/n-6 PUFA ratio in the diet reduces LPS from the rumen. Interestingly, we observed that DEGs associated with the taste receptor T2R65A were significantly enriched in the taste transduction pathway in the HN3 group, which has been shown to have a significant effect on feed intake and production traits [63], this is consistent with changes in the levels of orexins in plasma, which may be important in influencing the appetite of transition cows. Notably, these genes encoding proteins have been suggested to be expressed in taste bud cells of the human gustatory system but are limited in dairy cows. In addition, the GO analysis suggested that these altered pathways were mainly involved in lipid metabolism.

Therefore, to better understand how the n-3: n-6 PUFA ratio affects lipid metabolism in the liver, targeted metabolomic methods were initially used to identify the related metabolic pathway changes. Consistent with the transcriptome results, we found that the n-3: n-6 PUFA ratio affected liver lipid metabolism, including fat digestion and absorption, and insulin resistance. Additionally, NF-κB is a key mediator of the inflammatory response and can induce the expression of various proinflammatory genes [64]. The results from this study further suggest that the NF-κB signaling pathway is significantly down-regulated, which is consistent with earlier research findings that a high n-3/n-6 PUFA ratio in a diet can inhibit the activation of the NF-κB signaling pathway [65–67]. Interestingly, we also found that a high dietary n-3/n-6 PUFA ratio in the diet al.leviates insulin resistance in the postpartum period, which may be attributed to the upregulation of the taste transduction pathway in the liver, which ultimately creating positive feedback that downregulates insulin resistance. It is also consistent with previous findings that increasing the proportion of n-3 PUFA-rich foods significantly reduced the risk of developing type 2 insulin resistance [68, 69].

This study focused on the mechanism of alleviation of inflammatory responses in transition cows by regulating the n-3/n-6 PUFA ratio. Nevertheless, there are several limitations to this study. Firstly, while the n-3/n-6 PUFA ratio is the key dietary difference in this study, other secondary metabolites besides PUFAs may be introduced through extruded soybeans and extruded flaxseed. Since n-3 and n-6 PUFAs consist of various fatty acids with potentially different biological functions, further experiments could involve the targeted supplementation of one or more specific n-3 PUFAs in the diet to validate the results. Additionally, compared to the CON group, how rumen fluid bacteria influence plasma levels of appetite factors in transition cows is unknown and needs to be further explored in conjunction with bacterial isolation and culture experiments, at the same time, the expression levels of genes related to rumen epithelial barrier function need to be further verified. In the future, we will further explore the potential biological processes of the n-3/n-6 PUFA ratio in vitro and in vivo to explore more possibilities regarding the relationship between the n-3/n-6 PUFA ratio, lipid metabolism, and inflammatory responses.

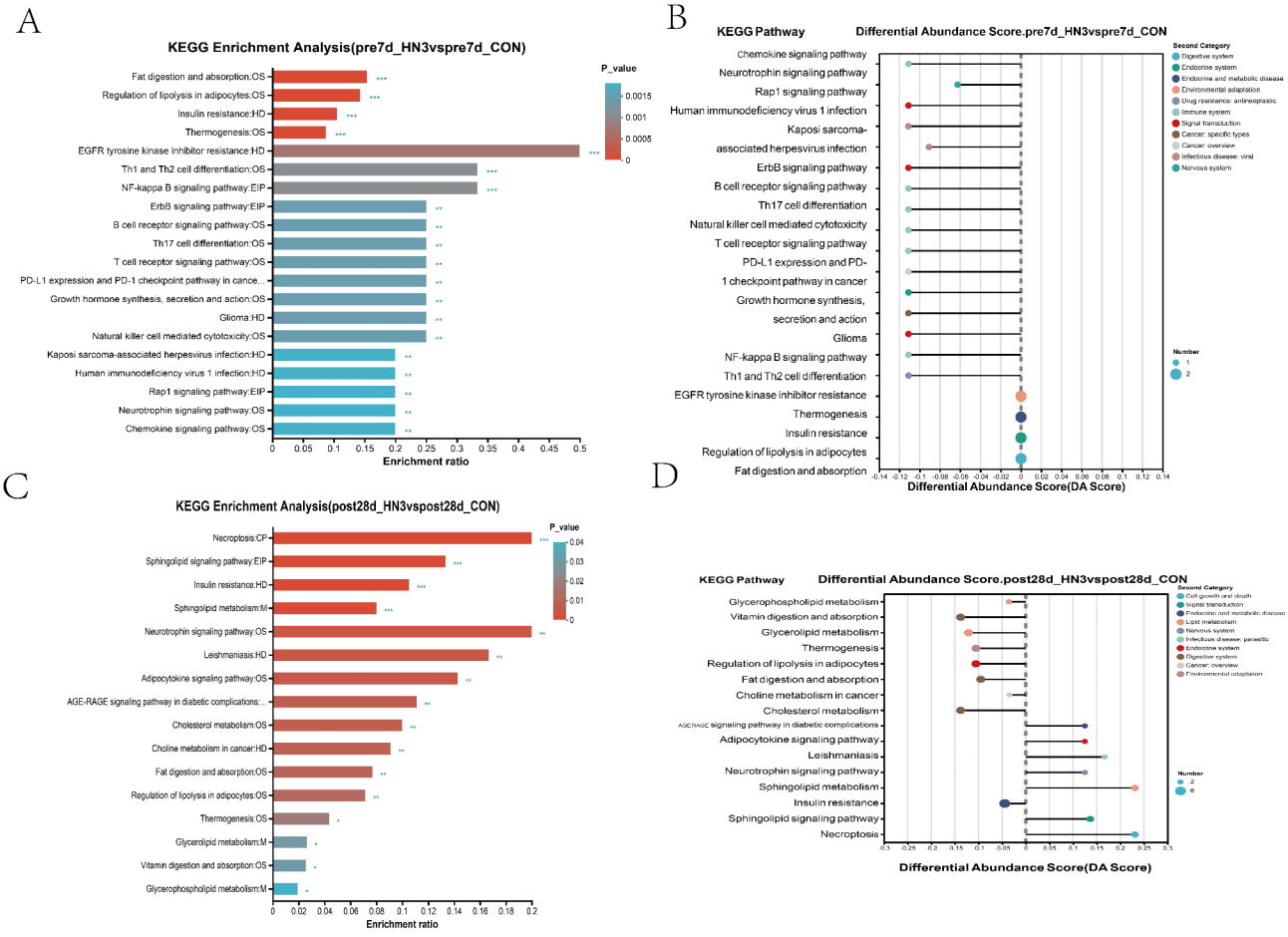

Fig. 4 Effect of n-3/n-6 PUFA ratio on the lipid metabolism pathway in the liver. The enriched pathways of differential metabolites in the prepartum and the postpartum as shown in (**A**) and (**C**), respectively. The vertical axis indicates the pathway name and the horizontal axis indicates the enrichment ratio, where a larger ratio indicates greater enrichment. Column color gradient indicates the significance of enrichment, asterisks mean statistically significant difference: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The KEGG pathway differential abundance score in the prepartum and the postpartum are shown in (**B**) and (**D**). The horizontal axis indicates the differential abundance score (DA Score) and the vertical axis indicates the KEGG metabolic pathway name. The DA Score reflects the overall change of all metabolites in the pathway, with the right side of the middle axis indicating the trend of up-regulation of the expression of all annotated differential metabolites, and the left side of the middle axis indicating the trend of down-regulation of the expression

Conclusions

The ratio of n-3/n-6 PUFAs in the diet plays a crucial regulatory role in the physiology and metabolism of transition cows, and this regulatory effect differs between the prepartum and postpartum stages. In the prepartum phase, a high n-3/n-6 PUFA ratio diet reduced the relative abundance of gram-negative bacteria in the rumen fluid and down-regulated the NF-κB signaling pathway in the liver; in the postpartum phase, high n-3/n-6 PUFA ratio in diet altered the level of appetite factor in plasma and increased milk protein, at the same time ameliorating the insulin resistance in the liver. Overall, these findings shed light on the potential mechanisms underlying the impacts of a high n-3/n-6 PUFA ratio in the diet of transition cows and provide substantial insights into the application of n-3 PUFA in commercial cow farms.

Abbreviations

DMI	Dry matter intake
NEB	Negative energy balance
PUFA	Polyunsaturated fatty acids
PYY	Peptide-YY
LPS	Lipopolysaccharide
LDA	Linear discriminant analysis
FDR	False discovery rate
PCA	Principal component analysis
LEfSe	Linear discriminant analysis effect size
DEGs	Differentially expressed genes
KEGG	Kyoto Encyclopedia of Genes and Genomes

Supplementary Information

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Supplementary Material 1

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Author contributions
X. Liu: Data analysis, Writing-Original draft preparation, Writing-Reviewing and editing; X. Zhang: Methodology, Writing- Reviewing and Editing; Q. He: Software; X. Sun: Experimental conducting, Investigation, Validation; W. Wang: Conceptualization, Methodology, Supervision; S. Li: Conceptualization, Methodology, Supervision, Funding acquisition.

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Data availability
The raw sequence data of liver transcriptome and 16 S rRNA for rumen bacteria were subjected to the Sequence Read Archive (SRA) of the NCBI under accession number PRJNA1111922 and PRJNA1083270, respectively.

Declarations

Ethics approval and consent to participate
The animal experiment was performed according to the ethical policies and procedures approved by the committee of animal welfare and animal experimental ethical inspection of China Agricultural University, Beijing, China (protocol number: CAU20201024-2). The animal experiment was conducted at Beijing Zhongdi Animal Husbandry Technology Co. Ltd. (39°30′ N, 116°33′ E). Written informed consent was obtained from the owners for the participation of their animals in this study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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