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Enhancing Holstein steers growth performance: oregano essential oil's impact on rumen development, functionality and microorganism

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Abstract

Background Dietary supplementation with oregano essential oil (OEO), a natural plant extracts, is an effective and acceptable method to improve growth, beef quantity and quality, but the undergoing mechanism in rumen has not yet been reported in Holstein steers. This study investigated the effects of oregano essential oil (OEO) on growth performance, fermentation parameters, digestive enzymes activity, rumen development and microbiota in Holstein steers. Eighteen steers were randomly divided into two groups ($n=9$) and fed either a basal diet (CCK) or the same diet supplemented with 20 g/(d·head) OEO (CEO) for 270 days.

Results OEO increased the rumen contents of volatile fatty acids (VFA, acetate ($P=0.011$), propionate ($P=0.008$), butyrate ($P=0.018$)) and digestive enzymes activity (cellulase ($P=0.018$), protease and β-glucosidase ($P<0.001$)), and improved rumen development (papillae width ($P=0.008$) and micropapillary density ($P=0.001$)), which reasons contribute to increase body weight (BW, $P=0.022$), average daily gain (ADG, $P=0.021$), carcass weight ($P=0.001$), dressing percentage ($P<0.001$), and net meat production ($P=0.001$) of steers. Meanwhile, metagenomic and metabolomic analysis revealed OEO significantly reduced abundance of rumen microorganisms, especially methanogenic archaea and viruses while beneficial bacteria (*Bifidobacterium*) and virulence factors were not affected. KEGG analysis revealed that OEO significantly reduces the host risk of disease, improves the digestive system, and reduces the energy basic metabolism level. A correlation analysis indicated fourteen kinds key microbiome and six downregulated metabolites interfere with each other and together influence the growth performance of steers.

Conclusion These results suggest that feed with 20 g/(d·head) OEO in steers diets could improve growth performance, and reduces virus abundance and disease risk. And the findings provide fundamental insights into OEO, as an alternative source of natural bioactive compounds, how effect on rumen development, composition and function of microorganisms.

Keywords Volatile fatty acids, Digestive enzymes activity, Rumen microbiome and metabolites, Growth performance

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Background

As consumption patterns have shifted, particularly regarding the increased value of consumer placed on beef requirement and quality. Thus, a substantial effort and prudent decision in the cattle production goes toward the improvement of beef quantity and quality via selection of additives in diets [1]. However, there is criticism on adding chemical additives for growth promoters in livestock feed due to harmful effects on consumers [2]. In addition, non-therapeutic using in large-scale animal production since 1938, antibiotics has become increasingly profitable for disease treatment and prevention, survival rate enhancement, feed efficiency, growth promotion, and labor reduction [3–6]. Alarmingly, over 70% of antimicrobials produced globally are administered to animals, a trend that is on the rise, which lead to antimicrobial-resistant infections and environment pollution, prompting more countries to ban their use [7–9]. Consequently, there is a strong incentive to select natural and acceptable by the public additives for beef cattle practitioners and researchers to optimize both the yield meat and health of bulls under limited dietary raw material conditions.

Oregano essential oil (OEO) was extracted from *Origanum vulgare*, the main components of OEO are terpenes (Carvacrol and Thymol, ≥75 ~ 85%) with several biological functions including broad-spectrum antimicrobial and insect repellent, antioxidant, and anti-inflammatory activities [10, 11]. OEO is traditionally used in perfumes, flavors and medicines against pruritus, headaches and depression [12–14]. OEO is also used to promote different livestock health and performance, like as improving milk production of cows [15], intestinal antioxidative capacity and immunity of chickens [16], abundance of intestinal microbiota and reducing diarrhea of calves [17]. However, although the application of OEO has a long history, the exact mechanism of action is largely unknown to date, especially as a phytogenic feed additive intervene rumen activity to improve growth performance in Holstein steers.

Production performance and health of ruminants is directly dependent on rumen regulatory strategies [18]. A complete symbiosis dependence exists between the host and microorganisms, where microbial communities are primarily responsible for digestive and nutrient absorptive functions in ruminants and form a highly complex and anaerobic microbial ecosystem [19, 20]. Rumen microbiome performs a multitude of functions, such as host dependent on the resided in the upper digestive tract microbial community to degrade and ferment the ingested plant, which provides more than 66% energetic requirements with volatile fatty acids (VFA) [21]. Furthermore, composition and proportion of VFA balance the environment for microbial development and fermentation, to regulate immune responses, and protecting against pathogenic bacteria [22], and improve the development of the rumen epithelium by modulating gene regulation [23, 24]. In addition, rumen microbiome is also associated with the appetite and secretion of digestive enzymes to promote feed conversion ratio and production, which improved economic benefits [25]. Therefore, investigating whether OEO supplementation alters the ruminal development, VFA, digestive enzymes, microbiome and metabolome is highly relevant to further promote the growth performance of steers.

Here, we investigated the effects of OEO on growth performance, rumen development, VFA, digestive enzymes, microorganisms and metabolites in Holstein steers. We hypothesized that feeding steers OEO would improve rumen development, digestive function and alter rumen microbiome, and thereby affect growth performance. The objectives of our study were to: (1) examine the alterations in the rumen composition and function of microorganisms and metabolites upon OEO treatment, and (2) utilize metagenomics and metabolomics to determine the impact of microbial and metabolite variations on host growth performance. Based these findings to provide an understanding of the regulation of rumen development and functionality after OEO treatment in steers.

Table 1 Comparative analysis of production performance

Items	Groups[1]		*P*-Value
	CCK	CEO	
Body weight (BW), kg	600.84 ± 19.67[b]	661.49 ± 12.95[a]	0.022
Average daily gain (ADG), kg/d	0.95 ± 0.06[b]	1.13 ± 0.03[a]	0.021
Feed intake (FI), kg/d	17.56 ± 0.60	17.51 ± 0.69	0.942
Dry matter intake (DMI), kg/d	10.45 ± 0.25	10.45 ± 0.23	0.990
Feed/gain ratio (F/G)	11.46 ± 0.85[a]	9.27 ± 0.30[b]	0.035
Carcass weight, kg	331.14 ± 10.24[b]	389.79 ± 6.32[a]	0.001
Dressing percentage, %	57.36 ± 0.47[b]	60.86 ± 0.29[a]	< 0.001
Net meat weight, kg	258.55 ± 7.93[b]	309.33 ± 6.26[a]	0.001

[1] CCK: castrated bulls with basal diet group; CEO: castrated bulls with the same diet adding to 20 g per steer/day oregano essential oil; values presented as mean ± standard error ($n = 6$)

[a, b] Different superscripts within a row indicate significant differences between two groups following EO treatment ($P \leq 0.05$), as analyzed by independent samples t-tests

Results

OEO treatment improved growth performance of steers

The body weight (BW, $P = 0.022$), average daily gain (ADG, $P = 0.021$), carcass weight ($P = 0.001$), dressing percentage ($P < 0.001$) and net meat weight ($P = 0.001$) were significantly higher in the CEO group than the CCK group. Conversely, the feed/gain ratio (F/G, $P = 0.035$) was significantly lower, whereas feed intake (FI) and dry matter intake (DMI, $P = 0.990$) did not differ significantly between the groups (Table 1).

OEO treatment affected fermentation parameters and enzyme activities of steers

We investigated fermentation parameters and enzyme activities in the rumen of steers (Table 2). The results showed that acetate ($P = 0.011$), propionate ($P = 0.008$), butyrate ($P = 0.018$), cellulase ($P = 0.018$), protease ($P < 0.001$) and β-glucosidase ($P < 0.001$) in CEO group were higher than in the CCK group. In contrast, lipase ($P < 0.001$) in the CEO group were lower than in the CCK group. Valerate ($P = 0.194$) and xylanase ($P = 0.250$) levels did not exhibit significant differences between the two groups.

OEO treatment promoted the development of the rumen epithelium

The width and density of rumen papillae were measured to investigate the effects of OEO on rumen development (Fig. 1). The rumen papilla width ($P = 0.008$) and micropapillary density ($P = 0.001$) were higher in CEO than in CCK.

OEO treatment reduced the harmful microbiome and interfered with its function

Metagenome sequencing was performed to understand the effects of OEO treatment on the rumen microbiome. An average of 74,792,855.00 and 67,062,200.33 raw reads in the CCK and CEO groups were obtained, respectively. After excluding low-quality and N-containing reads, 73,283,668.00 and 65,569,598.33 clean reads were analyzed, accounting for 97.98% and 97.78% of the raw reads in CCK and CEO, respectively. The optimized reads obtained for subsequent analysis after removing the host genome sequence were 61,910,355.33 and 54,218,683.33, accounting for 82.81% and 80.80% of the raw reads for CCK and CEO, respectively. Sequence reads were reliable, and could be used for subsequent analysis (Table S1). The rumen microbial Venn chart (Fig. 2A) showed that there were 5,622 shared species, 682 and 376 species in the CCK and CEO, respectively. Analysis of similarities (ANOSIM) showed that microbial species were clustered into significantly different groups ($P = 0.022$, Fig. 2B). The dominant bacteria in the rumen were found to belong to Bacteroidetes (CCK: 43.83%, CEO: 43.08%) and Firmicutes (CCK:36.91%, CEO: 36.90%). The abundance of 11 phyla in the CCK group was higher than that in the CEO group, whereas opposite results were obtained for 4 phyla ($P \le 0.05$) (Table S2). There were 344 genera with significant variations. Among 15 genera showing the highest level of variation, the abundance of 10 genera in the CCK group was higher than that in the CEO group ($P \le 0.05$), whereas five genera showed the opposite trend between the two groups ($P \le 0.05$, Fig. 2C). A total of 31,485 microbial species were identified, of which 1,934 yielded significantly different abundances. The dominant bacteria were *Clostridiales_bacterium* (CCK: 8.388%, CEO: 7.254%), *Bacteroidales_bacterium* (CCK: 5.062%, CEO: 3.965%), *Rikenellaceae_bacterium* (CCK: 4.165%, CEO: 2.719%), *Bacterium_P3* (CCK: 1.733%, CEO: 2.709%), and *Prevotella_ruminicola* (CCK: 2.060%, CEO: 2.014%, Fig. 2D). The archaeal differential analysis revealed that the abundance of 51 species of archaea was significantly different between two groups. The abundances of 41 archaea among them were found to be higher in CCK higher than in CEO, whereas those of 10 archaea were lower ($P \le 0.05$). The abundance of 1,741 species of bacteria was significantly different between the two groups. Of these, abundances of 1,280 bacterial species were higher in CCK than in CEO, where those of 461 bacterial species were lower ($P \le 0.05$). The abundances of 75 species of eukaryota were significantly different between the two groups: 63 species were more abundant in CCK than in CEO, and 12 kinds were less abundant in CCK than in CEO ($P \le 0.05$). The abundances of 64 species of viruses were significantly different between the two groups: 52 viral species were more abundant in CCK than in CEO, and 12 viruses were less abundant in CCK than in CEO ($P \le 0.05$, Table S3). These findings indicate that OEO could effectively decrease the rumen microbiome.

To explore the intervention of OEO for rumen microbial function, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that there was obvious clustering of microbial function between the two groups (Fig. 3A). Six pathways were annotated at the first level: metabolism, genetic information processing, environmental information processing, cellular processes, human diseases, and organizational systems (Fig. 3B). At the second level, 46 pathways were observed, the top five were global and overview maps, carbohydrate

Table 2 Comparative analysis of fermentation parameters and enzyme activities

Items	Groups[1]		*P*-Value
	CCK	CEO	
Acetate, ug/g	162.72 ± 5.65^{b}	202.02 ± 9.23^{a}	0.011
Propionate, ug/g	71.80 ± 3.98^{b}	107.28 ± 8.16^{a}	0.008
Butyrate, ug/g	33.34 ± 3.84^{b}	51.13 ± 3.95^{a}	0.018
Valerate, ug/g	12.69 ± 1.00	14.91 ± 1.14	0.194
Lipase, U/mg	8.50 ± 0.37^{a}	5.23 ± 0.29^{b}	< 0.001
Cellulase, U/mg	76.20 ± 12.73^{b}	123.98 ± 10.80^{a}	0.018
Protease, U/mg	1.77 ± 0.09^{b}	2.96 ± 0.07^{a}	< 0.001
Xylanase, U/mg	3.92 ± 0.07	4.36 ± 0.33	0.250
β-glucosidase, U/mg	21.66 ± 0.62^{b}	33.60 ± 1.58^{a}	< 0.001

[1] CCK: castrated bulls with basal diet group; CEO: castrated bulls with the same diet adding to 20 g per steer/day oregano essential oil; values presented as mean ± standard error ($n = 6$)

[a, b] Different superscripts within a row indicate significant differences between two groups following EO treatment ($P \le 0.05$), as analyzed by independent samples t-tests

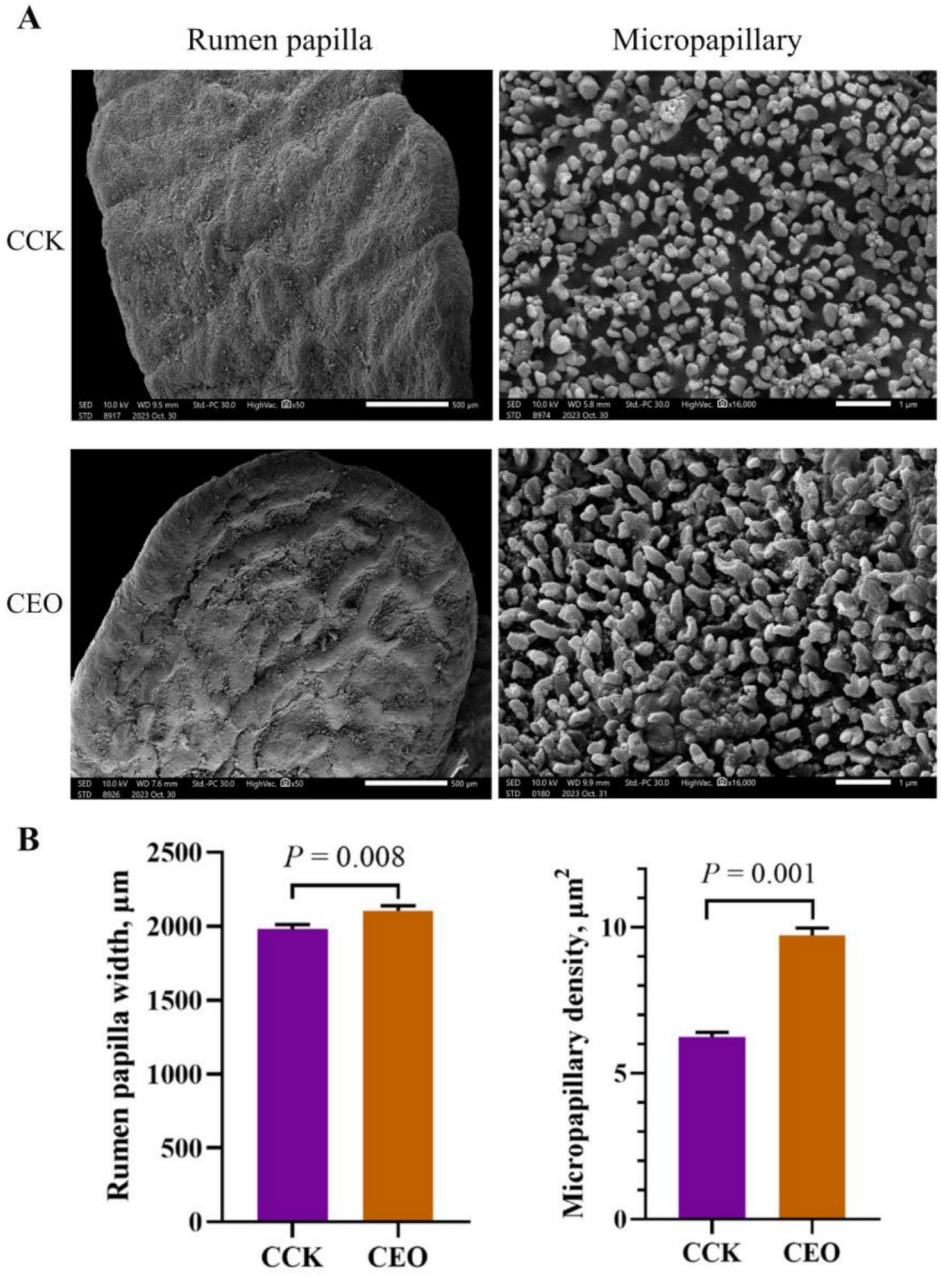

Fig. 1 Electron microscope scanning of rumen tissue. (**A**) Scanning electron microscope image of the rumen; (**B**) Compare of rumen papilla width and micropapillary density; CCK: castrated bulls with basal diet group; CEO: castrated bulls with the same diet adding to 20 g per steer/day oregano essential oil ($n = 6$)

metabolism, amino acid metabolism, energy metabolism, and the metabolism of cofactors and vitamins. Notably, there were five pathways with significant differences ($P \le 0.05$), and basal metabolism was clustered in the CCK group, whereas the digestive system was enhanced in the CEO group (Table S4). A total of 330 third-level pathways were observed, 23 of which were significantly enriched ($P \le 0.05$). Thirteen pathways were significantly enriched in CCK, mainly in diseases and microbial, carbohydrate, and amino acid metabolism, whereas 10 pathways were significantly enriched in CEO, mainly in amino acids, fatty acids, proteins, and lipoarabinomannan biosynthesis (Fig. 3C, Table S5). Furthermore, the effect of OEO on disease, probiotics and virulence factor database was analyzed. There were 43 diseases, 11 of which showed significant differences in both groups, and the addition of OEO was resistant to 10 diseases, including maintenance of the gut microenvironment,

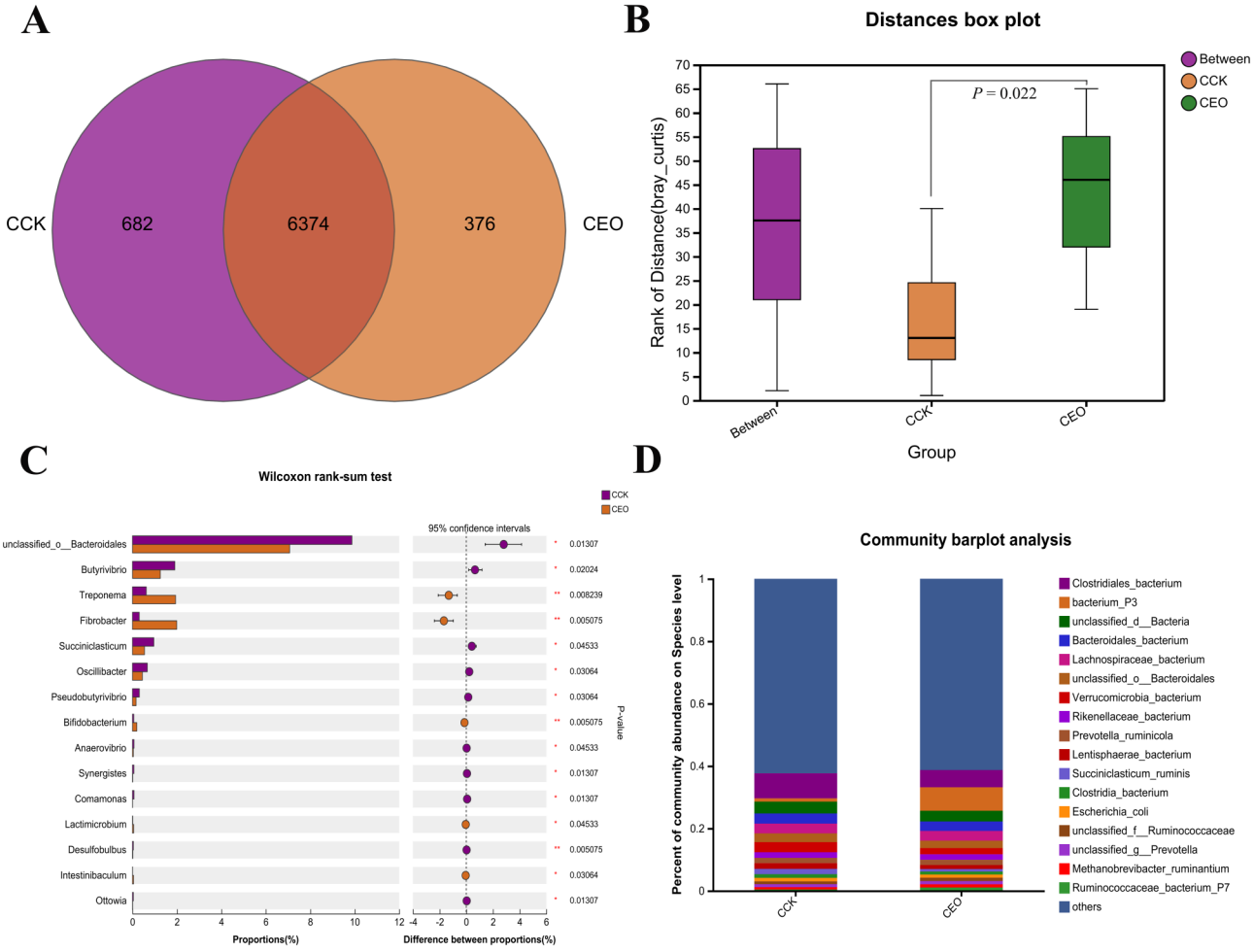

Fig. 2 Rumen microbial composition and differences. (**A**) Rumen microbial Venn chart; (**B**) ANOSIM analysis chart between two groups; (**C**) Differences between CCK and CEO at the genus level; (**D**) Dominant strains of CCK and CEO at the species level; CCK: castrated bulls with basal diet group; CEO: castrated bulls with the same diet adding to 20 g per steer/day oregano essential oil ($n = 6$)

endocrine, nutritional, and metabolic diseases, and animal survival (Fig. 3D). In addition, there were 116 types of probiotics between CCK and CEO, 15 of which were significantly different, and the relative abundance of *Bifidobacterium* increased significantly after the addition of OEO ($P \le 0.05$, Fig. 3E). In particular, analysis of the virulence factor database (VFDB) revealed that the virulence factors were not significantly different between the two groups (Table S6). These findings suggest that OEO may be improved the beneficial microorganisms, host digestive system, reduced basic metabolism, and enhanced disease resistance, while did not contribute to VFDB development.

OEO treatment changed rumen metabolome of steers

To fully characterize the impact of changes in composition and function of rumen microbiome, we performed a metabolite profiling of steers rumen fluid between two groups using untargeted liquid chromatography-mass spectrometry (LC-MS). In total, 662 metabolites were identified. The orthogonal projections to latent structure-discriminate analysis (OPLS-DA) indicated a clearly separated cluster ($R^2X = 0.381$, $R^2Y = 0.954$, $Q = 0.657$), suggesting that the two groups had differential metabolites in the rumen contents and that the OPLS-DA models were reliable and could be used for further analysis (Fig. 4A & B). A total of 116 metabolites were found to be significantly different upon screening of the concentrations of rumen metabolites, as evidenced by fold change (FC, $FC \ge 2$ & $FC \le 0.5$) and variable important for the projection (VIP, $VIP \ge 1$) results. These included 68 upregulated and 48 downregulated metabolites (Fig. 4C). Quinoline-4-carboxylic acid and 4-Pyridoxic Acid were significantly upregulated, whereas glycine deoxycholic acid, gamma-cholic Acid, 2-Aminoethanesulfonic Acid, Carnitine C6:0, carnitine isoC4:0 and Carnitine C4:0 was significantly downregulated when $|Log_2FC| > 2.5$ was used as criteria to determine significance (Fig. 4D).

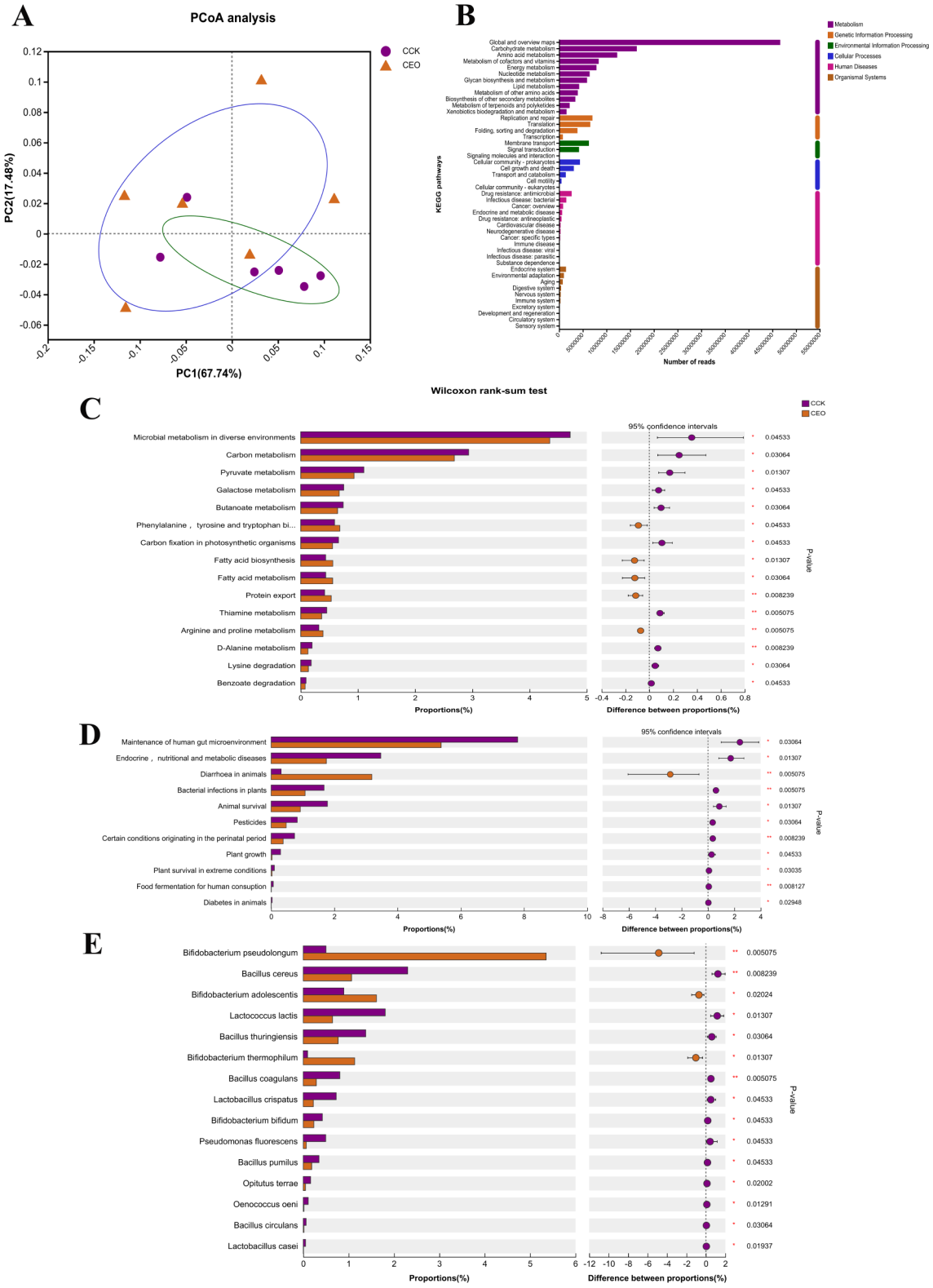

Fig. 3 Rumen microbiome function and differences. (**A**) Microbiome function PCoA analysis; (**B**) First level KEGG pathway; (**C**) Third level differentially significant KEGG pathway; (**D**) Comparison of disease between two groups; (**E**) Comparison of probiotics between two groups; CCK: castrated bulls with basal diet group; CEO: castrated bulls with the same diet adding to 20 g per steer/day oregano essential oil ($n = 6$)

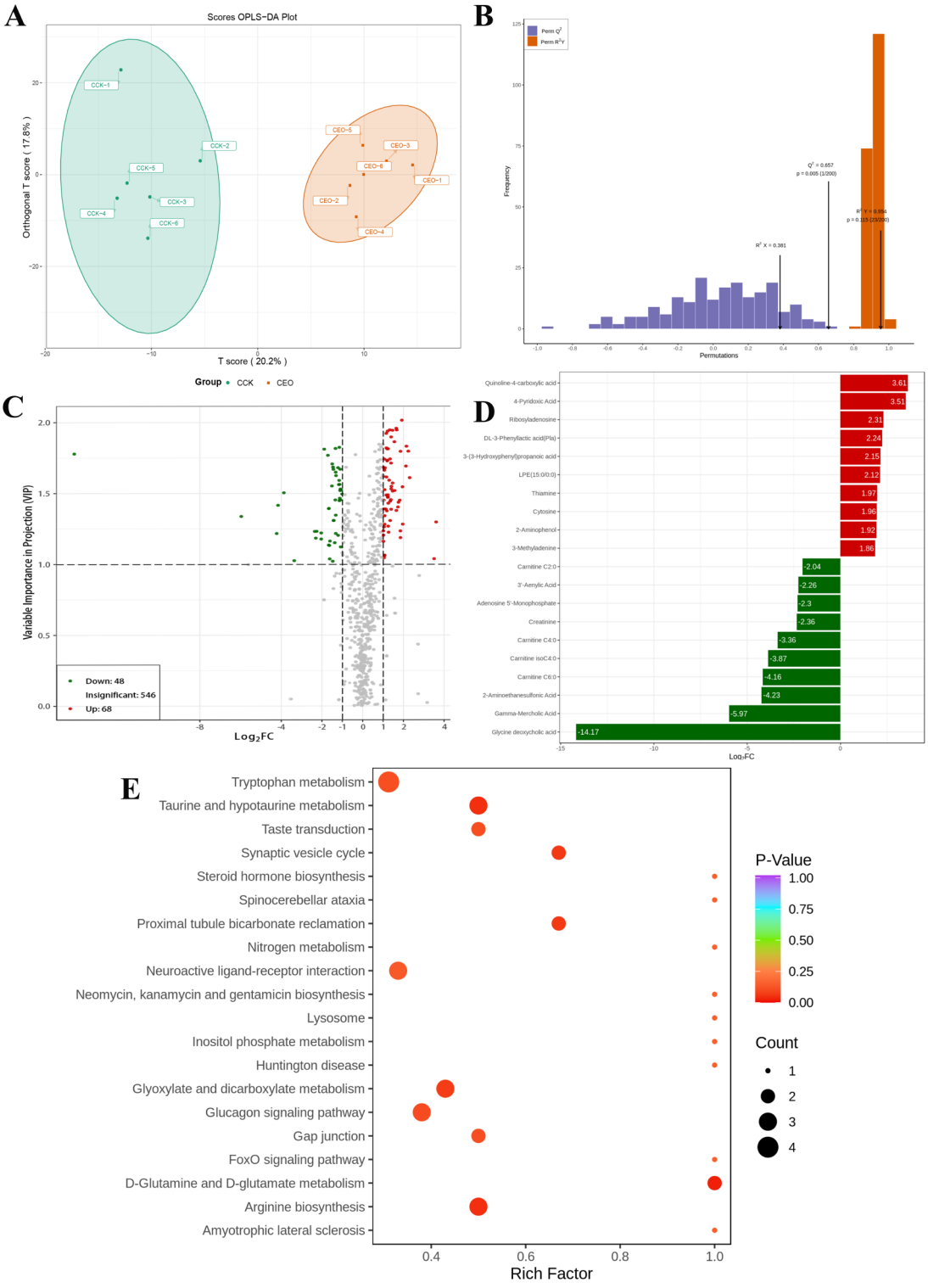

Fig. 4 Rumen metabolite composition and function. (**A**) OPLS-DA analysis; (**B**) OPLS-DA model chart; (**C**) Differential metabolite volcano plot; (**D**) Fold change in the top 20 different metabolite bar charts; (**E**) KEGG functional enrichment analysis of differential metabolites; CCK: castrated bulls with basal diet group; CEO: castrated bulls with the same diet adding to 20 g per steer/day oregano essential oil ($n = 6$)

KEGG pathway analysis revealed that 116 metabolites were significantly enriched in 75 pathways, mainly those related to tryptophan metabolism, taurine and hypotaurine metabolism, neuroactive ligand-receptor interaction, glyoxylate and dicarboxylate metabolism, glucagon signaling, d-glutamine and d-glutamate metabolism, Histidine metabolism and arginine biosynthesis (Fig. 4E).

Systematic correlations across phenotype, microbiome, and metabolome revealed OEO-mediated changes

The linkage effect between metabolome and microbiome was detected by first combining the two omics datasets. Then, we identified the top 15 microorganisms and metabolites with the largest linkage effects using two-way orthogonal partial least squares (O2PLS) analysis (Fig. 5A). Fourteen significantly different microbial species ($P = 0.036$) were used for subsequent analyses. Six

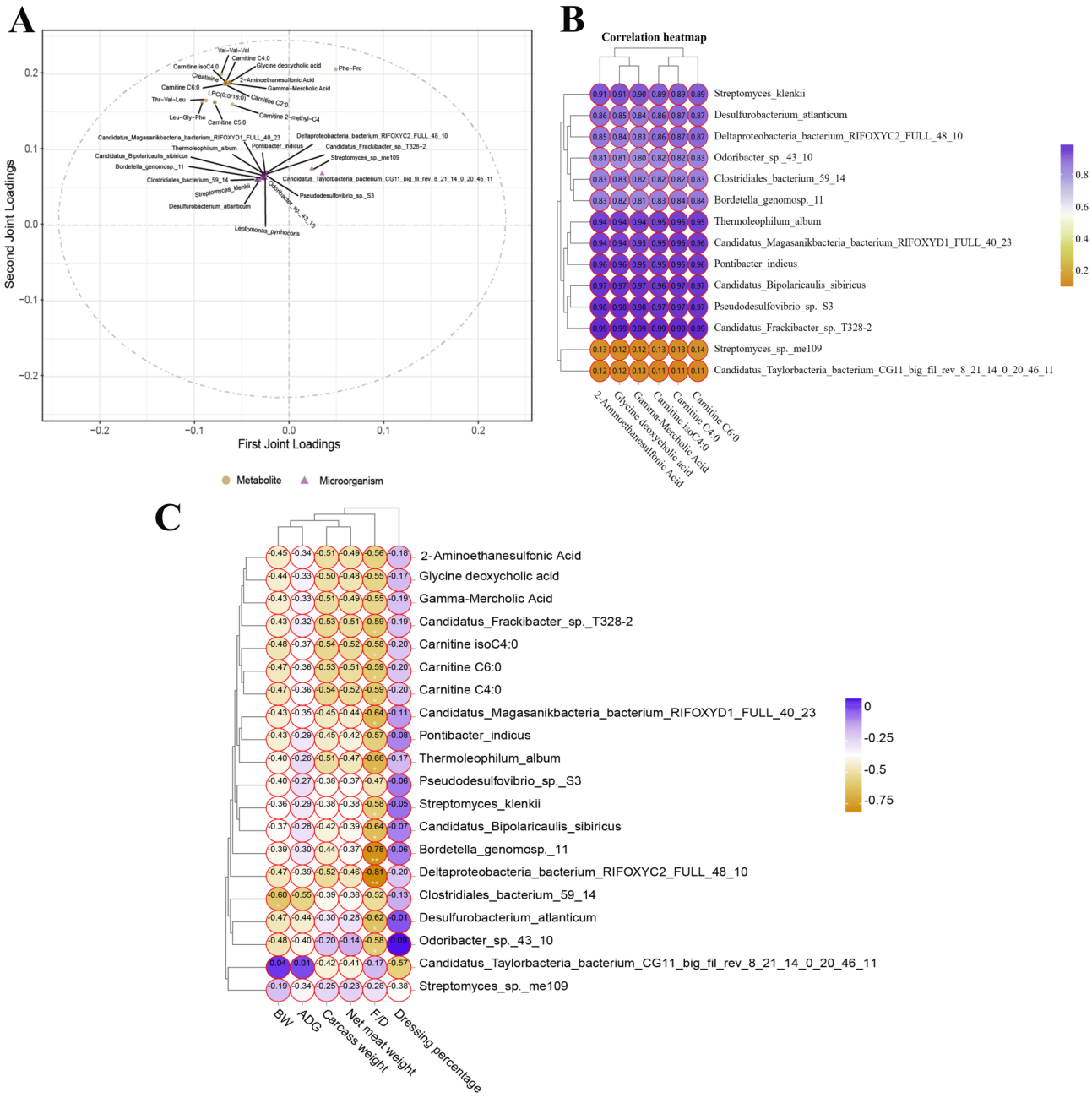

Fig. 5 Combined analysis of metagenome and metabolome. (**A**) O2PLS analysis of the metagenome and metabolome; (**B**) Target metabolite and microorganism correlation analysis; (**C**) Correlation analysis of growth performance, metabolites, and microorganisms. CCK: castrated bulls with basal diet group; CEO: castrated bulls with the same diet adding to 20 g per steer/day oregano essential oil ($n = 6$)

significantly downregulated metabolite (2-Aminoethanesulfonic Acid, Carnitine isoC4:0, glycine deoxycholic acid, gamma-cholic acid, carnitine C6:0, and Carnitine C4:0) were used for subsequent analyses by using FC < 0.1 as filtering criteria.

Correlation analysis was used to further explore the effects of the screened 6 metabolites and 14 microorganisms on the growth performance of castrated bulls. The results revealed that 6 metabolites were positively correlated with 12 microorganisms ($P < 0.05$), except for *Streptomyces_sp._me109* and *Candidatus_Taylorbacteria_bacterium_CG11_big_fil_rev_8_21_14_0_20_46_11* (Fig. 5B). *Clostridiales_bacterium_59_14* was negatively correlated ($P = 0.037$) with BW. F/G was negatively correlated with 3 down-regulated metabolites (Carnitine isoC4:0 ($P = 0.049$), Carnitine C6:0 ($P = 0.044$) and Carnitine C4:0 ($P = 0.043$)) and 9 microbial species (*Streptomyces_klenkii* ($P = 0.048$), *Thermoleophilum_album* ($P = 0.020$), *Desulfurobacterium_atlanticum* ($P = 0.033$), *Odoribacter_sp._43_10* ($P = 0.046$), *Candidatus_Bipolaricaulis_sibiricus* ($P = 0.026$), *Candidatus_Magasanikbacteria_bacterium_RIFOXYD1_FULL_40_23* ($P = 0.024$), *Candidatus_Frackibacter_sp._T328-2* ($P = 0.042$), *Bordetella_genomosp._11* ($P = 0.003$), and *Deltaproteobacteria_bacterium_RIFOXYC2_FULL_48_10* ($P = 0.001$)). No significant correlations with the other indicators were obtained (Fig. 5C).

Discussion

As consumers become more aware of the safety and health of beef, and the international commitment to eliminating antibiotics in livestock production for safety reasons is commendable, leading to traditional additive (chemical component) and antibiotics were not accepted by public for its potential security threat. Therefore, identifying additives, green organic without residues and accepted by the public, is an urgent issue and current research hotspot [26]. This study explores the potential of OEO to improve growth performance and health by interfering with rumen development and function in steers. We observed significant increases in acetate, propionate, and butyrate concentrations after adding OEO, VFA contributed for host up to 65% of energy requirements [21]. Additionally, VFA was also beneficial for the rumen environment, epithelial cell development (increasing papilla width and density), and microflora stability to contribute to the absorption of nutrients by the host [24, 27]. Acetate also plays a regulatory role in body weight by influencing energy metabolism [28]. Specifically, acetate can crossed the blood-brain barrier via monocarboxylate transporters, and increases parasympathetic nervous system activation, which leads to increased glucose-stimulated insulin secretion, ghrelin secretion, promoted hyperphagia, and energy retention to increase body weight [29]. Previous studies [30] and Our results also confirmed that the addition of OEO increased growth performance, and ruminal papillae width and density in steers. This result may be attributed to the high VFA concentration [31]. In addition, the content of digestive enzymes (cellulase, protease and β-glucosidase) was found to be higher in CEO group than in CCK group. Cellulase can degrade the lignin-hemicellulose-cellulose complex of plants. This loosening effect can facilitate the attachment of microorganisms to the substrate, leading to faster growth of microbial populations and increased fiber degradation into monosaccharides, which is conducive to host absorption [32, 33]. Traditionally, proteases are used for the degradation of proteins into small peptides that can be absorbed by the gastrointestinal epithelium [34]. However, recent studies have shown that proteases play major roles in many other physiological functions as well [35]. Similarly, β-glucosidase can break down cellulose into glucose to provide energy for the host, and to improve the utilization of lignocellulose (cellulose, hemicellulose and lignin) feed by plants [36]. Therefore, the addition of OEO increased digestive enzyme content, contributing to the degradation of macromolecules in the diet. These factors may explain the improved production performance of the steers upon OEO supplementation.

The addition of OEO can effectively reduce rumen microflora, especially viruses. Microorganisms participate in complex host-microbial interactions during degradation, fermentation, and transformation in diet contents [37]. This interaction does not only help produce high-quality proteins and sustain rural livelihoods, but also possibly contribute to food security [38]. Similar to previous metagenomic studies in the rumen, bacteria are the main components of rumen microorganisms, and the dominant bacteria in the rumen are Bacteroidetes and Firmicutes, which facilitate functional homeostasis and anaerobic digestion [39, 40]. Firmicutes to Bacteroidetes ratio is often used to assess host weight gain since the ratio is linked to energy metabolism [41]. This suggests that bacteria may contribute to a higher level of promoting host growth performance. Effective reduction in rumen microflora (archaea, bacteria, eukaryota and viruses) may occur upon OEO supplementation, as OEO is a hydrophobic molecule that is permeable through the cell membrane and causes expansion of the cellular content [14]. Here, death of the bacteria may occur through the drainage of crucial molecules and ions from the bacterial cell.

In this study, the abundances of archaea, especially *Methanobrevibacter*, *Methanocorpusculum*, *Methanomethylovorans* and others associated with methane biosynthesis, decreased significantly upon OEO addition in the diets, which convert hydrogen and carbon dioxide

into methane to be excreted to the outside by belching or venting and do not convert into propionic acid for host energy [42]. This may eventually contribute to decrease atmospheric pollution and increase host growth. In addition, OEO can significantly inhibit viral species (CCK:52, CEO:12) through viral envelope disruption, capsid disintegration, inhibition of viral replication, or inhibition of viral binding to host cell receptors [43]. However, there was no such significant effect on the relative abundance of beneficial bacteria such as *Bifidobacterium*, which have physiological functions for host health, including biological barrier, nutritional, antitumor, immune enhancement, improving gastrointestinal function, and anti-aging [44]. Moreover, OEO reduced the disease risk, increased the amino acids, fatty acids and proteins metabolism rates, and improved gastrointestinal digestion and absorption levels. Previous studies have argued that highly abundant microorganisms play an important role in promoting livestock production [26]; however, we speculate the low abundance (0.00046%) of *Thermoleophilum_album* contributed significantly to the F/D of steers in this study. These results showed that OEO could not only selectively reduce abundance of rumen microorganisms to save energy (increasing VFA and decreasing archaea), but also maintain health (increasing disease resistance and decreasing virus) to promote growth in steers.

The addition of OEO reduces the risk of disease, and reduces energy consumption by altering rumen metabolism [45]. 40 metabolite types were derived from amino acids and their metabolites, 20 from organic acids and their derivatives, and 56 from others (Table S7), which OEO treatment may exert significant regulatory effects on amino acid metabolism in the rumen metabolites of steers. Here, quinoline-4-carboxylic acid and 4-pyridoxic acid were found to be significantly upregulated metabolites. Quinoline-4-carboxylic acids are a group of compounds associated with antiviral, anti-inflammatory, antimicrobial, anti-atherothrombosis, antiemetic, anxiolytic, antimalarial, and antileishmanial activities [46]. With a stable supply of vitamin B6, vitamin B6 is converted into pyridoxal, and then converted into 4-pyridoxic acid and hydrogen peroxide by aldehyde oxidase, which enhance the host anti-inflammation, antioxidant and immune levels [47, 48]. While glycine deoxycholic acid, gamma-mercholic Acid, 2-aminoethanesulfonic acid, carnitine C6:0, carnitine isoC4:0, and carnitine C4:0 was significantly down-regulated. Glycine deoxycholic acid and gamma-mercholic acid participate in enterohepatic circulation to regulate glucose homeostasis, energy expenditure, and lipid metabolism [49]. Glycine deoxycholic acid also increases the abundance of *B. wadsworthia* in the mucosa, which could promote inflammation [50]. 2-Aminoethanesulfonic Acid decreased blood lipid metabolic levels through the upregulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase, low-density lipoprotein receptors, and cholesterol 7 alpha-hydroxylase expression, along with the downregulation of serum aspartate aminotransferase, alanine aminotransferase, and C-reactive protein levels [51]. Carnitine, a water-soluble quaternary amine that is highly enriched in skeletal and cardiac muscles, is synthesized from the essential amino acids lysine and methionine and is used for fat and lipid metabolism in all cell types. Carnitine is mainly involved in the oxidation of fatty acids to ATP to provide energy for host basic metabolism, and also used as a dietary supplement for weight loss [52]. Comprehensive metabolomic analysis showed enhanced host health and reduced energy metabolism efficiency after the addition of OEO in steers.

Our findings provide a much-needed basis to identify natural and acceptable additive, and facilitates the identification of biomarkers upon OEO supplementation. However, the study still has several limitations. This study was conducted on the most widely farmed breed of Holstein cattle worldwide, and generalizing our findings to other breeds may require additional considerations. Meanwhile, the experimental animals were all castrated, and therefore application to other sexes may be limited. Furthermore, the exact mechanism of action of OEO need to further research.

Conclusions

This study elucidated the OEO improved host growth performance by regulating structure and function of ruminal microorganisms. Adding 20 g/(d·head) OEO not only improved rumen function and development, selectively increased the abundance of *Bifidobacterium* while reduce methanogenic archaea and viruses, but also reduce the risk of disease and basal energy metabolism in Holstein steers. In addition, screened out key biomarkers (fourteen microorganisms and six metabolites) regulating growth performance of steers. Interestingly, low-abundance bacteria were found to play an important role in feed conversion rate. These observations reveal that OEO can be used as a plant-derived feed additive for beef cattle practitioners choosing to promote beef cattle growth performance.

Methods

Experimental design and animal management

Eighteen healthy Holstein steers, aged 300 days (castrated at 60 days), were selected from a commercial bull farm (Huarui Ranch, Zhangye, Gansu, China). The steers, with similar BW (mean ± standard error: 350.32 ± 4.41 kg), were randomly divided into two treatment groups ($n = 9$), with equal numbers of each bull being kept in individual stalls. The steers of the CCK group were fed a basal diet, while the steers of the CEO group were fed a basal diet

Table 3 Composition and nutrient levels of experimental diets (DM basis)

Items	Fattening stages				
	I	II	III	IV	V ~ IX
	0 ~ 30 d	31 ~ 60 d	61 ~ 90 d	91 ~ 120 d	121 ~ 270 d
Ingredients (%)					
Corn silage	45.00	40.00	30.00	25.00	20.00
Whole cottonseed	0	0	0	0	10.78
Flattened corn	14.84	10.00	8.00	5.00	10.00
Corn	25.17	41.36	51.06	59.93	54.36
Soybean meal	8.21	3.20	0	2.00	0
Rapeseed meal	0	0.54	5.17	3.57	0
Cottonseed meal	0	2.00	2.00	1.00	1.00
Pea protein powder	3.49	0	0	0	0
Fatty acid calcium	0	0	0	0	1.00
$CaHPO_4$	1.12	0.80	1.10	1.28	0.65
NaCl	0.62	0.30	0.47	0.50	0.47
$NaHCO_3$	1.11	1.00	1.28	1.00	1.15
MgO	0	0.19	0.28	0.16	0.13
Premix[1]	0.44	0.61	0.64	0.56	0.46
Total	100	100	100	100	100
Nutrition levels[2]					
CP, %	11.94	10.95	10.80	10.60	9.80
TDN, %	72.10	76.54	77.51	78.74	80.52
NEm, MJ/kg	7.44	7.97	8.14	8.31	8.53
NEg, MJ/kg	4.92	5.31	5.47	5.61	5.80
Ca, %	0.61	0.50	0.59	0.63	0.37
P, %	0.31	0.33	0.36	0.35	0.34

[1] Contained the following per kg of premix: VA 4,000,000 IU, VD3 600,000 IU, VE 120,000 IU, Mn 3340 mg, Fe 4800 mg, Zn 12,600 mg, Cu 3140 mg, Se 60 mg, I 90 mg, Co 22 mg, nicotinic acid 5970 mg, biotin 200 mg

[2] Crude protein (CP), Calcium (Ca) and Phosphorus (P) were measured values according to AOAC (2023), while total digestible nutrient (TDN), net energy for maintenance (Nem) and net energy for gain (Neg) were calculated values according to NRC (2016)

supplemented with 20 g/(d·head) OEO. The OEO was acquired from Ralco Agriculture Inc. (Marshall, MN, USA), and the 20 g/(d·head) OEO as per the manufacturer's recommendation, preparation and composition of OEO have been previously reported [53]. The OEO was weighed and mixed evenly with 1 kg corn and topdressed onto the feed in each morning, and the CCK group only received 1 kg of corn without OEO. The experiment lasted for 270 days, all steers were weighed at the beginning and end of the experiment, and the ADG was calculated for the entire experimental period. The total mixed ration (TMR) was collected and weighed at 7:00 the next morning to calculate the feed intake and DMI for each bull. The F/G was calculated as the ratio of FI to BW. The TMR was adjusted every 30 days according to BW changes, and the TMR composition is shown in Table 3 [54], which met the nutritional requirements for beef cattle [55]. The diet of each stage was analyzed for dry matter (DM, drying at 65 ∘C for 48 h), crude protein (CP), calcium (Ca) and phosphorus (P) according to the AOAC International [56], and total digestible nutrient (TDN), net energy for maintenance (NEm) and net energy for gain (NEg) were calculated according to previous report [55]. Briefly, $TDN = tdNFC + tdCP + 2.25\ \ tdFA + tdNDF - 7$, where tdNFC is the total of digestible non-fiber carbohydrates, tdCP is the total of digestible crude protein, tdFA is the total of digestible fatty acid, and tdNFD is the total of digestible neutral detergent fiber. $Nem = [0.0007 \times (20 - T_p) + 0.077] \times BW^{0.75}$, where T_p is the environment temperature of pasture, and $BW^{0.75}$ is the empty state body weight of beef cattle. $Neg = 0.0635 \times EBW^{0.75} \times EBG^{1.097}$, where EBW is the energy of empty state weight of beef cattle, and EBG is the energy of beef cattle gain weight. During the experiment, all steers were fed TMR twice daily at 08:00 and 16:00 and had ad libitum access to diet and water.

Sample collection

At the end of the feeding experiment, six steers were randomly selected from each group based on median BW. After fasting for 12 h, the bulls were transported to a commercial abattoir for slaughter (bolt stunning and jugular vein exsanguination). After slaughter, the carcass weight and net meat percentage were measured and calculated according to a previously described method [26]. Immediately after opening the abdominal cavity,

rumen contents were mixed, collected into four 5 mL sterile tubes from the left dorsal sac, and stored at -80 °C for further metagenomic and metabolomic analyses. At the same time, rumen tissue (2×2 cm^2) specimen was immediately removed from the dorsal sac and reserved in glutaraldehyde solution, stored at 4 °C for scan electron microscopy examination. In addition, another 15 mL of rumen contents of each bull were collected and stored in -20 °C refrigerator for ruminal fermentation parameters and enzymic activity analysis.

Electron microscope scanning of the rumen tissue

Rumen tissue specimens for electron microscopy were prepared as previously reported [26]. Briefly, rumen tissue (1×1 cm^2) was washed with phosphate buffered saline three times for 10 min each time, fixed with 1% osmic acid for 1 h, and then washed three times for 10 min each time. The specimens were then dehydrated in 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol for 15 min each. The specimens were lightly adhered to a conductive adhesive and ion-sputtered. Images were captured using a scanning electron microscope (JSM-IT700HR, InTouchScope™, Tokyo, Japan). Statistical analysis of rumen papillary width and micropapillary density was conducted using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Measurement of fermentation parameters and activity of digestive enzymes

Metaphosphorylated rumen contents were used to determine VFA using gas chromatographic method [57]. Briefly, ruminal fluid was centrifuged at 5400 rpm for 10 min, the supernatant was carefully collected and filtered through a 0.45 μm syringe filter into a vial for gas chromatography (GC). VFA content was determined using a TRACE-1300 series GC ultra-gas chromatograph (Thermo Scientific, Milan, Italy). The enzyme activities, including lipase, cellulase, protease, xylanase and β-glucosidase were determined using commercial assay kits (Biosino Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's instructions.

Metagenome sequencing and bioinformatics analysis

The total DNA of rumen microbes was extracted using a Soil DNA Kit (MOBIO, Carlsbad, CA, USA). The DNA concentration and purity were determined using a TBS-380 fluorometer (Turner Biosystems, Sunnyvale, CA, USA) and NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE, USA), respectively. DNA was fragmented to approximately 400 bp using a Covaris M220 (Gene Company Limited, Hong Kong, China) for library construction. Sequencing was performed the Illumina NovaSeq6000 platform (Illumina Inc., San Diego, CA, USA), and low-quality sequencing reads (length < 50 bp, quality value < 20, or N bases) were removed using FAST (version 0.20.0) [58]. Reads were aligned to the Bos Taurus reference genome assembly using BWA (version 0.7.9a) and the data were assembled using MULTIPLE MEGAHIT (Version 1.1.2) [59]. Overlapping sequences lengths ≥ 300 bp were selected as the final assembling result and used for further gene annotation, the best candidate open reading frames (ORFs) were predicted using Metagene [60]. Predicted ORFs with length ≥ 100 bp were retrieved. Cluster analysis of non-redundant gene catalogs with sequence homology and 90% coverage was performed using CD-HIT (version 4.6.1). Then, sequences of non-redundant gene catalog were aligned with the NCBI NR database with BLASTP (version 2.2.28 +, the best match e-value cutoff: 1e − 5) to obtain annotation results and species abundance [61]. Lastly, annotated against the KEGG database with BLAST search (version 2.2.28 +, the best match e-value cutoff: 1e − 5) [62].

Metabolome measure and bioinformatics analysis

After thawing, 50 mg rumen contents were weighed into 2 mL centrifuge tube and 500 μL of 70% methanol internal standard extract was added at 4 ℃. After vibration mixing for 3 min, the tube was left at -20 °C for 30 min, then centrifuged at 12,000 rpm for 10 min at 4 °C. Then, 250 μL of supernatant was centrifuged at 12,000 rpm for 5 min at 4 °C. Next, 150 μL of supernatant was transferred into the corresponding injection vial for subsequent analysis. The sample was analyzed in ultra-performance liquid chromatography (UPLC, Shim-pack UFLC SHIMADZU CBM30A) and Tandem mass spectrometry (MS/MS, QTRAP® 6500+, SCIEX, Framingham, MA, USA) devices [63]. Based on the LC/MS data, the extraction ion chromatographic peaks of all metabolites were integrated with MultiQuant software (Applied Biosystems, Foster, MA, USA) and the MetWare database (MWDB) and then corrected according to the chromatographic peaks [64]. FC (FC ≥ 2 and FC ≤ 0.5) and VIP (VIP ≥ 1) were used to screen the relative concentrations of rumen metabolites to identify differential metabolites. The identified metabolites were annotated using KEGG database and then mapped to the KEGG pathway database.

Statistical analysis

The data of growth performance, rumen fermentation parameters, enzyme activities and rumen papillae were statistically analyzed using independent samples t-tests in the SPSS software (version 25.0; IBM Corp., Armonk, NY, USA). All data are presented as mean ± standard error, and statistical significance was set at $P \leq 0.05$. GraphPad Prism 8 (GraphPad Software, LLC, San Diego, CA, USA) was used to generate statistical maps. The relative contents of the compounds were analyzed

using MultiQuant software, and differences in metabolites between the groups were determined by OPLS-DA. Enrichment analysis of metabolic pathways (MPEA) was performed using MetOrigin [65]. O2PLS analysis was performed using OmicShare Tools (https://www.omicshare.com/tools/Home/Soft/o2pls). Relationship analysis was conducted using Pearson's correlation.

Abbreviations

ADG	Average daily gain
BW	Body weight
DM	Dry matter
DMI	Dry matter intake
F/G	Feed/gain ratio
FC	Fold change
FI	Feed intake
GC-MS	Gas Chromatography-Mass Spectrometer
KEGG	Kyoto Encyclopedia of Genes and Genomes
MWDB	MetWare database
Neg	Net energy for gain
NEm	Net energy for maintenance
OEO	Oregano essential oil
ORFs	Open reading frames
TDN	Total digestible nutrient
TMR	Total mixed ration
VFA	Volatile fatty acids
VIP	Variable importance in projection

Supplementary Information

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Supplementary Material 1

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Author contributions

Y. Huang and Z. Lei: Writing– review & editing, Writing– original draft, Visualization, Validation, Methodology, Investigation, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. S. Cheng, J. Shi and P. He: Writing– review & editing, Validation, Methodology, Investigation, Data curation, Conceptualization. Y. Ma, R. Yang, X. Zhang and Y. Cao: Writing– review & editing, Project administration, Methodology, Validation, Supervision, Resources. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethic approval

All experiments were approved by the Animal Care Committee of Gansu Agricultural University (730070, Lanzhou, China) under approval number GSAU-Eth-AST-2022-035 and conducted in accordance with the regulations and guidelines established by the committee.

Competing interests

The authors declare no competing interests.

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