Breed Differences in the Expression Levels of gga-miR-222a in Laying Hens Influenced H_2S Production by Regulating Methionine Synthase Genes in Gut Bacteria

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Research

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Abstract

Background: The microbiota in the cecum of laying hens is critical for substance metabolism and odor gas production. Recent studies have suggested that host miRNAs can regulate gene expression in the gut microbiota. The expression profiles of host-derived miRNAs in the cecal content of two laying hen breeds, Hy-line Gray and Lohmann Pink, which have dissimilar H_2S production, were characterized, and their possible effects on H_2S production by regulating the expression of related genes in the microbiota were demonstrated.

Results: The differential expression of microbial serine O-acetyltransferase, methionine synthase, aspartate aminotransferase, methionine-gamma-lyase and adenylylsulfate kinase between the two breeds resulted in lower H_2S production in the Hy-line hens. The results also demonstrated miRNA microvesicles in the cecal content of laying hens and found potential miRNA-target relationships between 9 differentially expressed miRNAs and 9 differentially expressed microbial genes related to H_2S production, among which gga-miR-222a targeted two methionine synthase genes, *Odosp_3416* and *BF9343_2953*. An *in vitro* fermentation experiment showed that gga-miR-222a upregulated the expression of these genes, which increased methionine concentrations but decreased H_2S production and soluble sulfide concentrations, indicating the potential of host-derived gga-miR-222a to reduce H_2S emission in laying hens.

Conclusion: These findings identify both a physiologic role by which miRNA shapes the cecal microbiota of laying hens and a strategy to use host miRNAs to manipulate the microbiome and actively expressed key microbial genes to reduce H_2S emission and breed environmentally friendly laying hens.

Background

The laying hen industry is an important livestock sector that produces eggs as a major protein sources for human consumption. Nutrient digestion in laying hens is characterized by inadequate enzymatic hydrolysis in the foregut, followed by further microbial fermentation in the cecum. An increasing number of studies have proven that this ‘bacterial organ’ plays a vital role in host metabolism, immunity and disease [1–3]. Bacteria ferment undigested feed components to generate volatile fatty acids (VFAs), amino acids, ammonia (NH_3), hydrogen sulfide (H_2S) and other metabolites [4, 5]. Furthermore, NH_3 and H_2S are the two main odorous gases in poultry houses and represent a great loss in nutrients and environmental pollution that is a public concern [6, 7]. NH_3 accounts for the largest proportion of odor gas in livestock and poultry facilities. In addition, H_2S is also a predominant compound because its concentration is second to that of NH_3, but the odor thresholds of it is significantly lower than NH_3 which also contributes to odor in the farm environment [8, 9]. In addition to its adverse effects on air quality, H_2S has also been reported to cause intestinal diseases [10]. Therefore, reducing H_2S besides mitigating the environmental problems will also have a positive effect on the health of animals. Several recent studies have explored nutritional manipulations; for example, probiotic inclusion or protein reduction in the diet

have been used to regulate the gut microbiota to mitigate H_2S emission in animals [11, 12]. However, there are a few disadvantages to nutritional manipulations. For instance, the supplementation of probiotics must be continuous to guarantee the sustained reduction effect of H_2S emission. In view of this, breeding laying hens with a low-H_2S emission “cecal microbiota structure” could be a better and permanent measure for H_2S reduction and environmentally friendly culture in the poultry industry. However, the first key point in breeding low-H_2S emission laying hens is to understand the regulatory relationship between the host and its cecal microbiota.

Numerous factors influence the composition and function of the gut microbiota. For instance, the composition of the gut microbiota is shaped by the host’s genetic background and to some extent can be transiently altered by diet, the environment and disease states [13, 14]. MicroRNAs (miRNAs) are a group of noncoding RNAs of ~ 22 nucleotides (nt), that are known for their sequence-specific regulatory function that operates by targeting the 3' untranslated region of mRNAs in the cytoplasm [15]. Increasing evidence has demonstrated that miRNAs also exist extracellularly and circulate in body fluids in the form of exosomes and microvesicles. Secreted miRNAs have been isolated from blood, milk and even stool and urine [16–20]. Some studies have characterized miRNAs as potential markers of tumorigenesis in human stool [21–23]. Interestingly, recent studies have also revealed that host-derived miRNAs could serve as an important crosstalk channel between the host and the intestinal bacterial population. Liu et al. (2016) found that the host could modulate the gut microbiota through intestinal epithelial cell-secreted miRNAs, which enter gut bacteria and directly regulate bacterial gene expression. Other studies reported that plant-derived miRNAs could be taken up by gut bacteria and shape the gut microbiota [24, 25]. Therefore, we hypothesize that there might be cross-regulation between host-derived miRNAs and the cecal microbiota in laying hens. In addition, whether the host-derived miRNAs regulate microbiota abundance or the expression levels of bacterial function genes, which influence the structure of the microbiota and the metabolism function, and finally lead to H_2S production differences in different breeds of laying hens, is still unknown.

In our previous study, we found a significantly higher daily H_2S production in Lohmann laying hens than Hy-line Gray laying hens (the daily H_2S production per kg average daily feed intake was 7.75 and 4.17 mg for Lohmann and Hy-line, respectively) [26]. However, whether the H_2S emission difference between the two breeds of laying hens was due to host miRNA regulation of the gut microbiota requires further investigation. Therefore, we determined the expression profiles of host-derived miRNAs in the cecal content of these two breeds of laying hens to find out the significantly different miRNAs between the two breeds of laying hens, and then predicted the target relationships between differentially expressed miRNAs and microbial genes related to H_2S production. Finally, the effect of selected targeted miRNA on H_2S production in laying hens was verified by an *in vitro* experiment. This work may unveil the interkingdom regulation relationships among miRNAs, cecal microbiota and H_2S production in laying hens, which provide a reference for the breeding of environmentally friendly laying hens. At the same

time, miRNAs, which have been proven to regulate the production of H_2S in the cecum of laying hens, could be used as a safe and clean additive for H_2S emission reduction in the future.

Results

Identification of miRNA profiles in the cecum of laying hens

The morphology of exosomes derived from laying hen cecal content was observed using TEM. Microvesicle-sized (approximately 100–200 nm in diameter) extracellular vesicles were present in the cecal content of Lohmann and Hy-line hens, but the morphological character of microvesicles was mostly the same in the two breeds (Fig. 2A and B). After high-throughput sequencing, we found 288 known miRNAs could be annotated on the miRBase online website, with gga-miR-7, gga-miR-21-5p, gga-miR-215-5p, gga-miR-26a-2-5p, gga-miR-26a-5p, gga-miR-200a-3p, gga-miR-194, gga-let-7a-5p, gga-let-7j-5p and gga-miR-148a-3p being the most abundant miRNAs. The information for all miRNAs is listed in Table S3. Of all the miRNAs, 10 were significantly differentially expressed between the two breeds: four miRNAs were expressed significantly higher, and six miRNAs were expressed lower in the Hy-line than in the Lohmann ($P < 0.05$) (Fig. 2C). The KEGG pathway annotations of target genes in the chicken genome of these 10 significantly expressed miRNAs are shown in Fig. S1. The target genes were mostly enriched in Metabolic pathways, Neuroactive ligand-receptor interaction, Focal adhesion, Endocytosis and Purine metabolism.

The expression of microbial genes related to H_2S production

After the annotation of microbial transcripts to KEGG pathways, we focused on two metabolism pathways involved in H_2S production, the cysteine and methionine metabolism pathway (map 00270) and the sulfur metabolism pathway (map 00920). The degradation of cysteine and methionine could result in the production of H_2S. As shown in Fig. 3A, L-cysteine is synthesized from L-serine and sulfide under the action of serine O-acetyltransferase and cysteine synthase, or from L-cystathionine under the action of cystathionine gamma-lyase. The degradation of cysteine finally forms pyruvate with the production of sulfite by aspartate aminotransferase, and sulfite can participate in sulfur metabolism to produce H_2S. The degradation of methionine by methionine-gamma-lyase can produce methanethiol (Fig. 3B), which can be converted into H_2S in subsequent processes. Another pathway for methionine degradation is the formation of S-adenosyl-L-methionine by S-adenosylmethionine synthetase without H_2S production. The synthesis of methionine includes two pathways: one pathway, from 4-methylthio-2-oxobutanoate, is catalyzed by aromatic-amino-acid transaminase. The other way is from L-cystathionine catalyzed by cystathionine beta-lyase and methionine synthase.

Comparative analysis revealed that the expression of serine O-acetyltransferase (cysE, EC: 2.3.1.30) and methionine synthase (metH, EC:2.1.1.13) in Hy-line hens was higher than that in Lohmann hens, but the expression of aspartate aminotransferase (aspB, EC:2.6.1.1) and methionine-gamma-lyase (MGL, EC:4.4.1.11) in Hy-line hens was lower than that in Lohmann hens ($P < 0.05$), indicating that there was a higher tendency for synthesis of cysteine and methionine but a lower tendency for their degradation and H_2S production in the cecum of Hy-line hens (Fig. 3D).

Sulfur metabolism is highly important pathway in the cecum of hens with H_2S being a key metabolite. The assimilatory reduction and dissimilatory reduction of sulfate lead to the production of sulfite and its eventual conversion into sulfide (Fig. 3C). We found a significantly lower expression of adenylylsulfate kinase (cysC, EC: 2.7.1.25) in the cecum of Hy-line hens ($P < 0.05$), which indicated a lower sulfide production there than in Lohmann hens (Fig. 3E).

In the next step, we explored the differentially expressed genes and their source bacteria of these five differentially expressed enzymes in the above two metabolism pathways and found 13 microbial genes differentially expressed between the two breeds and mostly expressed by the genus *Bacteroides* (Table 1).

Table 1
Differentially expressed genes (DEGs) and source bacteria of differentially expressed enzymes

Enzymes	DEGs(log_2 Fold change)	Source bacteria
cysE	*Ddes_0279* (6.35)	*Desulfovibrio desulfuricans*
	CK3_01000 (5.31)	Unclassified *Clostridiales*
	SELR_06150 (4.94)	*Selenomonas ruminantium*
	Bache_0784 (2.36)	*Bacteroides helcogenes*
metH	*Odosp_3416* (8.48)	*Odoribacter splanchnicus*
	Bacsa_0021 (5.67)	*Bacteroides salanitronis*
	BF9343_2953 (5.31)	*Bacteroides fragilis* NCTC9343
aspB	*BVU_0144* (-5.37)	*Bacteroides vulgatus*
	Bache_2087 (-4.20)	*Bacteroides helcogenes*
	PRU_1300 (-3.66)	*Prevotella ruminicola*
	OBV_25710 (-3.27)	*Oscillibacter valericigenes*
MGL	*GFO_2175* (-5.68)	*Gramella forsetii*
cysC	*Mmc1_2549* (-4.76)	*Magnetococcus marinus*

Target prediction of miRNAs

In total, we found that 9 differentially expressed miRNAs had target sites with 9 differentially expressed genes related to H_2S production by miRanda (Fig. 4). In particularly, we found that gga-miR-222a had a target site associated with two methionine synthase genes, *Odosp_3416* (expressed by the bacterium *Odoribacter splanchnicus*) and *BF9343_2953* (expressed by the bacterium *Bacteroides fragilis* NCTC 9343) (Fig. 5). Considering the high expression levels of gga-miR-222a, *Odosp_3416* (read counts = 138 and 225 for Hy-line and Lohmann) and *BF9343_2953* (read counts = 75 and 137), we suspected that the regulatory effect between them may be notable. Therefore, it was used as a miRNA-mRNA target for subsequent verification.

H_2S production in the fermentation experiment

After 24 h of fermentation, the total gas production by the Lohmann blank group was significantly higher than that by the Hy-line blank group ($P < 0.05$).

Furthermore, the total gas production of the gga-miR-222a treatment group in Lohmann treatment group was significantly lower than that of the Lohmann blank and control groups ($P < 0.05$), but this production did not differ among Hy-line groups (Fig. 6A). Less H_2S was produced in Hy-line hens than Lohmann hens ($P < 0.05$) (Fig. 6B). H_2S production in the Lohmann and Hy-line hens in the gga-miR-222a treatment groups was significantly lower than that in the blank and control groups ($P < 0.05$). Compared to the blank group, the amount of H2S produced by the Lohmann and Hy-line hens treated with gga-miR-222a was 22.88% and 26.33% lower, respectively.

The chemical indexes in the fermentation broth of each group are shown in Table 2. The concentration of soluble sulfide for the Hy-line samples was significantly lower than that for the Lohmann samples ($P < 0.05$), while the concentration of butyric acid in the Hy-line was significantly higher than that in Lohmann samples ($P < 0.05$). The concentration of soluble sulfide of the gga-miR-222a treatment group in Hy-line and Lohmann hens was significantly lower than those in the corresponding blank groups and control groups ($P < 0.05$), but the concentration of methionine of gga-miR-222a treatment group in Hy-line and Lohmann was significantly higher than those in the blank groups and control groups ($P < 0.05$).

Table 2

The comparison of fermentation incubation indexes [1]

Items	Ln	Lc	Lt	Hn	Hc	Ht
pH	7.57 ± 0.04	7.49 ± 0.02	7.61 ± 0.05	7.59 ± 0.01	7.55 ± 0.03	7.48 ± 0.03
S^{2-}, μg/g	15.06 ± 0.54^{a}	14.83 ± 0.65^{a}	12.50 ± 0.29^{b}	12.32 ± 0.57^{b}	12.99 ± 0.92^{ab}	9.84 ± 0.83^{c}
SO_4^{2-}, mg/g	256.94 ± 17.28	268.52 ± 35.37	221.30 ± 45.63	257.87 ± 49.42	287.96 ± 26.78	243.98 ± 40.90
Acetate, mmol/L	31.53 ± 2.00	30.19 ± 0.43	32.81 ± 1.46	32.45 ± 0.92	34.63 ± 1.00	34.15 ± 1.21
Propionate, mmol/L	15.51 ± 0.77	15.34 ± 1.52	15.21 ± 0.60	16.00 ± 0.50	16.58 ± 0.41	15.59 ± 0.33
Butyrate, mmol/L	8.33 ± 0.27^{c}	8.16 ± 0.25^{c}	9.10 ± 0.33^{bc}	10.81 ± 0.72^{a}	10.67 ± 0.55^{a}	10.17 ± 0.27^{ab}
Total VFAs, mmol/L	55.37 ± 3.00	53.69 ± 1.43	57.11 ± 2.36	59.26 ± 1.47	61.88 ± 0.89	59.91 ± 1.66
Methionine, μg/mL	246.21 ± 9.41^{c}	245.88 ± 7.51^{c}	280.39 ± 2.89^{ab}	257.70 ± 9.65^{bc}	258.92 ± 6.04^{bc}	299.12 ± 1.84^{a}

[1] Data are presented as means with their standard errors.

[a, b] Means within a row with different superscripts differ ($P < 0.05$).

Bacterial abundance and gene expression in fermentation broth

It was predicted that the methionine synthetase genes *Odosp_3416* (expressed by the bacterium *Odoribacter splanchnicus*) and *BF9343_2953* (expressed by the bacterium *Bacteroides fragilis* NCTC 9343) might be the target genes of gga-miR-222a. The abundances of *Odoribacter splanchnicus* and *Bacteroides fragilis* NCTC 9343 and the expression of the genes *Odosp_3416* and *BF9343_2953* were quantified to explore the effect of gga-miR-222a on bacterial abundance and gene expression (Fig. 7). The abundances of the two bacteria did not differ between the two breeds ($P>0.05$). After gga-miR-222a treatment, the abundances of the two bacteria increased slightly, but the difference was not significant ($P>0.05$). Meanwhile, the expression of the genes *Odosp_3416* and *BF9343_2953* in the Hy-line hens was significantly higher than that in the Lohmann hens ($P<0.05$). After gga-miR-222a treatment, the expression of these two genes was significantly increased in both breeds ($P<0.05$). The results showed that gga-miR-222a did not significantly affect the abundances of target gene related bacteria in the cecum of laying hens but could upregulate the expression of the target genes.

The effects of gga-miR-222a on *Bacteroides fragilis* NCTC9343

At 8 h, the bacterium *Bacteroides fragilis* NCTC9343 entered the logarithmic phase, and at 10 h, the strain entered the stable phase. gga-miR-222a significantly improved the abundance of *Bacteroides fragilis* NCTC9343 at 10 h (P<0.05) (Fig. 8A). In addition, gga-miR-222a also upregulated the expression of the *BF9343_2953* gene and increased the concentration of methionine in the medium (P<0.05). (Fig. 8B and C). To determine whether gga-miR-222a could be taken up by *Bacteroides fragilis* NCTC9343 and then play a series of regulatory functions inside the cell, we measured bacterial internalization of gga-miR-222a by *in situ* hybridization and TEM, and found that exogenous gga-miR-222a was selectively absorbed by *Bacteroides fragilis* NCTC9343 (Fig. 9).

Discussion

Although there is a known association among host genetic background, cecal microbiota structure and odor production by laying hens [27, 28], the potential mediators of this relationship remain unclear. Recently, some findings demonstrated that mammalian secreted miRNAs could regulate the expression of bacterial genes [29, 30]. Here, we presented the first insight into the characterization of miRNAs derived from the cecal content of laying hens and found that gga-miR-222a could reduce the production of H_2S by regulating the expression of cecal microbial methionine synthetase genes in the cecum of laying hens.

Differential expression of cecal microbial genes led to dissimilar H_2S production between the two breeds

In a previous study, we found that Hy-line hens exhibited lower H_2S production than Lohmann hens as a result of different microbiota structures related to H_2S production in the cecum [26]. However, due to the limitation of 16S rRNA sequencing, we did not annotate and identify pathways and genes related to bacterial sulfur metabolism. Transcriptomic sequencing can be more accurate than other methods to elucidating the functional makeup of a microbial community and allowing us to characterize potential miRNA interactions across the microbiome and transcriptome. The gene expression of the cecal microbiota was characterized for H_2S production related pathways using the metatranscriptome. The synthesis of cysteine and methionine requires the participation of sulfur, and related decomposition is accompanied by the release of sulfur [31]. Higher expression of serine O-acetyltransferase and methionine synthase but lower expression of aspartate aminotransferase and methionine-gamma-lyase in the Hy-line cecal microbiota community indicated that the Hy-line hens had a stronger ability to utilize sulfur for the synthesis of cysteine and methionine than the Lohmann hens.

Dissimilatory sulfate reduction is the exclusive sulfate reduction pathway for most sulfate-reducing bacteria (SRB), but assimilatory sulfate reduction can be carried out by most bacteria in the gut [32, 33]. The metatranscriptome showed that there was no significant difference in the expression of dissimilatory sulfate reduction pathway related genes. This may be because the abundance of SRB was low in the animal gut (approximately 0.028–0.097%) [12]. Low abundances of gut SRB led to a low and unobvious

differential expression of related genes. However, for the assimilatory sulfate reduction pathway, the gene expression of adenylsulfate kinase in the Hy-line hens was significantly lower than that in the Lohmann hens, indicating a more powerful transformation of sulfate to sulfide in the latter case.

Here, we found that the differentially expressed microbial genes in sulfur related metabolism pathways were the reason for dissimilar H_2S production between the Lohmann and Hy-line hens, but whether the host specifically regulates microbial genes by some cross-regulation factors is not clear. In this study, we identified cecal miRNAs and found that they could directly regulate specific bacterial gene expression and affect gut microbial growth to affect H_2S production in laying hens.

The miRNAs in the cecal content differed between the two breeds

miRNAs have not been previously characterized in the cecal content of laying hens. First, we demonstrated that microvesicles existed in the cecal content of Lohmann and Hy-line hens, but only 288 known miRNAs were sequenced. Owing to the bacterial RNA sequence accounted for the main proportion of total RNA in the cecal content of laying hens and some miRNAs may be degraded by the high temperature and high uric acid cecum environment [34], the number of types and abundances of sequenced miRNAs were relatively low. Only 10 miRNAs were differentially expressed between the Lohmann and Hy-line hens. The highly conserved and homologous characteristics of miRNAs may lead to a high similarity in miRNA types and abundances between two breeds [35]. Most of the chicken genome targets of these miRNAs were enriched in Metabolic pathways, Neuroactive ligand-receptor interaction, Focal adhesion, Endocytosis and Purine metabolism, but were not enriched in pathways related to cancer occurrence and disease formation, indicating that these miRNAs did not have a potentially negative effect on the host's normal life activities. This means that the application of these miRNAs to odor reduction may be harmless to the host itself.

Host-derived miRNAs targeted the genes of the cecal microbiota of laying hens

miRNA binds with mRNA to perform its regulatory functions. We predicted the possible target relationships between differentially expressed miRNAs and differentially expressed genes related to H_2S production. It was found that gga-miR-222a had a target relationship with the methionine synthetase genes *Odosp_3416* and *BF9343_2953* (expressed by *Odoribacter splanchnicus* and *Bacteroides fragilis* NCTC 9343, respectively). Therefore, gga-miR-222a may be a host regulator that affect H_2S emission in laying hens by regulating the production of methionine, a sulfur-containing amino acid. *In vitro* fermentation experiment and bacterial culture showed that gga-miR-222a could upregulate the expression of the genes *Odosp_3416* and *BF9343_2953*, and increase the abundance of *Bacteroides fragilis* NCTC 9343 in the logarithmic growth period (10 h), which resulted in a higher concentration of methionine but lower H_2S production and soluble sulfide concentration in fermentation broth and bacterial medium. The concentrations of gut soluble sulfide are positively correlated with the release of H_2S [36]. The decrease H_2S production and soluble sulfide concentration showed that gga-miR-222a reduced H_2S emission in laying hens.

The host-derived miRNA gga-miR-222a influenced H_2S emission in laying hens

Interestingly, we found that gga-miR-222a played a positive role in regulating the expression of the *Odosp_3416* and *BF9343_2953* genes, which was different from the results of most studies, which suggest that miRNA always inhibits the transcription of mRNA or directly degrades the sequence of the mRNA after binding with mRNA [37, 38]. However, some studies have shown that miRNA not always played a negative regulation on mRNA [24, 25, 39]. How miRNA regulates the expression of genes and affects bacterial growth may rely on the function of the genes targeted by the miRNA and the site at which miRNA binds mRNA. Binding between miRNA and bacterial transcripts of 16S rRNA, yegH, RNaseP and β-galactosidase genes upregulates the expression of these genes and promotes the growth of bacteria [29, 30]. In this study, we found that gga-miR-222a played a similar role in the regulation of methionine synthetase gene expression and promoted the abundance of bacteria in bacterial medium, especially in the logarithmic phase. After *in situ* hybridization, we found that exogenous gga-miR-222a could be selectively up-taken by *Bacteroides fragilis* NCTC 9343, indicating an intracellular cross-regulation role of gga-miR-222a. However, the increase of bacteria abundance in fermentation broth was not significant except for a slight rise after gga-miR-222a treatment. The reason for this discrepancy may be that the intestinal environment is more complex and there are many interfering factors, such as interactions among various microorganisms. This was the why conducting bacterial growth experiment in a pure culture environment was necessary.

Conclusions

In conclusion, this study found host-derived miRNAs in the cecum of laying hens for the first time and the expression profiles of miRNAs were different between different breeds. It was also demonstrated that gga-miR-222a regulated the expression of H_2S production related genes (*Odosp_3416* and *BF9343_2953*) to affect the production of H_2S in laying hens. Meanwhile, gga-miR-222a could enter *Bacteroides fragilis* NCTC 9343, which increased its abundance in the logarithmic period. Therefore, different profiles of host-derived miRNAs in different breeds of laying hens could affect the production of H_2S by regulating the gene expression of H_2S production related bacteria. Regulation of H_2S production in the cecum of laying hens by host miRNAs such as gga-miR-222a provides the possibility that if these miRNAs could be incorporated into the breeding of laying hens, they could provide a certain reference value for the selection of low odor yield and environmentally friendly laying hen breeds.

Methods

Animals and feeding

Approximately one hundred Hy-line Gray laying hens and one hundred Lohmann Pink laying hens were hatched and fed together at a local hatchery. To eliminate the confounding effects that might be caused by diet, age, weight and feeding environment. Thirty Hy-line Gray laying hens and thirty Lohmann Pink

laying hens at 28 weeks of age with similar weights (1.70 ± 0.02 and 1.71 ± 0.02, respectively for Hy-line and Lohmann) were selected and moved into twelve respiration chambers in an environmentally controlled room for a daily H_2S production measurement for the two breeds [26]. A commercial-type laying hen diet was fed to birds *ad libitum*, water was available at all times (Table S1), and a 12-h light cycle at 24 °C room temperature management schedule was used. At the end of the experiment, all birds were euthanized by cervical dislocation, and then the cecum was ligated at both sides and removed from the gastrointestinal tract. The contents were aseptically collected into an Eppendorf tube containing Bacterial Protect RNA reagent (Qiagen, Hilden, Germany) at an approximate 1:1 ratio (w/v), and immediately frozen at -80 °C until analysis.

Animal ethics statement

All animal experiments were approved by the Animal Experimental Committee of South China Agricultural University (SYXK2014-0136). All experimental steps were performed to decrease animal suffering as much as possible. After the experiment, the bodies of laying hens were incinerated.

The determination of exosomes in the cecum contents

Cecal contents from laying hens were suspended in PBS to 30 mg/ml, spun down at 10,000 ×g for 5 min to remove debris and then filtered through a 0.2 μm filter and the filtrates were observed by Thermo Fisher Talos L120C transmission electron microscope (Thermo Fisher Scientific, MA, US).

Extraction and analysis of miRNA in the cecum of laying hens

Total miRNA was extracted with the mirVana™ miRNA Isolation Kit (Austin, TX, USA) according to Liu et al. (2016). Briefly, approximately 100 mg cecal content was mixed adequately with 600 μL 1×DPBS, and the mixture was left at room temperature for 30 min, and then mashed to complete suspension. Then 600 μL acid-phenol: chloroform was added, and the samples were vortexed for 60 sec and then centrifuged for 15 min at 10,000 ×g to separate organic phases. The aqueous phase was recovered, and 1.25 volumes of 100% ethanol was added to the aqueous phase for final miRNA isolation. For each sample, a filter cartridge was placed into one of the collection tubes (supplied by the kit), and the sample was pipetted onto a filter and centrifuged for 90 sec at 10,000 ×g, and then the flow-through was discarded. The filter was washed with 700 μL miRNA Wash Solution 1 and then washed three time with 700/500/250 μL Wash Solution 2/3 (supplied by the kit). Finally, the filter was transferred into a fresh collection tube, and 50 μL nuclease-free water was applied to the center of the filter. The filter was incubated at room temperature for 10 min, centrifuged for 5 min at 8000 ×g to recover miRNA and then stored it at -80℃. Pooled miRNA was prepared by combining equal amounts of extracted miRNA from five birds of the same breed, which means that each breed was represented by six pooled miRNA

samples. miRNA libraries were constructed according to the TruSeq Small RNA Sample Preparation protocol. The raw sequence reads were obtained with an Illumina HiSeq^{TM} 2500 instrument (Illumina, San Diego, USA). FastQC was applied to obtain clean reads from the raw data by removing the joint sequences, low-quality fragments, and sequences <18 nucleotides (nt) in length. miRDeep2 was used to align the clean sequences to the miRBase database sequences (http://www.mirbase.org/).

The extraction and analysis of RNA of cecal microbiota

Cecal content aliquots (200 mg) were used for RNA extraction with the RNeasy® PowerMicrobiome Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The integrity and quantity of extracted RNA was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). Pooled RNA was prepared by combining equal amounts of extracted RNA from five birds of the same breed; thus, each breed was represented by six pooled RNA samples. RNA was subjected to standard Illumina library preparation with the TruSeq RNA Sample Prep Kit (Illumina, San Diego, USA), and rRNA was depleted with the Ribo-ZeroTM rRNA Removal Kit (Epicenter Biotechnologies, Madison, WI). Sequencing was performed on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, USA). Sequences were quality filtered and poor-quality bases of raw reads were removed by using Cutadapt (v1.9.1) software. A 10 bp window was moved across each sequence, and nucleotides in windows with a mean quality score < 20 were removed; reads with "N" bases (>10%) and lengths below 75 bp were discarded; primer sequences and adaptor sequences were also removed. Next, rRNA, tRNA and host reads were filtered using BWA (v 0.7.5). Putative mRNA reads were then assembled using the Trinity (v2.1.1) de novo assembler. Gene annotation was performed by searching against a protein non-redundant database (NR database), and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was conducted for gene function classification. After the comparation of transcript profiles between the two breeds, we focused on three pathways related to H_2S production, including cysteine and methionine metabolism, sulfur metabolism and butyrate metabolism, and the expression of microbial genes in these pathways between the two breeds was compared.

Target prediction of differentially expressed miRNAs

After the exploration of miRNA profiles in the cecal content of laying hens, significantly differentially expressed miRNAs between the two breeds were used for target prediction analysis for microbial significantly differentially expressed genes related to H_2S production. Bacterial mRNAs potentially targeted by miRNAs were identified by miRanda (http://www.microrna.org). Furthermore, a prediction of host genome genes targeted by of 10 differentially expressed miRNAs was also conducted by miRanda, and all the target genes were determined to be enriched by KEGG analysis.

In vitro fermentation experiment

Thirty Hy-line Gray and thirty Lohmann Pink laying hens at 28 weeks of age were sacrificed, and the ceca were ligated immediately. The cecal contents in the same breed group were pooled, thoroughly mixed and diluted in sodium and ammonia bicarbonate buffer solution (35.0 g $NaHCO_3$+4.0 g NH_4HCO_3 for a 1 L volume) at a ratio of 1:3 (W/V) [40]. The intestinal content–buffer mixture was blended for 60 sec in a blender after which the solution was squeezed through four layers of surgical gauze, and then mixed with a buffer mineral solution at a 1:2 ratio (V/V) at 40℃ under continuous flushing with CO_2. A corn-soybean basal laying hen diet was used as a substrate for fermentation.

Approximately 10 mL of the inoculum was added to a 100 mL gas syringe with 0.2 g of the substrate. Six groups with different treatments were designed, including the blank group (10 mL inoculum+ 0.2 g substrate+ 1 mL pure water), control group (10 mL inoculum+ 0.2 g substrate+ 1 mL control mimic at a final concentration of 2 μM) and treatment group (10 mL inoculum+ 0.2 g substrate+ 1 mL gga-miR-222a mimic at a final concentration of 2 μM) for each of the types of hens, Hy-line and Lohmann (Fig. 1). The miRNAs were supplied by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). After removing the air from the head-space of the syringe, the syringe was sealed with a clip and placed in a 42°C incubator, where it was rotated at 60 rpm for 24 h. At the end of incubation, the syringes were put into an ice box to stop further fermentation, the volume of gas produced in the head-space was recorded and the gas was transferred by syringes into a gas collection bag for H_2S analysis. Ten milliliters of fermentation broth was sampled and stored at -80℃ for chemical analysis. The quantity of H_2S in the syringes and the concentrations of soluble sulfide (S^{2-}) in the fermentation broth were determined using the methylene-blue colorimetric method. The pH value was determined using a pH meter (INESA Scientific Instrument, Shanghai, China). The concentration of sulfate radicals (SO_4^{2-}) was determined using the turbidimetric method. The concentrations of VFAs were determined using high-performance liquid chromatography. The concentrations of methionine in fermentation broth were detected by an automatic amino acid analyzer (Sykam, Munich, Germany).

Bacterial abundance and gene expression in fermentation broth

DNA was extracted from fermentation broth by using a QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The DNA was used to quantify the abundance of *Odoribacter splanchnicus* and *Bacteroides fragilis* NCTC 9343. RNA was extracted from fermentation broth using the RNeasy® PowerMicrobiome™ Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted mRNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit (TaKaRa, Kusatsu, Japan). The cDNA was used to quantify the expression of *Odosp_3416* and *BF9343_2953.* The primers were designed using the NCBI website with the total bacterial 16S rRNA gene as the reference gene (Table S2).

In vitro bacterial growth measurements

To further verify the effect of gga-mir-222a on the growth and metabolism of bacteria in a pure culture environment and considering that *Odoribacter splanchnicus* could not be cultured *in vitro*, we selected *Bacteroides fragilis* NCTC9343 cultured in anaerobic medium to investigate the effect of gga-miR-222a on the growth and metabolism of bacteria. The anaerobic bacterium *Bacteroides fragilis* NCTC9343 were cultured at 37ºC by inoculating 40 mL aliquots of anaerobic basal medium (Becton Dickinson and Company, Lincoln Park, USA) and then grown anaerobically in an anaerobic chamber. gga-miR-222a and the control mimic were supplied in the culture at a concentration of 2 μM. (RiboBio, Guangzhou, China). Growth was monitored as absorbance at 600 nm once per hour for up to 24 h with a spectrophotometer. The cultured bacterial cells were collected at 10 h and used for *BF9343_2953* gene expression measurement with the *Bacteroides fragilis* 16S rRNA gene as the reference gene. The concentrations of methionine in culture medium at 10 h were detected by an automatic amino acid analyzer (Sykam, Munich, Germany).

In situ hybridization detection of the uptake of gga-miR-222a

The bacterial cells of *Bacteroides fragilis* NCTC9343 were centrifuged at 12,000 ×g and washed twice with ice cold PBS. Then, the cells were fixed in 4% PFA/0.25% glutaraldehyde. A 5'-DIG and 3'-DIG dual labeled probe for gga-miR-222a was used for *in situ* hybridization. The detection of the uptake of gga-miR-222a by bacteria was imaged using a Thermo Fisher Talos L120C transmission electron microscope Thermo (Fisher Scientific, MA, US).

Statistical analysis

Comparisons among groups were performed using SPSS 22.0 for statistical analysis. Data are presented as the means with standard errors of the mean. Differences were considered significant if $P<0.05$.

Abbreviations

H_2S: Hydrogen sulfide; SO_4^{2-}: Sulfate radical; S^{2-}: Soluble sulfide; SRB: Sulfate-reducing bacteria; VFAs: Volatile fatty acids.

Declarations

Acknowledgments

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Authors' contributions

CH conducted experiment and manuscript writing. CH, SX, and XL involved in acquisition of funding, experimental design and review of the manuscript. JC and YY did sample collection. SX, JM, YW and YBW did manuscript correction. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol for this experiment was approved by the Animal Experimental Committee of South China Agricultural University (SYXK2014-0136).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

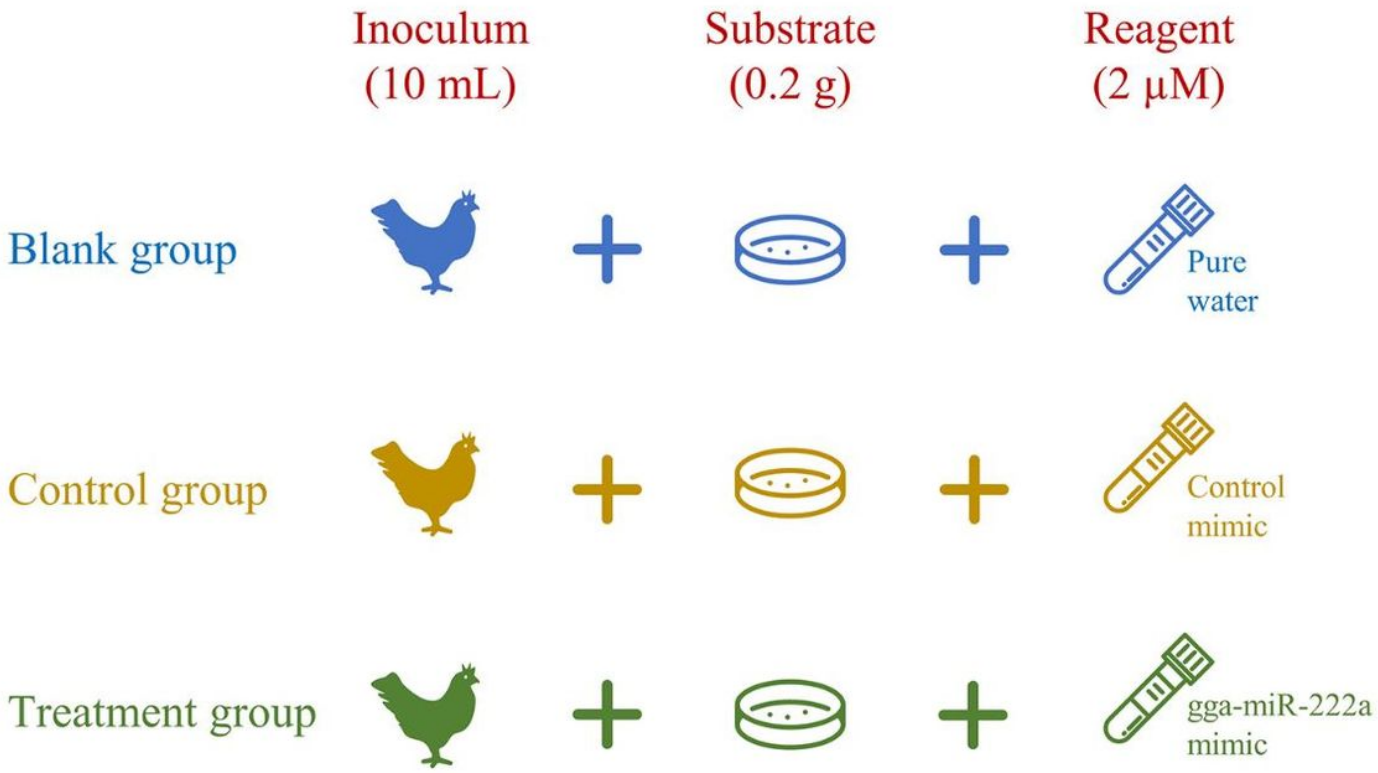

Figure 1

Division of experimental groups.

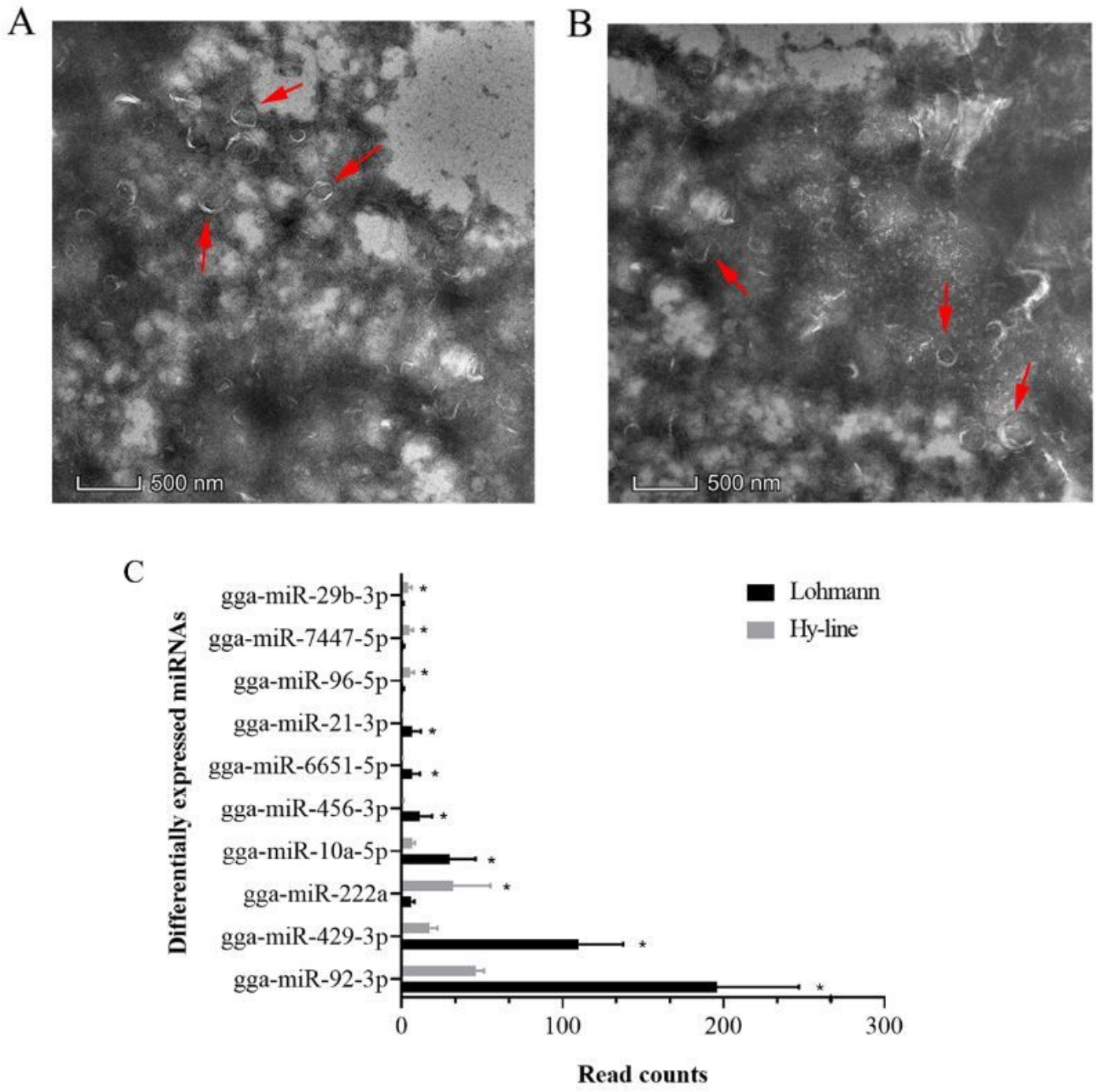

Figure 2

Morphological characteristics of microvesicles and significantly expressed miRNAs. (A) and (B) Morphological characteristics of microvesicles in Lohmann cecal content (A) and in Hy-line cecal content (B). Red arrows point to microvesicles. (C) differentially expressed miRNAs between the two breeds. * indicates a significant difference in miRNA between the two breeds ($P<0.05$).

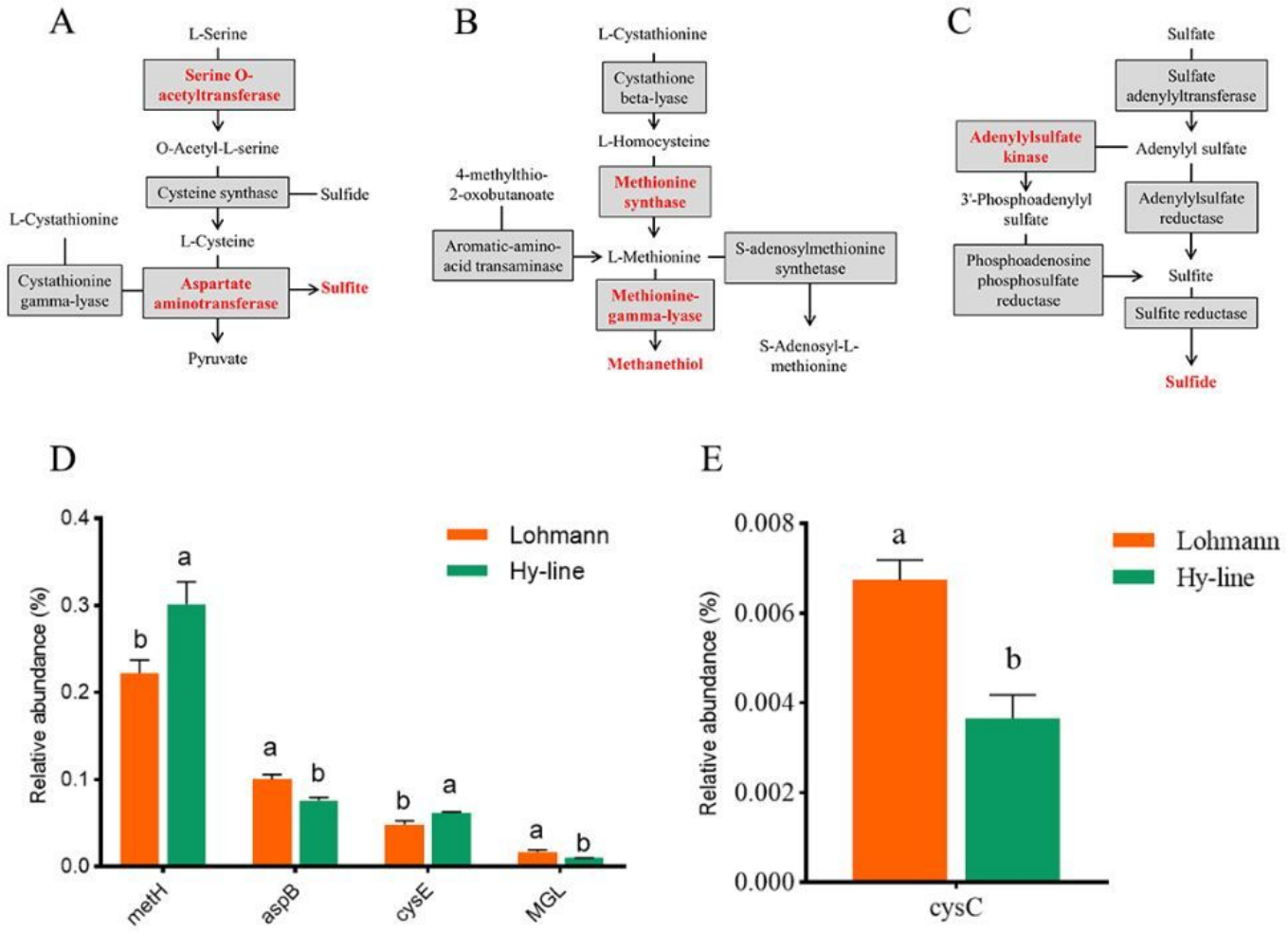

Figure 3

Expression of enzymes related to the cysteine and methionine metabolism and sulfate metabolism pathways. (A) and (B) Related enzymes (boxes) and intermediates in the cysteine and methionine metabolism pathway. (C) Related enzymes (boxes) and intermediates in the sulfate metabolism pathway. (D) and (E) Differentially expressed enzymes are marked in red in metabolic pathways. Different letters in different groups indicate a significant difference ($P<0.05$).

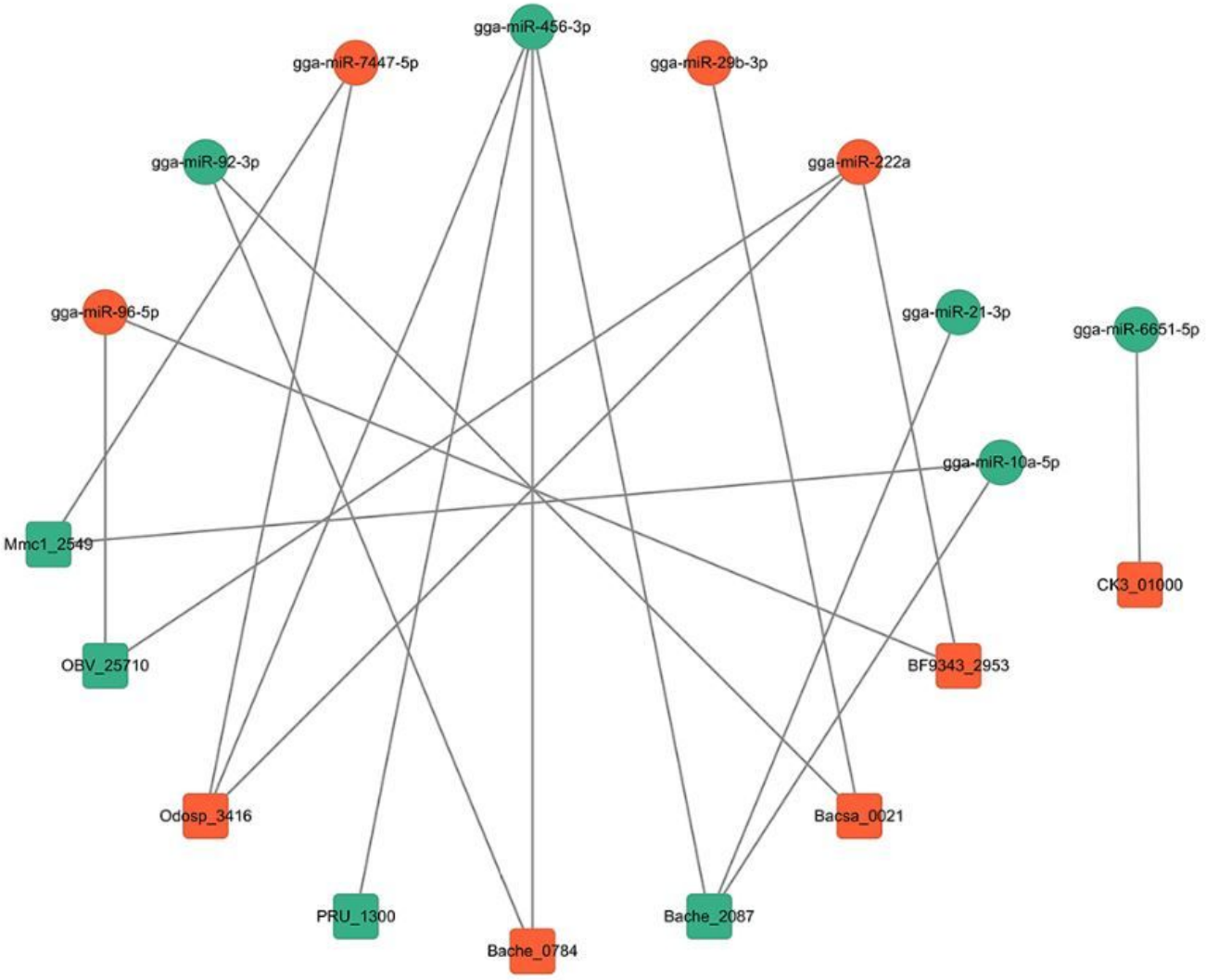

Figure 4

miRNAs and their target bacterial genes. Red represents significantly upregulated genes and miRNAs, while green represents significantly downregulated genes and miRNAs (Hy-line vs. Lohmann hens). Squares indicate bacteria genes, and circles indicate host miRNAs.

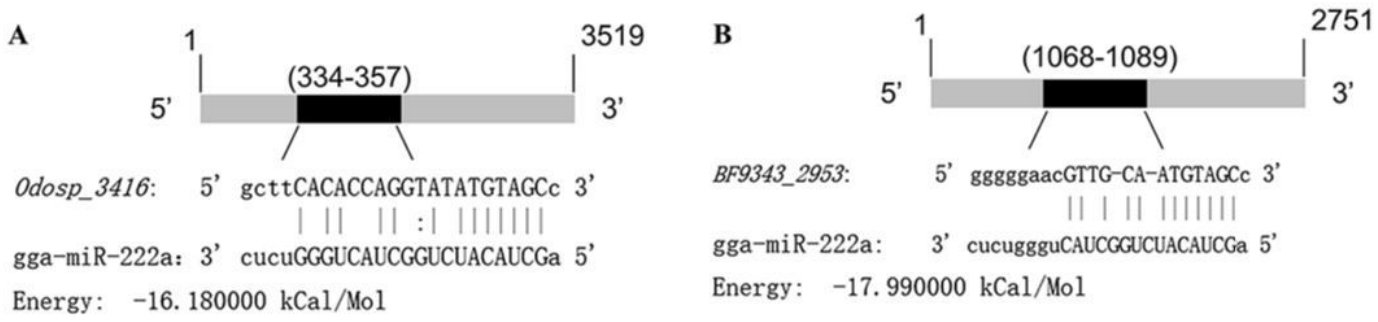

Figure 5

Sites where gga-miR-222a binds genes Odosp_3416 and BF9343_2954 (A) and (B) Binding sites of Odosp_3416 (A) and BF9343_2953 (B).

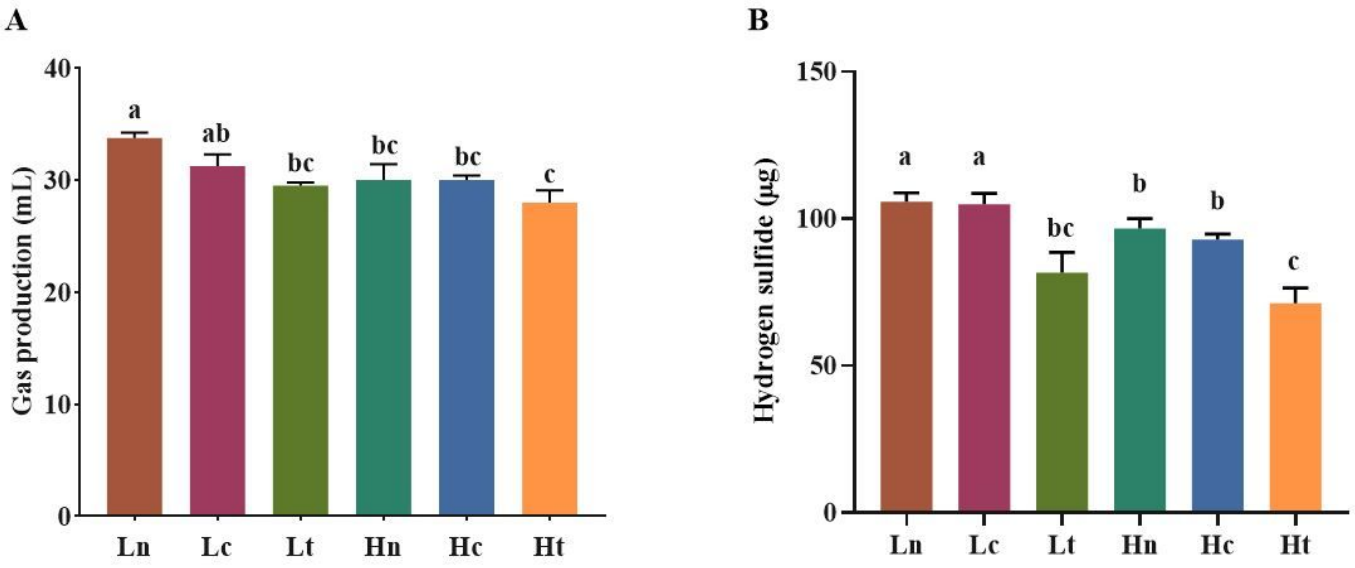

Figure 6

Total gas and H2S levels of each group after fermentation. (A) Total gas production. (B) H2S production. Different letters in different groups indicate a significant difference (P<0.05).

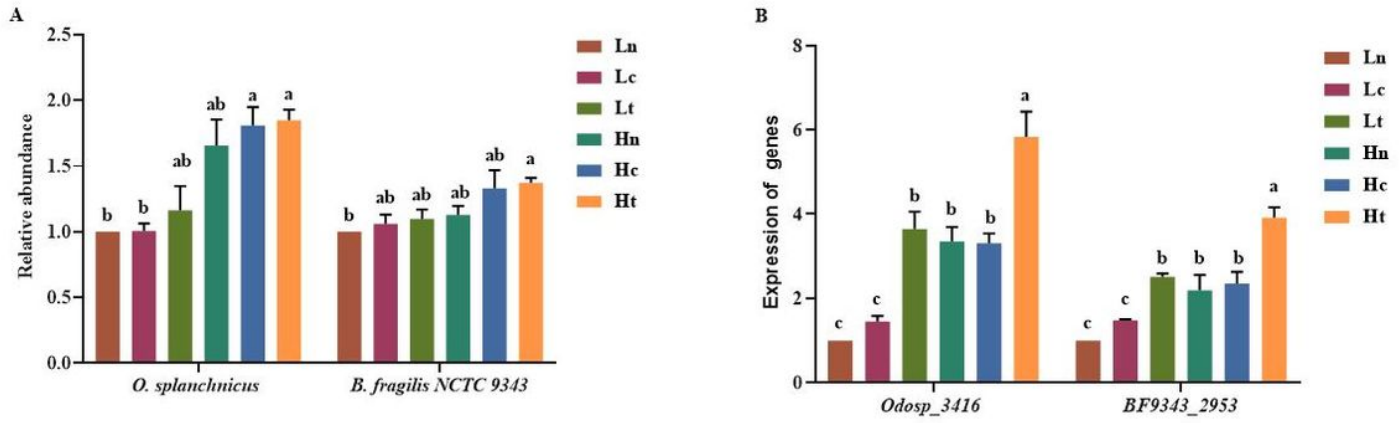

Figure 7

Abundances of bacteria and expression of genes of each group after fermentation. (A) Relative abundances of Odoribacter splanchnicus and Bacteroides fragilis NCTC9343. (B) Expression of genes Odosp_3416 and BF9343_2953. Different letters in different groups indicate a significant difference (P<0.05).

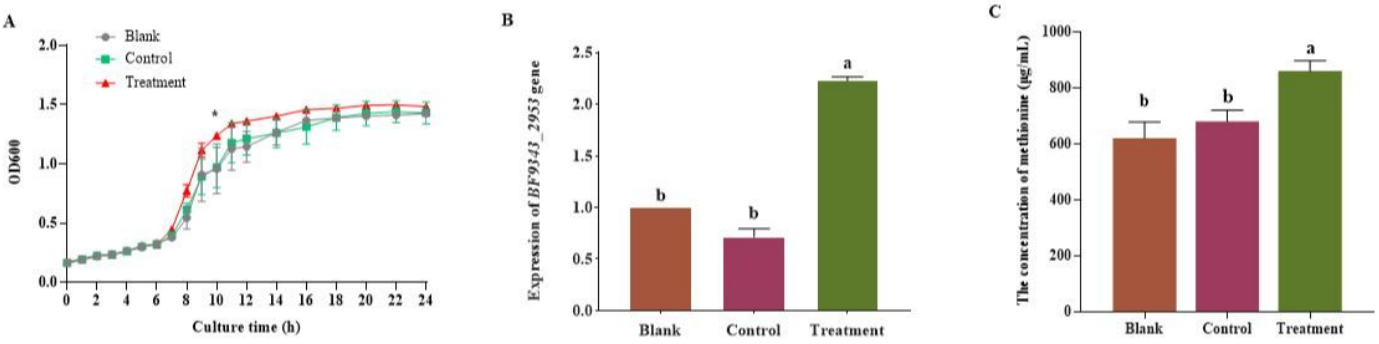

Figure 8

Effects of gga-miR-222a on the growth and metabolism of Bacteroides fragilis NCTC9343. (A) A growth curve of Bacteroides fragilis NCTC9343. * indicates a significant difference among groups (P<0.05). (B) Expression of the BF9343_2953 gene in bacterial medium. (C) Concentration of methionine in bacterial medium. Different letters in different groups indicate a significant difference (P<0.05).

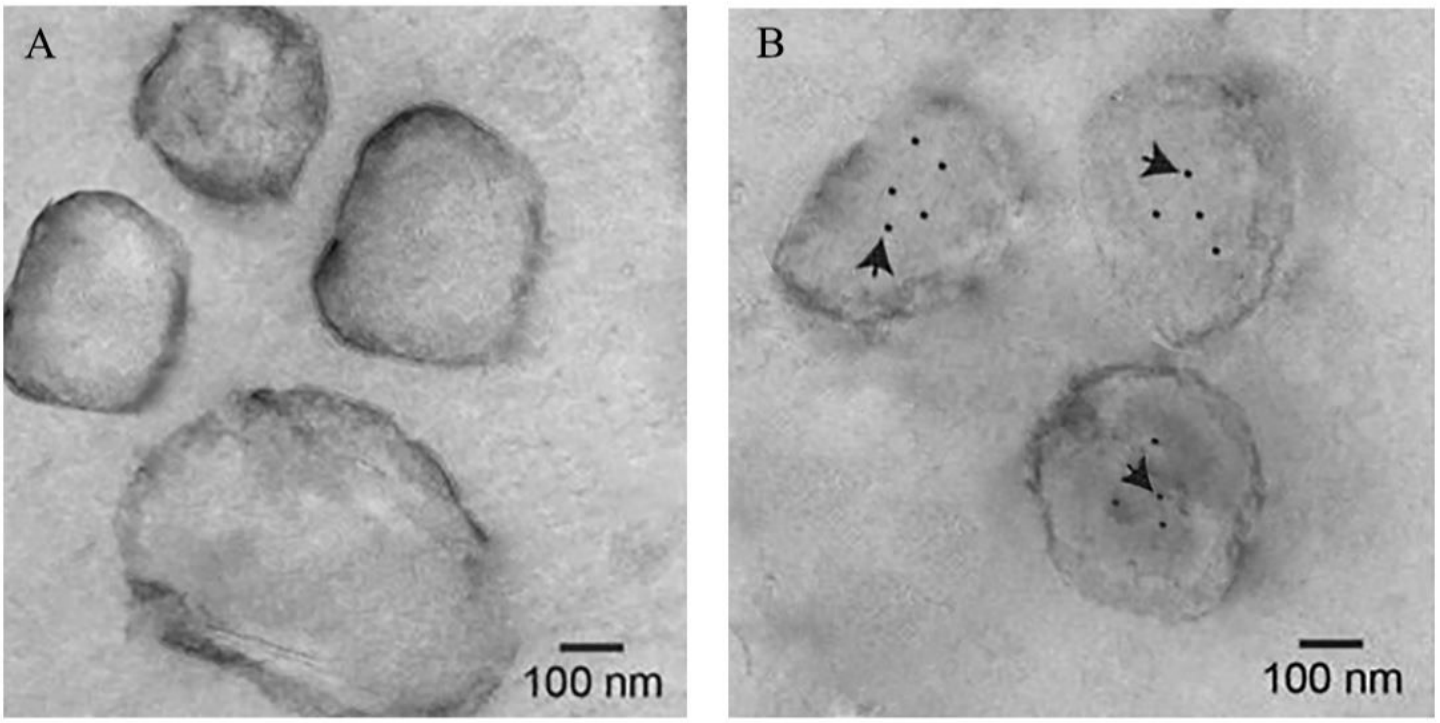

Figure 9

In situ hybridization map of gga-miR-222a internalized in Bacteroides fragilis NCTC9343. (A) and (B) In situ hybridization map of the miRNA control group (A) and gga-miR-222a group (B). Black dots and arrows indicate the site of hybridization.

Supplementary Files

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- Additionalfile2.xlsx
- Additionalfile1.docx