Breed Differences In The Expression Levels Of Gga-Mir-222a In Laying Hens Influenced H$_2$S Production By Regulating Methionine Synthase Genes In Gut Bacteria

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Abstract

Background: The microbiota in the cecum of laying hens was critical for host digestion metabolism and odor gas production. Recent studies have suggested that host miRNAs could 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 exosomes in the cecal content of laying hens and the potential miRNA-target relationships between 9 differentially expressed miRNAs and 9 differentially expressed microbial genes related to H_2S production were investigated, 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 one of the common nutrition sources for human consumption in daily life [1]. 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 [2–4]. Bacteria ferment undigested feed components to generate volatile fatty acids (VFAs), amino acids, ammonia (NH_3), hydrogen sulfide (H_2S) and other metabolites [5, 6]. As previous studies elucidated that 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 [7, 8]. Although, the NH_3 accounts are the largest proportion of odor gas in livestock and poultry facilities, and the accounts of H_2S is second to that of NH_3, the odor thresholds of H_2S is significantly lower than NH_3 which also contributes to the bad smell in the farm environment [9, 10]. In addition to its adverse effects on air quality, the early study reported that H_2S cause intestinal diseases in human [11], the recent study also showed that H_2S induced chicken pneumonia response [12], there is the possibility for H_2S to damage the health of breeding worker and animals. 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 [13, 14]. 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 [15, 16]. 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 [17]. 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 [18–22]. Some studies have characterized miRNAs as potential markers of tumorigenesis in human stool [23–25]. 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 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 [26]. Other studies reported that plant-derived miRNAs could be taken up by gut bacteria and shape the gut microbiota [27, 28]. 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) [29]. 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 transmission electron microscope (TEM). The exosome-sized (approximately 50–200 nm in diameter) extracellular vesicles were present in the cecal content of Lohmann and Hy-line hens, but the morphological character of exosomes was mostly the same in the two breeds (Fig. 1A and B). The existing of exosomes elucidated that there was the possible for the further miRNA sequencing to investigate the miRNA differences between the two breeds.

Based on the high-throughput sequencing, we found 288 known miRNAs could be annotated on the miRBase online website, and the information of miRNAs sequencing was listed in Table S1. Based on the DEGSeq R software statistical analysis, totally 10 miRNAs were significantly differentially expressed between the two breeds, the result of log2 (Fold change)⊠1 showed four miRNAs (gga-miR-222a, gga-miR-96-5p, gga-miR-7447-5p, gga-miR-29b-3p) were expressed significantly higher and six miRNAs (gga-miR-92-3p, gga-miR-429-3p, gga-miR-10a-5p, gga-miR-456-3p, gga-miR-6651-5p, gga-miR-21-3p) were expressed significantly lower in the Hy-line by the comparison of Lohmann ($P < 0.05$), in addition the top four high expression miRNAs were gga-miR-92-3p (Hy-line: 46 ± 11.47; Lohmann: 196 ± 113.67), gga-miR-429-3p (Hy-line: 17.5 ± 10.5; Lohmann: 110.167 ± 61.50), gga-miR-222a (Hy-line: 32.167 ± 51.547; Lohmann: 6 ± 4.80) and gga-miR-10a-5p (Hy-line: 6.833 ± 3.08; Lohmann: 30 ± 35.81) in Hy-line and Lohmann. (Fig. 1C). Thus the results indicated that the four miRNAs should be considered for the further analysis.

In addition, the KEGG pathway annotations of target genes in the chicken genome of these ten 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 Hs Production

The sequencing information of the metatranscriptomic was listed in Table S2, and the top 30 KEGG microbial function enrichment pathways were showed in Fig. S2. The 5 pathways of the 30 top pathways were showed the significant differences gene enrichment between Lohmann and Hy-line, the microbial genes enrichment abundance of two-component system was significantly higher in the Hy-line by the comparison of Lohmann ($P < 0.05$), and the microbial genes enrichment abundance of amino sugar and nucleotide sugar metabolism, cysteine and methionine metabolism, alanine aspartate and glutamate

metabolism and RNA degradation pathways were significantly higher in Lohmann by the comparison of Hy-line ($P < 0.05$). The result of transcriptomics also showed that totally the abundance of 22237 genes were significantly different between Lohmann and Hy-line ($P < 0.05$), in addition based on the combination analysis of the microbial gene enrichment pathway and the significantly different genes, 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). Based on the KEGG database recorded, the degradation of the sulfur-containing amino acid, cysteine and methionine, could result in the production of H_2S. As shown in Fig. 2A, L-cysteine was synthesized from L-serine and sulfide under the action of serine O-acetyltransferase and cysteine synthase, moreover, L-cysteine also could be synthesized from L-cystathionine by the regulation of cystathionine gamma-lyase. The L-cysteine degraded into pyruvate and sulfite through the regulation of aspartate aminotransferase, and sulfite could be the raw material for the production of H_2S through the sulfur metabolism pathway. In addition, the methionine could be degraded into methanethiol by methionine-gamma-lyase, which could be converted into H_2S in subsequent processes. Another pathway for methionine degradation was the formation of S-adenosyl-L-methionine by S-adenosylmethionine synthetase without H_2S production. Sulfur metabolism was the essential pathway in the cecum of hens which involved in the production of H_2S. The assimilatory reduction and dissimilatory reduction of sulfate promoted the production of sulfite and its eventual conversion into sulfide (Fig. 2A), thus the adenylylsulfate kinase was one of the key enzyme for the production of sulfide from sulfate, and it was worth to be tested in order to elucidate the H_2S production.

Through the analysis of annotation information of KEGG database, the present results indicated that O-acetyltransferase (cysE, EC: 2.3.1.30), methionine synthase (metH, EC:2.1.1.13), aspartate aminotransferase (aspB, EC:2.6.1.1), methionine-gamma-lyase (MGL, EC:4.4.1.11) and adenylylsulfate kinase (cysC, EC: 2.7.1.25) were the key enzymes for the production H_2S, and the gene expression level of these key enzyme was tested. The expression level of cysE and metH were both significantly higher in Hy-line hens than that in Lohmann hens ($P < 0.05$), the expression level of cysE was 0.061 ± 0.004 and 0.048 ± 0.010 in Hy-line and Lohmann respectively, and the expression level of metH was 0.302 ± 0.062 and 0.222 ± 0.036 in Hy-line and Lohmann respectively; but the expression of aspB and MGL were both lower in Hy-line hens than that in Lohmann hens ($P < 0.05$), the expression level of aspB was 0.081 ± 0.015 and 0.085 ± 0.020 in Hy-line and Lohmann respectively, and the expression level of MGL was 0.011 ± 0.002 and 0.014 ± 0.011 in Hy-line and Lohmann respectively. The present results indicated 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. 2B). In addition, we also found a significantly lower expression of cysC in the cecum of Hy-line hens by the comparison of Lohmann ($P < 0.05$), the cysC expression level was 0.004 ± 0.001 and 0.007 ± 0.001 in Hy-line and Lohmann respectively, the result also indicated the lower sulfide production in Hy-line than that in Lohmann hens (Fig. 2B).

Based on the analysis of the regulation enzyme involving H_2S production, totally, thirteen differentially expressed microbial genes which involved in the encoding of these five enzymes were found between the

two breeds, MGL and cysC only assigned to one gene and one genus, other three enzymes assigned several genes and genera, in addition most of the genes were assigned to the genus *Bacteroides* (Table 1).

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

Enzymes	DEGs(log2 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

Through the combination analysis of differentially expressed miRNAs (Fig. 1C) and differentially expressed genes which related to H_2S production (Table 1), in total, nine miRNAs could target 9 genes by using miRanda analysis (Fig. 3A). As above mentioned that gga-miR-222a and gga-miR-10a-5p were both high expression level miRNAs in Hy-line and Lohmann, however, the abundance of gga-miR-222a was significantly higher in Hy-line by the comparison of Lohmann, and the abundance of gga-miR-10a-5p was significantly lower in Hy-lin by the comparison of Lohmann, in addition, our previous study showed that the amount of H_2S production was obviously higher in Lohmann by the comparison of Hy-line (the daily H_2S production per kg average daily feed intake was 7.75 and 4.17 mg for Lohmann and Hy-line, respectively) [29], thus we concluded that gga-miR-222a was the potential additive candidate for the reduction of H_2S production in the cecum of laying hens, it was much worth to select gga-miR-222a for the further investigation rather than the selection of gga-miR-10a-5p. We found that gga-miR-222a could

target two genes that associated with methionine synthase, *Odosp_3416* (expressed by the bacterium *Odoribacter splanchnicus*) and *BF9343_2953* (expressed by the bacterium *Bacteroides fragilis* NCTC 9343) (Fig. 3B). The read count of *Odosp_3416* was 138 and 225 in Hy-line and Lohmann respectively, and the read count of *BF9343_2953* was 75 and 137 in Hy-line and Lohmann respectively, based on above results, we suspected that the regulatory effect of gga-miR-222a on the two gens may be notable. Therefore, the subsequent function verification of gga-miR-222a targeted with *Odosp_3416* and *BF9343_2953* was carried.

Hs Production Investigation In The Fermentation Experiment

After 24 h of fermentation, the amount of the total gas and H_2S production was tested in each experiment groups. The addition of gga-miR-222a could influence the total gas and H_2S production.

The total gas production was 33.75 ± 0.83 mL and 30 ± 0.71 mL in LB (Lohmann intestinal content broth added nothing) and HB (Hy-line intestinal content broth added nothing) respectively, the statistical analysis showed that the total gas production of LB was significantly higher than that in HB ($P < 0.05$), the result potentially indicated that the total gas production ability of Lohmann was higher than that of Hy-line. In present result, we found that the amount of total gas production was significantly decreased in LT (Lohmann intestinal content broth added gga-miR-222a, 29.5 ± 0.5 mL) by the comparison of LB ($P < 0.05$), however there was no significantly different between HT (Hy-line intestinal content broth added gga-miR-222a, 28 ± 1.87 mL) and HB, the result elucidated that gga-miR-222a could effectively decrease the total gas production in Lohmann rather than in Hy-line (Fig. 4A). In addition, there was no obvious difference between blank groups and control groups (LC and HC, intestinal content broth added miRNA control), the result suggested that the commercial synthesis gga-miR-222a was credible.

The effect of gga-miR-222a on the H_2S production was investigated, and the result was showed in Fig. 4B. The amount of H_2S was 105.747 ± 5.22 μg and 96.592 ± 5.84 μg in LB and HB respectively, and the statistical analysis showed that the amount of H_2S was significantly higher in LB by the comparison of HB ($P < 0.05$), the result indicated that the H_2S production ability of Lohmann was higher than that of Hy-line. It was worth to note that gga-miR-222a addition significantly decreased the amount of H_2S production in both fermentation broth of two breeds ($P < 0.05$), the amount of H_2S production was 81.553 ± 11.95 μg and 71.152 ± 8.94 μg in LT and HT respectively. By the comparison of blank groups, gga-miR-222a addition decreased H_2S production 22.88% and 26.33% in Lohmann and Hy-line intestinal content broth respectively. The present result suggested that there was the potential ability for gga-miR-222a to decrease the H_2S production of the intestinal content.

The chemical indexes of the fermentation broth of each group are shown in Table 2. The concentration of soluble sulfide (S^{2-}) was significantly lower in HB (12.32 ± 0.98 μg/g) than that of LB (15.06 ± 0.94 μg/g)

($P < 0.05$), in addition, the S^{2-} concentration also significantly lower in the gga-miR-222a addition groups (LT, 12.50 ± 0.50 μg/g and HT, 9.84 ± 1.44 μg/g) by the comparison of blank groups (LB and HB) ($P < 0.05$). The concentration of methionine in the fermentation broth was also confirmed due to methionine was the crucial amino acid to donate the sulfur for the form of H_2S through the microbial metabolism. Here, the addition of gga-miR-222a also significantly increased the concentration of methionine in LT (280.39 ± 5.78 μg/mL) and HT (299.12 ± 3.68 μg/mL) by the comparison of LB (246.21 ± 18.83 μg/mL) and HB (257.70 ± 19.30 μg/mL) respectively ($P < 0.05$).

Table 2
The comparison of fermentation incubation indexes [1]

Items	LB	LC	LT	HB	HC	HT
pH	7.57 ± 0.08	7.49 ± 0.03	7.61 ± 0.10	7.59 ± 0.02	7.55 ± 0.05	7.48 ± 0.05
S^{2-}, μg/g	15.06 ± 0.94^a	14.83 ± 1.12^a	12.50 ± 0.50^b	12.32 ± 0.98^b	12.99 ± 1.59^{ab}	9.84 ± 1.44^c
SO_4^{2-}, mg/g	256.94 ± 29.93	268.52 ± 61.26	221.30 ± 79.04	257.87 ± 85.95	287.96 ± 46.39	243.98 ± 70.84
Acetate, mmol/L	31.53 ± 4.01	30.19 ± 0.96	32.81 ± 3.25	32.45 ± 1.85	34.63 ± 2.23	34.15 ± 2.70
Propionate, mmol/L	15.51 ± 1.54	15.34 ± 3.40	15.21 ± 1.35	16.00 ± 1.00	16.58 ± 0.91	15.59 ± 0.74
Butyrate, mmol/L	8.33 ± 0.54^c	8.16 ± 0.56^c	9.10 ± 0.73^{bc}	10.81 ± 1.44^a	10.67 ± 1.22^a	10.17 ± 0.60^{ab}
Total VFAs, mmol/L	55.37 ± 6.00	53.69 ± 3.18	57.11 ± 5.29	59.26 ± 2.94	61.88 ± 1.98	59.91 ± 3.70
Methionine, μg/mL	246.21 ± 18.83^c	245.88 ± 15.01^c	280.39 ± 5.78^{ab}	257.70 ± 19.30	258.92 ± 12.08	299.12 ± 3.68^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

DNA was extracted from fermentation broth by using a QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, briefly 1 mL fermentation broth was centrifuged at 20,000 × g for 1 min at 4°C to collect the precipitate (approximately 200 mg) for the DNA extraction, and the DNA purification following steps was referenced the protocol of manufacturer. RNA extraction of

fermentation broth followed the protocol of RNeasy® PowerMicrobiome™ Kit (Qiagen, Hilden, Germany), briefly, 1−2 mL fermentation broth was centrifuged at 20,000 × g for 1 min at 4℃ to collect the precipitate (approximately 200−250 mg) for the RNA extraction, and the RNA purification following steps was referenced the protocol of manufacturer.

The DNA was used to quantify the relative abundance of *Odoribacter splanchnicus* and *Bacteroides fragilis* NCTC 9343. Briefly, the primers of these two bacteria was designed by using the software Primer 3, the sequences of the two bacteria was referenced the 16S rRNA sequencing in the web of NCBI, the details of the primers were showed in Table S5, the primer of bacterial16S rRNA was referenced previous study [45]. q-PCR was used to confirm the relative abundance of the two bacteria, the q-PCR reaction steps were followed the protocol of SYBR® Green PCR Kit (SYBR, Japan) (Table S6). The relative abundance of the two bacteria was calculated as $2^{\triangle Ct}$, where $\triangle Ct$ represents the difference in the Ct value for the 16S rRNA gene minus that for the genes [46].

Extracted RNA was reverse transcribed into cDNA by 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 S5). q-PCR was used the confirm the relative expression level of the two genes, the q-PCR reaction steps were followed the protocol of SYBR® Green PCR Kit (SYBR, Japan) with a little modification (Table S7). The relative expression level of the two genes was calculated as $2^{\triangle Ct}$, where $\triangle Ct$ represents the difference in the Ct value for the 16S rRNA gene minus that for the genes [46].

In vitro bacterial growth measurements

The anaerobic bacterium *Bacteroides fragilis* NCTC9343 were cultured at 37℃ by inoculating 40 mL aliquots of anaerobic basal medium (Becton Dickinson and Company, Lincoln Park, USA) and then grown anaerobically in an anaerobic chamber (Mitsubishi Gas Chemical Company, Inc. Tokyo, Japan). 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 tested as above mentioned.

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

The data of the comparison of fermentation incubation indexes, the comparison of the relative abundance of miRNA, bacteria and genes, gas production, H_2S production and growth curve were examined by analysis of variance (ANOVA) with Statistical Package for the Social Sciences (SPSS) software, version 22.0. Significant differences between the means were determined by Tukey's test. Differences were considered significant at P⬚0.05.

The metatranscriptomic results of each sample was analyzed by HTSeq software, and the model used was union, the number of genes in different expression levels and the expression level of individual genes were statistically analysed. In general, the value of FPKM is 0.1 or 1 as the threshold for determining whether genes are expressed. The software DESeq was used for normalization of the read counts from analysis of genes expression levels [47].

The expression level of miRNA was calculated by the TPM formula (normalization read counts= (readCount*1,000,000)/libsize)), libsize was the sum of the read count of all miRNAs.

The Investigation Of Gga-mir-222a Effectiveness

In order to further understand whether the effects of gga-miR-222a were really influenced the methionine synthetase genes carried bacteria, the culture medium experiment was applied to reach the goal. Because of the relative abundance of *Odoribacter splanchnicus* was significantly lower than that of *Bacteroides fragilis* NCTC9343 in both breeds (Fig. S3), thus *Bacteroides fragilis* NCTC9343 was selected for the following experiment. At 8 h, the bacterium *Bacteroides fragilis* NCTC9343 reached the logarithmic phase, and at 10 h, the strain reached a plateau. In the gga-miR-222a addition group, this miRNA significantly improved 29.04% of the abundance of *Bacteroides fragilis* NCTC9343 at 10 h by the comparison of blank ($P< 0.05$) (Fig. 6A). In addition, gga-miR-222a addition also significantly enhanced 2.23-fold of the expression level of gene *BF9343_2953* of *Bacteroides fragilis* NCTC9343 by the comparison of control ($P < 0.05$) (Fig. 6B), and the concentration of methionine in the medium also was significantly increased 38.71% with the addition of gga-miR-222a by the comparison of blank ($P< 0.05$) (Fig. 6C).

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, by the comparison of control (Fig. 7A), the exogenous gga-miR-222a was selectively absorbed by *Bacteroides fragilis* NCTC9343 in the gga-miR-222a addition group (Fig. 7B).

Discussion

Although there is a known association among host genetic background, cecal microbiota structure and odor production by laying hens [30, 31], the potential mediators of this relationship remain unclear. Recently, some findings demonstrated that mammalian secreted miRNAs could regulate the expression of bacterial genes [26, 32]. 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 [29]. 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 [33]. 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 [34, 35]. The metatranscriptome showed that there was no significant difference in the expression of dissimilatory sulfate reduction pathway related genes. The reason could be due to the abundance of SRB was low in the animal gut (approximately 0.028−0.097%) [14]. 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 [36], 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 [37]. 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 [38]. 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 [39, 40]. However, some studies have shown that miRNA not always played a negative regulation on mRNA [26, 27, 41]. 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 [31, 32]. 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, the present 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 trough the gene expression regulation in the 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±0.02kg and 1.71±0.02kg, 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 [29]. Water and the commercial-type laying hen diet were fed to birds *ad libitum* (Table S3), 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 liquid nitrogen and stored 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

The exsomes purification referenced Liu, briefly, 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) [26].

Extraction and analysis of miRNA in the cecum of laying hens

Total miRNA was extracted by using mirVana™ miRNA Isolation Kit (Austin, TX, USA) according to Liu [26]. Briefly, approximately 100 mg cecal content was mixed adequately with 600μL 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, then centrifuged for 5 min at 8000 ×g to recover miRNA and then stored at -80°C. 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 by using RNeasy® PowerMicrobiome Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 200mg sample was placed into PowerBead Tubes. Cells were lysed according to the kit manual by adding 650μL Solution PM1 with β-mercaptoethanol and 100μL phenol/chloroform/isoamylalcohol, followed by vortexing for 10 minutes at maximum speed using a 24-sample vortex adapter (Kelly Bell, Jiangsu, China), Centrifuged at 13,000×g for 1 min at room temperature (15-25°C). Transfer the supernatant to a clean 2 ml Collection Tube. The following RNA purification was referenced the protocol of the manufacturer. 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 homogenization of equal amounts 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 comparison 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, the significantly different expression miRNAs between the two breeds were used for the target prediction analysis of microbial significantly different expression genes which related to H_2S production. The target relationship between Bacterial mRNAs and miRNAs were identified by using miRanda (http://www.microrna.org). Furthermore, a prediction of host genome genes which was targeted by the 10 different expression miRNAs were also conducted by miRanda, and all the target genes were determined to be enriched by KEGG analysis.

In vitro fermentation experiment

The *in vitro* was referenced Menke and Steingass [42], briefly, thirty Hy-line Gray and thirty Lohmann Pink laying hens at age of 28 weeks were sacrificed respectively, and the caeca were ligated immediately. The cecal contents in the same breed group were pooled, then thoroughly mixed with the fermentation buffer solution which was pre-heat at 39°C as the fermentation broth. The fermentation buffer solution was homogenization by 474mL deionized water, 237mL macro element solution (per 1000mL, Na_2HPO_4 5.7 g, KH_2PO_4 6.2 g, $MgSO_4 \cdot 7H_2O$ 0.6 g), 237mL buffer solution (per 1000mL, 35.0 g $NaHCO_3$, 4.0 g NH_4HCO_3), 0.12 trace element solution (per 100mL, $CaCl_2 \cdot 2H_2O$ 13.2 g, $MnCl_2 \cdot 4H_2O$ 10.0 g, $CoCl_2 \cdot 6H_2O$ 1.0 g, $FeCl_2 \cdot 6H_2O$ 0.8 g), 1.22mL resazurin and 50mL reductant (per 50mL, 1mol/L NaOH 2.0 mL, $Na_2S \cdot 9H_2O$ 335 mg). The intestinal content-buffer mixture was blended for 60 sec in a blender after the solution was filtered through four layers of surgical gauze, and then mixed with the fermentation buffer solution at a 1:2 ratio (V/V), flushed with CO_2 at 40°C in order to eliminate all the O_2 in the solution. A corn-soybean basal laying hen diet was used as a substrate for fermentation.

After the air in syringe was eliminated from the head-space, approximately 10mL fermentation broth (FB) was added to a 100 mL gas syringe with 0.2g of the substrate. Three groups with different treatments for each breed were designed, the blank group (10mL FB+0.2g substrate+1mL pure water), control group

(10mL FB+0.2g substrate+1mL control mimic at a final concentration of 2μM) and treatment group (10mL FB+0.2g substrate+1mL gga-miR-222a mimic at a final concentration of 2μM) (Table. S4). The miRNAs applied in present study were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Then, these syringes were sealed with clips and placed in incubator and rotated at 42°C, 60rpm for 24h. At the end of incubation, the syringes were put on the ice to stop the fermentation, the gas production was recorded as the volume of head-space of syringe and the gas was also injected into a gas collection bag for H_2S analysis. Ten milliliters of fermentation broth was sampled and stored at -80°C for chemical analysis. The quantity of H_2S of the gas sample and the concentrations of soluble sulfide (S^{2-}) of the fermentation broth were determined using the methylene-blue colorimetric method, briefly, the adsorption liquid (per 1000mL, $3CdSO_4 \cdot 8H_2O$ 4.3g, NaOH 0.3g, ammonium polyvinyl phosphate 10g) was mixed with gas (10mL adsorption liqiud) and fermentation broth (adsorption liquid : fermentation broth, V/V=9:1), then followed the steps mentioned in previous studies [43, 44]. The pH value was determined using a pH meter (INESA Scientific Instrument, Shanghai, China) [29]. The concentration of sulfate radicals (SO_4^{2-}) was determined using the turbidimetric method [29]. The concentrations of VFAs were determined using high-performance liquid chromatography [29]. The concentrations of methionine in the fermentation broth were tested by using automatic amino acid analyzer (Sykam, Munich, Germany), briefly, 1mL fermentation broth was mixed with 10mL hydrochloric acid solution (6mol/L), 3-4 drops of phenol liquid were added to the above mixture solution, then put the tube which contained the mixture solution on ice for 3-5min. The tube which contained the mixture solution was oxygen-free by the treatment of nitrogen flushing, then put the tube into air oven at 110°C for 22h. After cooling the mixture solution at room temperature, the mixture solution was constant volume as 50mL after filtering by using filter paper. The 15mL filtering solution was dried at 40-50°C, and the deposition was washed with the deionized water twice and dried again. Then the deposition was diluted by 1mL 0.02mol/L hydrochloric acid solution, after filtering 0.22μm membrane the concentration of methionine was tested by automatic amino acid analyzer.

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, briefly 1mL fermentation broth was centrifuged at 20,000×g for 1 min at 4°C to collect the precipitate (approximately 200mg) for the DNA extraction, and the DNA purification following steps was referenced the protocol of manufacturer. RNA extraction of fermentation broth followed the protocol of RNeasy® PowerMicrobiome™ Kit (Qiagen, Hilden, Germany), briefly, 1-2mL fermentation broth was centrifuged at 20,000×g for 1 min at 4°C to collect the precipitate (approximately 200-250mg) for the RNA extraction, and the RNA purification following steps was referenced the protocol of manufacturer.

The DNA was used to quantify the relative abundance of *Odoribacter splanchnicus* and *Bacteroides fragilis* NCTC 9343. Briefly, the primers of these two bacteria was designed by using the software Primer 3, the sequences of the two bacteria was referenced the 16S rRNA sequencing in the web of NCBI, the

details of the primers were showed in Table S5, the primer of bacterial16S rRNA was referenced previous study [45]. q-PCR was used to confirm the relative abundance of the two bacteria, the q-PCR reaction steps were followed the protocol of SYBR® Green PCR Kit (SYBR, Japan) (Table S6). The relative abundance of the two bacteria was calculated as $2^{\triangle Ct}$, where $\triangle Ct$ represents the difference in the Ct value for the 16S rRNA gene minus that for the genes [46].

Extracted RNA was reverse transcribed into cDNA by 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 S5). q-PCR was used the confirm the relative expression level of the two genes, the q-PCR reaction steps were followed the protocol of SYBR® Green PCR Kit (SYBR, Japan) with a little modification (Table S7). The relative expression level of the two genes was calculated as $2^{\triangle Ct}$, where $\triangle Ct$ represents the difference in the Ct value for the 16S rRNA gene minus that for the genes [46].

In vitro bacterial growth measurements

The anaerobic bacterium *Bacteroides fragilis* NCTC9343 were cultured at 37°C by inoculating 40 mL aliquots of anaerobic basal medium (Becton Dickinson and Company, Lincoln Park, USA) and then grown anaerobically in an anaerobic chamber (Mitsubishi Gas Chemical Company, Inc. Tokyo, Japan). 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 10h 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 10h were tested as above mentioned.

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

The data of the comparison of fermentation incubation indexes, the comparison of the relative abundance of miRNA, bacteria and genes, gas production, H_2S production and growth curve were examined by analysis of variance (ANOVA) with Statistical Package for the Social Sciences (SPSS) software, version 22.0. Significant differences between the means were determined by Tukey's test. Differences were considered significant at P⎕0.05.

The metatranscriptomic results of each sample was analyzed by HTSeq software, and the model used was union, the number of genes in different expression levels and the expression level of individual genes were statistically analysed. In general, the value of FPKM is 0.1 or 1 as the threshold for determining whether genes are expressed. The software DESeq was used for normalization of the read counts from analysis of genes expression levels [47].

The expression level of miRNA was calculated by the TPM formula (normalization read counts= (readCount*1,000,000)/libsize)), libsize was the sum of the read count of all miRNAs.

Abbreviations

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

Declarations

Availability of data and materials

miRNA and metatranscriptomic sequence data are deposited in the NCBI Sequence Read Archive (SRA) database (PRJNA665380 and PRJNA666118)

Authors' contributions

SX and CH conducted experiment and manuscript writing. CH, SX, and XL involved in acquisition of funding, experimental design and review of the manuscript. RW analysed the data and 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
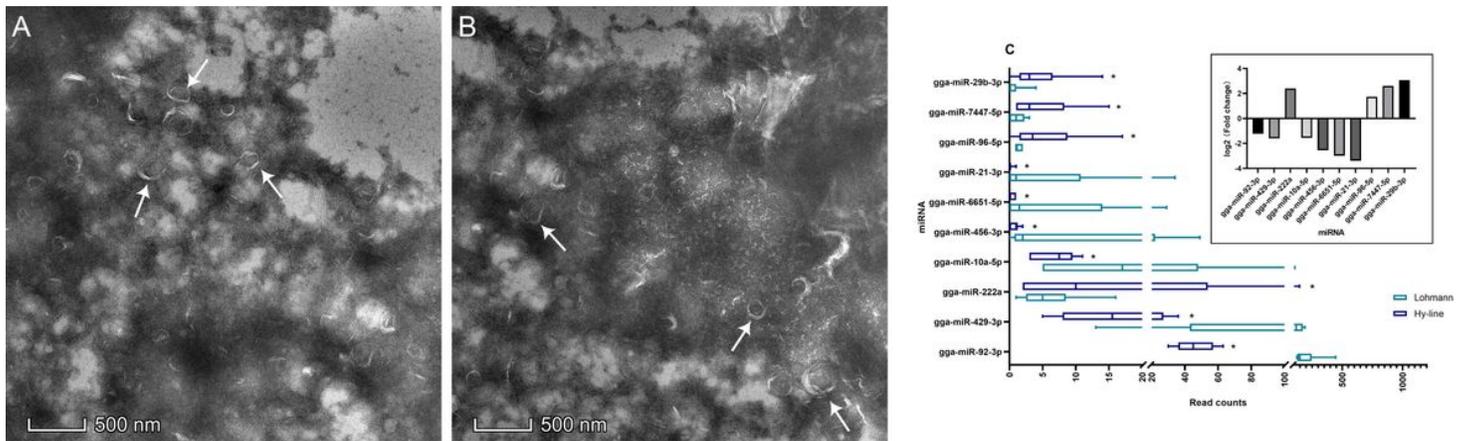
Figure 1

Morphological characteristics of exosomes and significantly expressed miRNAs. Fig1. A and B were the morphological characteristics of exosomes in Lohmann cecal content (A) and in Hy-line cecal content (B). White arrows point to exosomes. C was the ten differentially expressed miRNAs between the two breeds. * indicates a significant difference of miRNA between the two breeds, significant differences between the means were determined by Tukey's test. Differences were considered significant at P⬚0.05, the bar graph in Fig1. C was the expression level of these 10 miRNAs, the bars on the "0" X axis means up-regulation in the Hy-line by the comparison of Lohmann, and the bars under the "0" X axis means down-regulation in the Hy-line by the comparison of Lohmann.
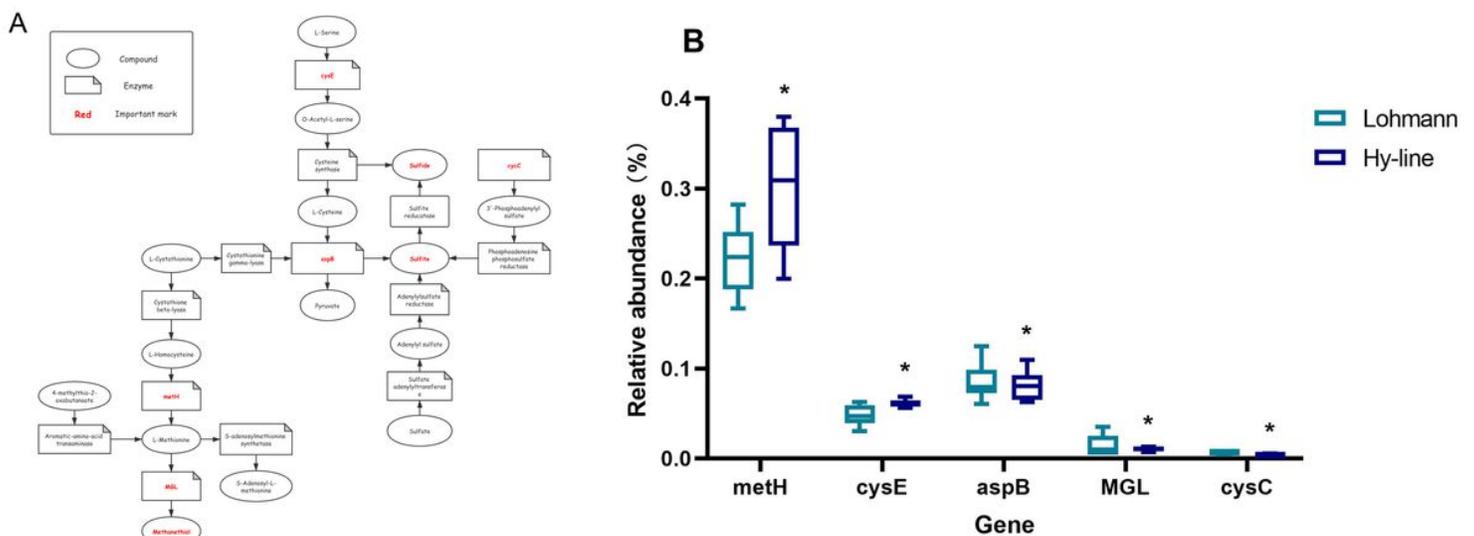
Figure 2

Expression of enzymes regulation genes related to the cysteine and methionine metabolism and sulfate metabolism pathways. A was the related enzymes and intermediates in the cysteine and methionine metabolism pathway and the sulfate metabolism pathway. B was the differentially expressed genes which were marked in red in metabolic pathways. * indicates a significant difference of genes relative abundance between the two breeds, significant differences between the means were determined by Tukey's test. Differences were considered significant at P⩽0.05.
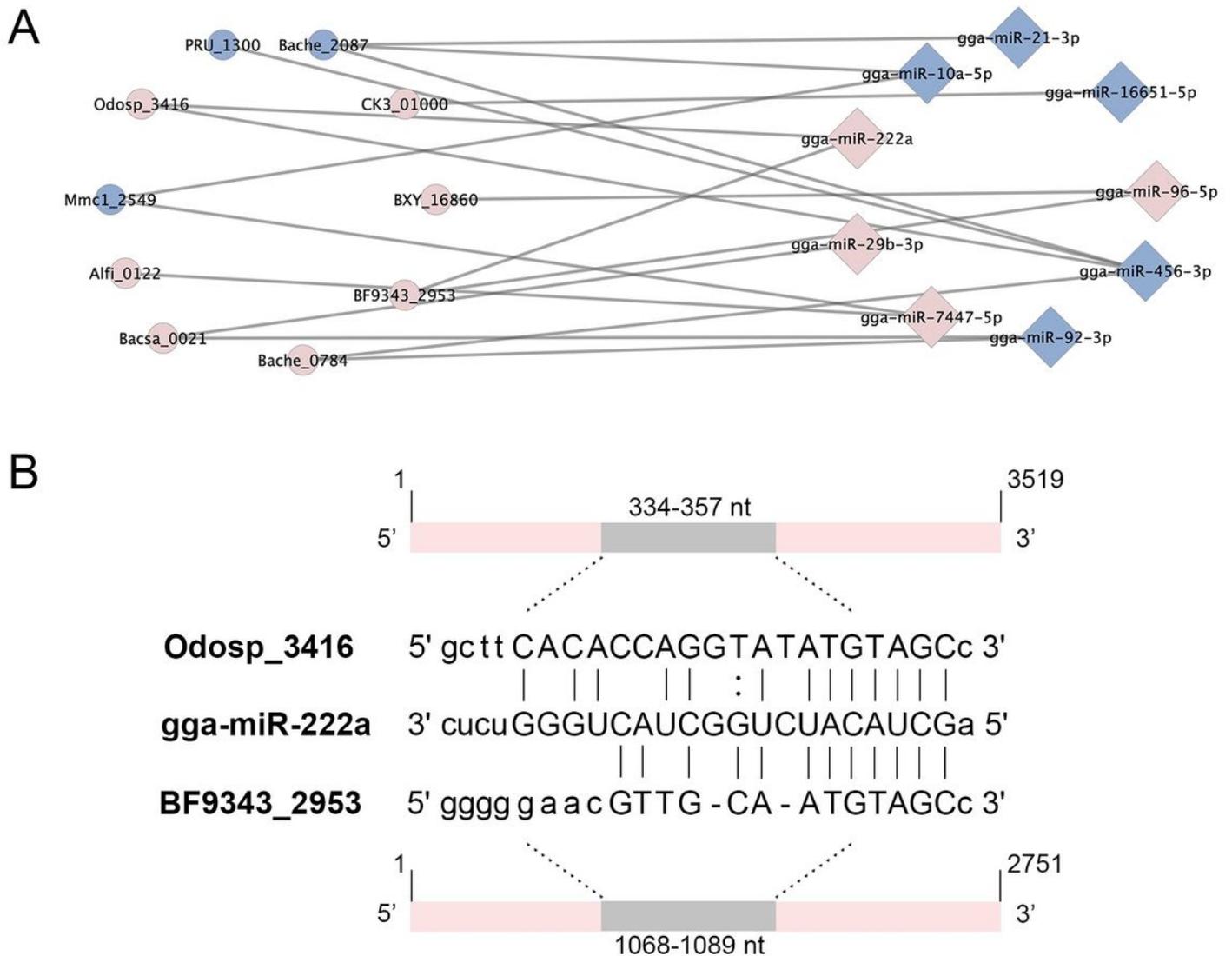

Figure 3

The correlation and target sites analysis between miRNA and the bacterial genes. In the A, pink color represents significantly upregulated genes and miRNAs in the Hy-line by the comparison of Lohmann, while blue color represents significantly downregulated genes and miRNAs in the Hy-line by the comparison of Lohmann. Squares indicate bacteria genes, and circles indicate host miRNAs. B showed the target sites between gga-miR-222a and genes Odosp_3416 and BF9343_2953.
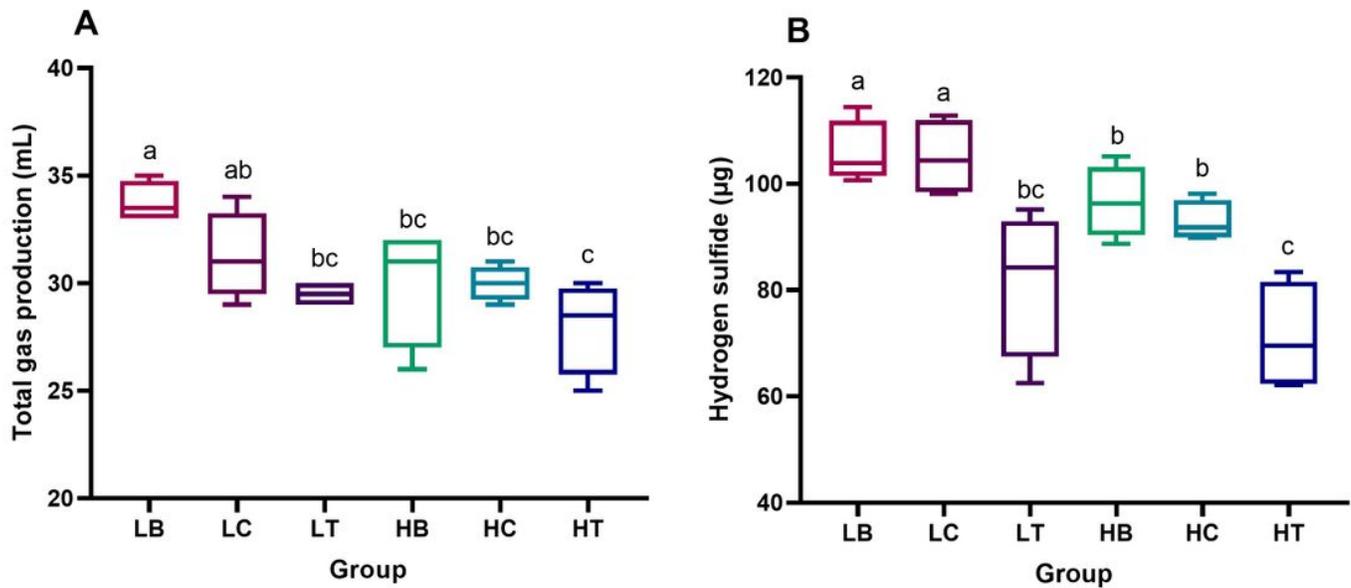
Figure 4

The amount of total gas and H2S production in fermentation experiment of each group. A showed the amount of total gas production, and B showed the amount of H2S production. Different letters indicated the significant difference of the amount of total gas and H2S production among the groups, significant differences among the means were determined by Tukey's test. Differences were considered significant at P◻0.05.
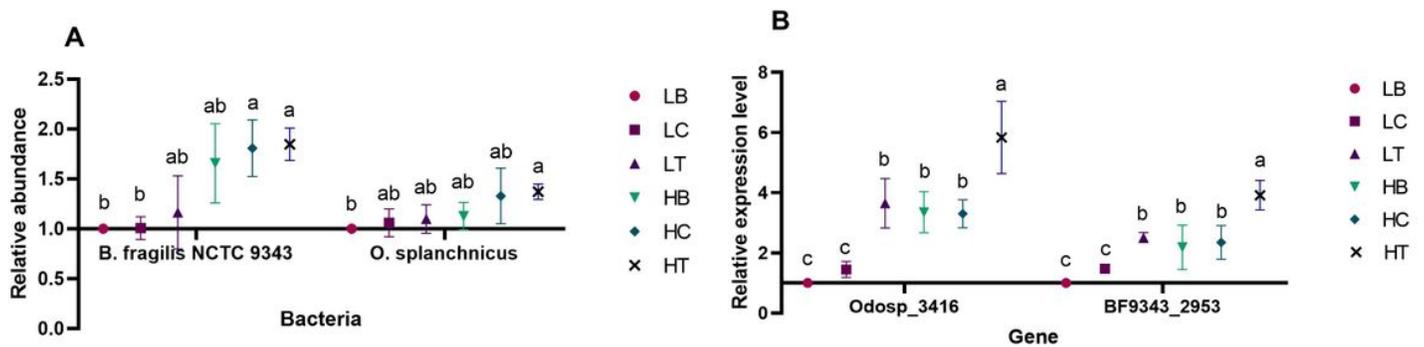

Figure 5

The relative abundance and expression level of bacteria and genes in fermentation experiment of each group. A showed the relative abundances of Odoribacter splanchnicus and Bacteroides fragilis NCTC9343 in each group, the relative abundance of group LC, LT, HB, HC and HT was showed as the comparison result based on the reference of the relative abundance of group LB, and the relative abundance of LB was set as 1. B showed the relative expression level of genes Odosp_3416 and BF9343_2953 in each group, the relative abundance of group LC, LT, HB, HC and HT was showed as the

comparison result based on the reference of the relative abundance of group LB, and the relative abundance of LB was set as 1. Different letters in different groups indicate a significant difference, significant differences among the means were determined by Tukey's test (P<0.05).
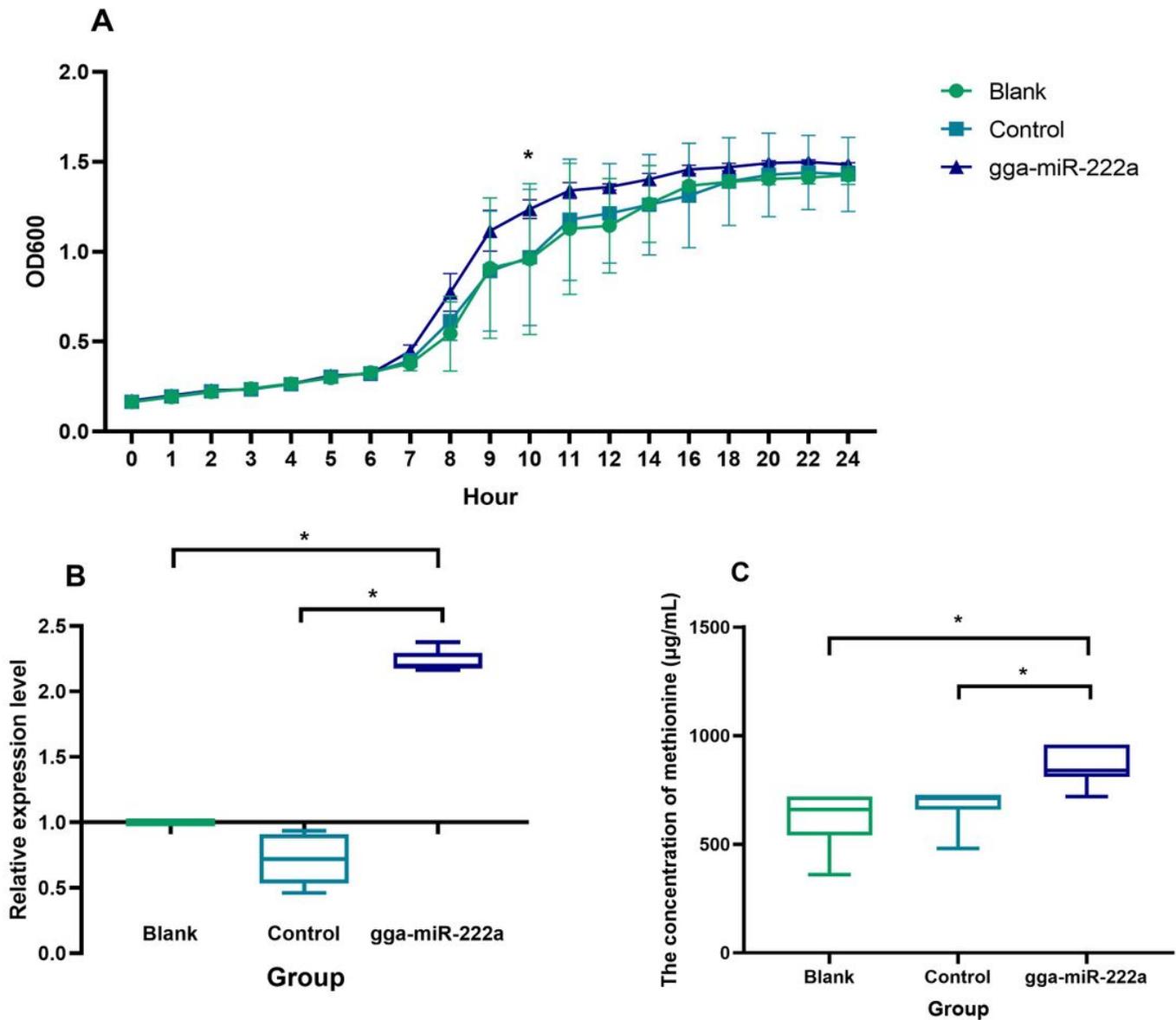
Figure 6

Effects of gga-miR-222a on the growth and metabolism of Bacteroides fragilis NCTC9343. A showed the growth curve of Bacteroides fragilis NCTC9343. B showed the expression of the BF9343_2953 gene in bacterial medium, the relative abundance of group Control and gga-miR-222a was showed as the comparison result based on the reference of the relative abundance of group Blank, and the relative abundance of Blank was set as 1. C showed the concentration of methionine in bacterial medium. In the figures, * indicates a significant difference among groups, significant differences among the means were determined by Tukey's test (P<0.05).
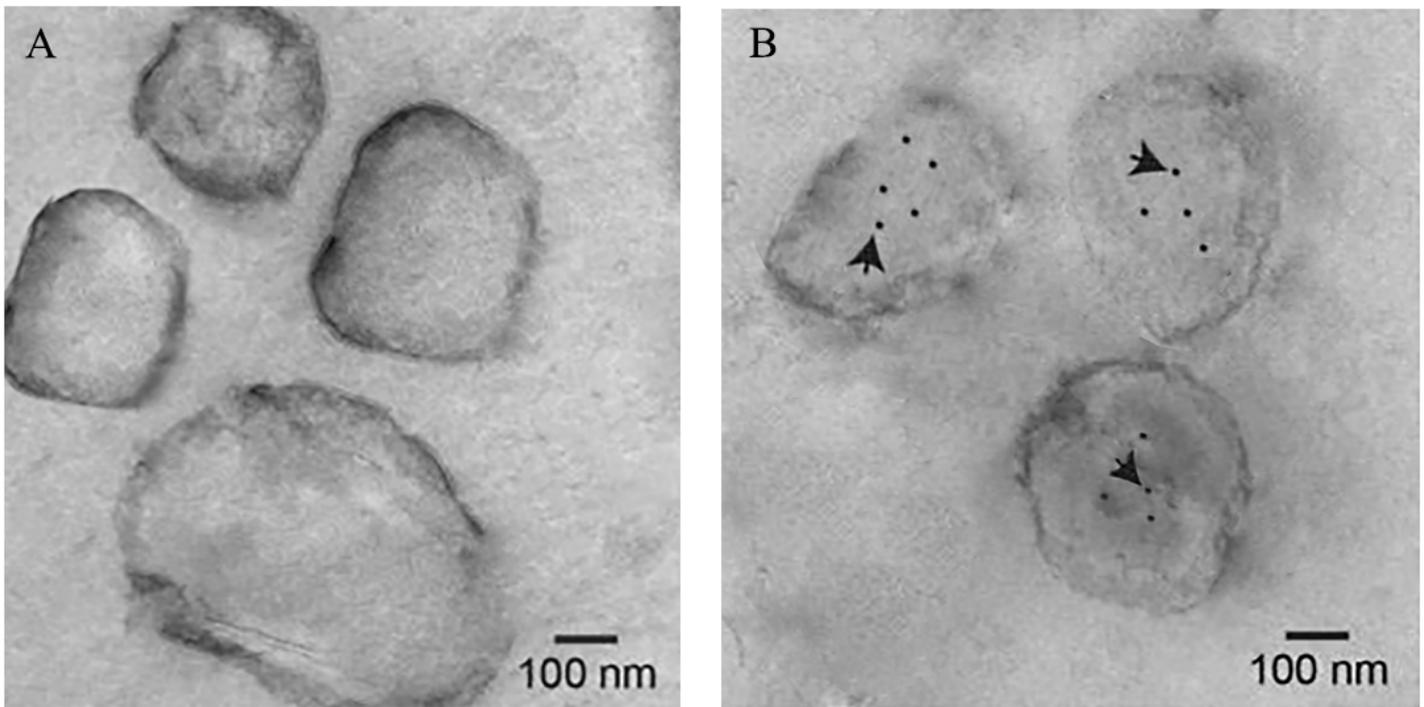
Figure 7

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

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Fig.S3.jpg
- Fig.S2.jpg
- Fig.S1.tif
- TableS.docx