Gut microbiome analysis in human living close to livestock at Mlati district, Sleman, Yogyakarta

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ABSTRACT

Background: Transmission of pathogenic and non-pathogenic microorganisms from animals to humans can affect the composition of gut bacteria (gut microbiome) in humans and can also be a potential source of transmission of zoonotic diseases. Therefore, this study aimed to analyze the gut microbiome interactions between the human and the livestock.

Method: This study was a cross-sectional study conducted in Mlati District, Sleman, Yogyakarta. The fecal sample was used to identify parasites by using trichrome staining. Then NGS selected samples were used to analyze the gut microbiome using Illumina high throughput 16S rRNA region V3-V4 sequencing.

Result: The analysis showed that Bacteroidetes was prevalent in the HCA group (57%), while Firmicutes (70%) were commonly found in HNC. Regarding the potential parasitic infection, direct microscopic examination and trichrome staining showed Blastocystis sp and Entamoeba coli cyst was found in several subjects in HCA groups, with prevalence rate at 7.69% and 2.5%. Meanwhile, no parasite was found in HNC feces.

Conclusion: There was a significant difference in the gut microbiome and parasitic infections between the HCA and HNC groups, with Blastocystis sp and Entamoeba coli as the most prevalent parasites.

Keywords: livestock, gut microbiome, 16S rRNA gene, high throughput sequencing.


INTRODUCTION

Zoonotic infections are caused by various microorganisms, such as bacteria, viruses, and parasites.¹ Transmission of pathogenic microorganisms from animals to humans can occur through direct contact, indirect contact, vector-borne, and foodborne.² The prevalence of zoonotic infections in humans is estimated to be at 2.5 billion cases per year and responsible for 2.7 million deaths worldwide.³ The previous study has shown that the risk of acquiring zoonotic diseases is higher in communities living close to livestock or with wildlife.³

The transmission of pathogens from animals to humans could alter the gut bacteria community's composition (gut microbiome) in humans.⁴,⁵ The composition of the gut microbiome is also closely related to overall health. A balanced gut microbiome composition plays an important role in maintaining health, especially from the immune system and metabolic-endocrine regulations.⁶ On the other hand, its imbalance can result in several health problems, one of which is the increased risk of infection by microorganisms.²

The gut microbiome in the healthy population is usually dominated by the families of Firmicutes (Lachnospiraceae and Ruminococcaceae), Bacteroidetes (Bacteroidaceae, Prevotellaceae, and Rikenellaceae), Proteobacteria, and Actinobacteria (Bifidobacteriaceae). The dominant bacterial species are Lactobacillus sp, Streptococcus salarius subsp. Thermophilus, Enterococcus faecium, Streptococcus diaacetylactis, Streptococcus intermedius, and Bifidobacteria sp. Changes in the gut microbiome composition are associated with various diseases such as diabetes, inflammatory bowel disease, obesity, asthma, rheumatoid arthritis, and increased susceptibility to infections.⁷,⁸

Because the gut microbiome can be influenced by the environment, this study aimed to evaluate the gut microbiome among people living close to Livestock in Mlati District, Sleman Yogyakarta.

METHODS

Statement of ethics
This study was conducted and carried out in accordance with the recommendations of the Health Research Ethics Commission of the

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Received: 2022-07-15
Accepted: 2022-09-08
Published: 2022-10-12

1390 Open access: www.balimedicaljournal.org
Sample Collection
Samples were collected from the livestock area, Mlati District, Sleman Regency, Yogyakarta Special Region. Laboratory examinations for fecal specimens are carried out at the Parasitology Laboratory and Biomedical Laboratory, Faculty of Medicine, Sebelas Maret University. Fresh stool samples were taken in the morning after the barn was cleaned. Samples were collected within half an hour after the aseptic cleaning process. Stool samples were frozen at cryogenic temperature for immediate storage and then frozen to -80°C before further processing and analysis.

DNA extraction
Genomic DNA extraction was carried out using the QIAamp® Stool Mini Kit (Qiagen, Germany) according to the manufacturer’s recommendations. Nanodrop UV-Vis Spectrophotometer (Thermo Fisher Scientific) was used to determine the amount of genomic DNA.

PCR 16S rRNA and Genetic Sequencing
To characterize the resident microbial communities, we amplified and analyzed the 16S rRNA gene from the V3-V4 region with forward primers (CCTAYGGGRBGCASCAG) and inverted primers (GGACTACNNGGGTATCTAAAT). The amplification reaction mixture’s final volume was 50 ml, containing 5 ml of microbial genomic DNA, 5 ml of each primer, 25 ml of KAPA HiFi HotStart Ready Mix (KAPA Biosystems), and 10 ml of ddH2O. The following PCR program was used to obtain the product: 95°C for 10 minutes, followed by 25 cycles at 94°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 30 seconds, and a final elongation step at 72°C for 5 minutes. The product DNA fragments were 410 bp in size and analyzed using agarose gel electrophoresis to ensure the product’s quality and quality. The PCR product was then purified using AMPure XP beads according to the manufacturer’s instructions. Before sequencing, TruSeq DNA PCR-free Sample Preparation Kit (Illumina, USA) was used to create a sorting library. Samples were sorted using the Illumina HiSeq 2500 (Illumina) platform with 250 bp, pair running mode.

Sequence Processing and Analysis
The raw readings of the Illumina MiSeqPE250 were cleaned and assembled in the following steps. Barcodes and primary sequences were cut and raw labels were obtained and performed using FLASH (Magoc & Salzberg 2011) and QIIME (Version 1.7.0). UCHIME algorithm was used to remove chimeric sequences from raw tags and effective tags were obtained. We assessed community composition by classifying sequences (Uparsev7.0.1001) into operational taxonomic units (OTUs) determined by whether the 16S rRNA sequence similarity was greater than or equal to 97%. Finally, species annotation was performed using the GreenGene Database with the RDP classifier algorithm (Version 2.2). Microbial communities’ alpha diversity was determined using different indices (Chao1, ACE, Shannon, Simpson, Coverage) and calculated by QIIME (V1.7.0).

Furthermore, bacterial communities’ diversity was analyzed using rarefaction and rank-abundance plots and displayed using R software. Tukey and Wilcoxon tests were used for statistical analysis. Differences between samples were assessed using Principal Component Analysis (PCA), Principal Coordinate Analysis (PCoA), and non-metric multidimensional scale (NMDS) and displayed using R software. Distance-based methods, such as the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to perform cluster analysis based on the similarity and inequality of bacterial communities among samples. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to detect statistically significant differences in species between study groups. The next-generation sequencing dataset has been stored in the NCBI read sequence archive (SRA) with accession number: PRJNA511517.

RESULTS AND DISCUSSION
Respondents of this study consisted of 2 groups, namely 39 people in the group who were close to livestock (HCA) and their livestock (ANL) and ten people from the group far from livestock (HNC). Groups who lived far from livestock (HNC) were selected with the following criteria: residents of the Mlati District area, do not have cattle or goats, and have no history of livestock contact.

Table 1 shows that all respondents in this study were men with an average age above 50 years (53.62 ± 10.57). The HCA group’s average income was Rp. 500,000- Rp 1,000,000, while those in the HNC group were above Rp. 1,000,000. Respondents’ Body Mass Index (BMI) was on a normal scale in both groups.

Gut Microbiome analysis living at group close to livestock (HCA) and far from livestock (HCN)
Gut microbiome analysis was carried out on ten people from the HCA group and their livestock (ANL), which were selected based on randomization from 39 people living close to livestock. The randomization was conducted by taking the lottery number 10 times. Meanwhile, the analysis was also performed on ten people from the HNC group. The categorization of the sample and groups is described in Table 2.

Sequencing data overview
The total high-quality reads of 30 samples from HCA, ANL, and HNC stool samples as described above resulted in 4,764,330 samples. The total high-quality reads were further classified into 2,774 Operational Taxonomy Units (OTU) with a similarity of 97%.

Based on the OTU data, a rarefaction curve was generated (Figure 1). The shape of the curves that tend to be flat indicates that the sequencing data reflects the diversity of gut microbiome samples in the HNC, ANL, and HNC groups. Meanwhile, the rank abundance curve suggests that the ANL (livestock) group’s sample wealth is greater than HCA and HNC. Saturated rarefaction curves and the species richness
index shows that the sampling process is carried out comprehensively. In the rank abundance curve, the curve's wider range and smoothness represent a higher rank abundance. According to the curves, the ANL group (red color) has a higher quantity of bacterial species than the HNC group. On the other hand, the rank abundance curve (Figure 1B) is used to analyze community diversity, which shows the evenness and abundance of species in groups.

Differences of the Gut microbiome of the exposed group and the group without exposure to livestock Composition and number of microbionts

Based on the whole OTU species' annotation results in fecal samples in HCA and HNC were then classified into the appropriate bacterial domains, which included 24 phyla, 53 classes, 35 orders, 105 families, and 197 genera. Figure 2 shows the distribution of the gut microbiota composition at the phylum level. The dominant phylum in the HCA group was Bacteroidetes (57%) and Firmicutes (26%), while in the HCN group, both phyla were also dominated, but Firmicutes were the most dominant one (70%). Both Proteobacteria and Actinobacteria comprised only a minor portion of the whole microbial community.

The results of the OTU analysis are shown in Figure 3. In general, the bacteria that dominate the gut microbiome in all groups was Firmicutes phyla. The HCA group has a smaller proportion of the phylum Firmicutes compared to the HNC group. The dominant phyla include Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria.

According to the average abundance level microbial family in HCA, livestock (ANL), and HNC Group, the top 5 were Ruminococcaceae (29.2%), Lachnospiraceae (19.8%), Prevotellaceae (14.1%), Planococcaceae (4.0%), and Erysipelotrichaceae (3.2%). While in the HCA group, 72 bacterial families were identified from the sample with the top 10 including Ruminococcaceae (16.89%), Prevotellaceae (29.61%), Lachnospiraceae (24.40%), Planococcaceae (24.40%), Enterobacteriaceae (5.04%), Erysipelotrichaceae (4.62%), Succinivibrionaceae (2.79%), Bifidobacteriaceae (2.87%), Lactobacillaceae (1.19%), Rikenellaceae (0.52%) and Bacteroidaceae (0.47%). Lachnospiraceae, Ruminococcaceae, Planococcaceae, and Erysipelotrichaceae are members of the phylum Firmicutes, while Succinivibrionaceae, Enterobacteriaceae are included in the Proteobacteria phylum. Meanwhile, Bifidobacteriaceae are included in the phylum Actinobacteriaceae and

Table 1. General characteristics of respondents HNC (N=10).

<table>
<thead>
<tr>
<th>Variable</th>
<th>HCA (N=39)</th>
<th>HNC (N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n(%)</td>
<td>n(%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>39 (100)</td>
<td>10 (100)</td>
</tr>
<tr>
<td>Age (Years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50 years</td>
<td>13 (33.33)</td>
<td>-</td>
</tr>
<tr>
<td>-&gt; 50 years</td>
<td>26 (66.67)</td>
<td>10 (100)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Literate</td>
<td>1 (2.56)</td>
<td>-</td>
</tr>
<tr>
<td>Primary school</td>
<td>20 (51.28)</td>
<td>5 (50)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>18 (46.15)</td>
<td>5 (50)</td>
</tr>
<tr>
<td>Income Level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 500.000</td>
<td>13 (33.33)</td>
<td>-</td>
</tr>
<tr>
<td>500.000-1.000.000</td>
<td>6 (15.38)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>&lt;1.000.000</td>
<td>20 (51.28)</td>
<td>8 (80)</td>
</tr>
</tbody>
</table>

Note: HCA: group who live close to livestock; HNC: Group who live away from livestock

Table 2. Feces sample data and categorization.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Group</th>
<th>Sample Name</th>
<th>Group</th>
<th>Sample Name</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>A001</td>
<td>HCA</td>
<td>HA001</td>
<td>ANL</td>
<td>B001</td>
<td>HNC</td>
</tr>
<tr>
<td>A003</td>
<td>HCA</td>
<td>HA003</td>
<td>ANL</td>
<td>B002</td>
<td>HNC</td>
</tr>
<tr>
<td>A005</td>
<td>HCA</td>
<td>HA005</td>
<td>ANL</td>
<td>B003</td>
<td>HNC</td>
</tr>
<tr>
<td>A014</td>
<td>HCA</td>
<td>HA014</td>
<td>ANL</td>
<td>B004</td>
<td>HNC</td>
</tr>
<tr>
<td>A015</td>
<td>HCA</td>
<td>HA015</td>
<td>ANL</td>
<td>B005</td>
<td>HNC</td>
</tr>
<tr>
<td>A023</td>
<td>HCA</td>
<td>HA023</td>
<td>ANL</td>
<td>B006</td>
<td>HNC</td>
</tr>
<tr>
<td>A026</td>
<td>HCA</td>
<td>HA026</td>
<td>ANL</td>
<td>B007</td>
<td>HNC</td>
</tr>
<tr>
<td>A028</td>
<td>HCA</td>
<td>HA028</td>
<td>ANL</td>
<td>B008</td>
<td>HNC</td>
</tr>
<tr>
<td>A031</td>
<td>HCA</td>
<td>HA031</td>
<td>ANL</td>
<td>B009</td>
<td>HNC</td>
</tr>
<tr>
<td>A032</td>
<td>HCA</td>
<td>HA032</td>
<td>ANL</td>
<td>B010</td>
<td>HNC</td>
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</table>

Table 3. Alpha Diversity Index antara kelompok HCA dan HNC.

<table>
<thead>
<tr>
<th>Alpha Diversity Index</th>
<th>observed_species</th>
<th>Shannon</th>
<th>Simpson</th>
<th>Chao1</th>
<th>ACE</th>
<th>goods_coverage</th>
<th>PD_whole_tree</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCA</td>
<td>1.017±</td>
<td>5.757.7</td>
<td>0.9344</td>
<td>1.140.420</td>
<td>1.174.077</td>
<td>0.9974</td>
<td>71.002.1</td>
</tr>
<tr>
<td>HNC</td>
<td>1.133.5</td>
<td>±126.48</td>
<td>±650.76</td>
<td>±156160.5</td>
<td>±161445.3</td>
<td>±0.00009</td>
<td>±10698.72</td>
</tr>
<tr>
<td>HCA &amp; HNC</td>
<td>0.059</td>
<td>0.00938</td>
<td>0.007</td>
<td>0.201</td>
<td>0.236</td>
<td>0.054</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Note: HCA: people live close to livestock; HNC: people live far away from livestock
can be further determined by measuring the distances of the weighted unifrac and unweighted unifrac. This distance is used to determine the coefficient of difference between samples and to form a heat map. Thus, the intragroup differences were slight but not significantly greater in the HNC group than in the HCA group (unifrac unifrac $P = 0.2619$, weighted unifrac $P = 0.1342$).

The interaction of the gut microbiome in HCA, ANL, and HNC groups was determined using phylogenetic analysis, which studies the similarities between samples and clustering analysis to determine the clustering tree. The method used was the Unweighted Pair-group with Arithmetic Mean (UPGMA) method, a hierarchical grouping method that is widely used in ecology for sample classification. The closest distance samples are grouped to form a new node (a new sample). The point of branching is $1/2$ of the two original samples.

**DISCUSSION**

In various studies related to microbiome gut analysis, most microbiota analyses did not use Next Generation Sequencing...
Figure 3. Gut microbiome composition in the HCA, ANL, and HNC groups at the phylum (A) and genus (B) levels.

Figure 4. UPGMA clustering analysis based on the Weighted Unifrac (A) and un-Weighted Unifrac (B) distance matrices.

(NGS), mainly due to cost-effectiveness issues. However, gut microbiome analysis is important because of its role as a mediator in the digestive system, immune system modulators, and pathogen control. The gut microbiome also plays a role in the host's defense aspect against invading pathogens, both through its microbial antagonistic effect and by the maturation of the intestinal immune system. The byproducts of bacterial metabolism are known to have an anti-microbial effect, contributing to pathogen inhibition in the host by forming surfactants.13

The protective mechanism of the normal gut microbiome is mainly through the effect of infection or inflammation. The balance of the microbiome (bacteria and viruses) can skew the inflammatory tendency. Skewing the inflammatory response may increase the risk of infection due to invading pathogens or the gut microbiome's pathological growth in the gut microbiota community. The infection will occur when the infectious agents penetrate the body's defense system, such as mucosal immunity or resistance to colonization by the normal gut microbiome.14,15 Colonization of the intestinal mucosa by enteric pathogenic bacteria induces an inflammatory
exposure to livestock. Our reports showed that the exposed and unexposed group gut microbiome's core bacteria consisted of the phylum Firmicutes, Actinobacteria, Tenericutes, Proteobacteria, and Bacteroidetes. Although it comprised a large fraction of total microbial diversity in both groups, the phylum composition was starkly different in both groups. Nevertheless, the population of bacteria in the human intestine is dynamic and influenced by several factors, such as genetics, environment, diet, age, drugs, and disease. The gut microbiome's existence is strongly influenced by several factors, both external and internal, such as the use of antibiotics, prebiotics, and probiotics. The gut microbiome also has a very important function for human health. Gut microbiome Firmicutes and Bacteroidetes play an important role in controlling and regulating physiological processes. If Firmicutes' ratio exceeds Bacteroidetes, the bacteria will convert lactate into butyrate, which benefits the mucosal defense system due to improved mucin synthesis and tight junctions formation. On the other hand, if the ratio of Bacteroidetes is higher, then the substrate (lactic acid) will be directed to succinate, acetate, and propionate metabolic pathways. Changes in the composition and function of the gut microbiome of the intestine will disrupt intestinal permeability, which enables lipopolysaccharide (LPS) to enter the blood circulation and triggers low-grade systemic inflammation.

Regardless of the findings, this study focused only on rural people who lived near livestock. Comparing these findings with gut microbial diversity in urban populations would provide a better picture to understand how the environment and animals’ presence alter the microbial diversity in the human population. Also, evaluating the baseline metabolic indicators will provide a link between the microbial diversity with the population metabolic tendency, which would help community health officials determine the risk level of a population toward chronic diseases.

**CONCLUSION**

To conclude, our findings indicated that the living distance from livestock alters response, which may decrease the gut microbiome's viability. For example, the presence of Enterobacteriaceae is known to cause total gut microbiota diversity due to inflammatory reaction induced by its presence. Analyzing stool samples by targeting the 16S rRNA gene using the Next-Generation Sequencing (NGS) method allows us to reach a consensus on the gut microbiota composition. The microbiota in healthy adults' large intestine is dominated by two phyla, namely Firmicutes and Bacteroidetes. On the other hand, the small intestine's microbiota composition is mainly composed of Streptococcus, Clostridium, and Veillonella.

This is the first study that analyzed the diversity of gut microbiome communities by using Illumina-based high throughput 16S rRNA sequencing in local people of a district in Java, according to their exposure to livestock. Our reports showed that the exposed and unexposed group gut microbiome's core bacteria consisted of the phylum Firmicutes, Actinobacteria, Tenericutes, Proteobacteria, and Bacteroidetes. Although it comprised a large fraction of total microbial diversity in both groups, the phylum composition was starkly different in both groups. Nevertheless, the population of bacteria in the human intestine is dynamic and influenced by several factors, such as genetics, environment, diet, age, drugs, and disease. The gut microbiome's existence is strongly influenced by several factors, both external and internal, such as the use of antibiotics, prebiotics, and probiotics. The gut microbiome also has a very important function for human health. Gut microbiome Firmicutes and Bacteroidetes play an important role in controlling and regulating physiological processes. If Firmicutes' ratio exceeds Bacteroidetes, the bacteria will convert lactate into butyrate, which benefits the mucosal defense system due to improved mucin synthesis and tight junctions formation. On the other hand, if the ratio of Bacteroidetes is higher, then the substrate (lactic acid) will be directed to succinate, acetate, and propionate metabolic pathways. Changes in the composition and function of the gut microbiome of the intestine will disrupt intestinal permeability, which enables lipopolysaccharide (LPS) to enter the blood circulation and triggers low-grade systemic inflammation.

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gut microbial diversity by changing the proportion of Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. The phylogenetic analysis also revealed a possible microbiome transmission from animals to people who live near livestock.

ETHICS APPROVAL
This study was conducted and carried out in accordance with the recommendations of the Health Research Ethics Commission of the Faculty of Medicine (KEPK) (No. 351 / UN27.06 / KEPK / EC / 2019).

AUTHOR CONTRIBUTION
All authors contributed equally in research and writing process of this article

CONFLICT OF INTEREST
All authors declared that there is no conflict of interest regarding this article

FUNDING
This research was self-funded and no third-party funding involved in this research.

REFERENCES
20. Valdes AM, Walter J, Segal E, Spector TD. Role of the gut microbiota in nutrition and health. BMJ. 2018;361:36-44. doi:10.1136/bmj.k2179