Comparative gut microbiota analysis of industrially polluted and cleaner regions

N S Pandupuspitasari[1], F A Khan[2], B W H E Prasetiyono[1], A Muktiani[1], Widiyanto[1]

[1] Animal Science Department, Faculty of Animal and Agricultural Sciences, Universitas Diponegoro
[2] Zoology Department, Faculty of Sciences, University of Central Punjab, Pakistan

Abstract

Ruminants gut microbiota is critical for fermentation and in turn it produce volatile fatty acids and other products which serve as major source of energy for ruminants. Gut microbiome composition varies with several factors such as diet, supplementary nutrients, environmental conditions, altitude and latitude. Industrial effluents disturbs the healthy microflora present in the gut of ruminants hence results in low productivity, quality of economic products and diseases in ruminants. The comparison of gut microbiota from selected industrially polluted and cleaner regions can provide important information and way to protect animals from the harmful effects for industrial pollutants. Environmental contaminants like heavy metals, persistence organic pollutants, pesticides, and food additives have been found to alter the structure of gut microbiota resulting in physiological dysfunction in the host. Total of 40 Dairy Cattle from four different zones, three of them are located in clean zones and one is located in polluted zone. NGS techniques such as Shotgun Metagenomic reveal the microbiota identification and functionally annotate those microbiota. Fecal samples were collected for detecting gut microbiota using Shotgun Metagenomic. *Prevotella paludivivens* were found in clean zone as much as 69%, and absent in polluted zone. The relative plenty of *Prevotella* was shown to be positively associated with NH3-N and butyrate concentrations. The higher butyrate content indicates a faster rate of fiber fermentation. The research helps to discover novel and potent prebiotics and probiotics that can be used in feed manipulation to improve the health and productivity of ruminants in industrially polluted areas.

Introduction

Microbial communities are omnipresent impacting hosts responses [1,2]. Gut microbiota has been implicated in several biological processes across different species wherein its role in dairy cows milk production and quality is of interest to scientific community [3]. Microbial products helps in food fermentation, methane and nitrogen emissions as well as in fibre-breakdown [1,4,5]. Volatile fatty acids are compounds formed by the process of microbial fermentation in the gut of ruminants that provides 70% energy source to the host [1,3,5,6]. *Selenomonas ruminantium* is responsible for propionate to succinate conversions in host animals. *Succinovibrinonaceae positively* impacts methane emissions [1,3,7] while *Methanobacter* converts methane to formate. *Bifidobacterium, Ruminococcus* and *Fibrobacter succinogen a gram negative bacteria* helps in cellulose digestion.

Host's metabolism influences the milk protein yield. *Fibrobacter* degrade the feed and produce products that are utilized by the host for metabolic pathways, for example *Clostridium sticklandii* produces NH3 whereas *Rosebura speciesresults* helps in butyrate production [8,9,10,11,12]. These end products are absorbed by host and transported to liver where gluconeogenesis occurs and glucose is produced. In dairy cows large amount of metabolites in blood stream leads to enhanced gluconeogenesis, in fact feed containing carbohydrates provides only 10% of body glucose requirements while remaining 90% are met by the process of gluconeogenesis, where propionate (a substrate) is converted through mitochondrial enzymes into oxaloacetate, which is metabolized to produce glucose that is influenced by a variety of gut microbiota such as *Solenomonas rucminantium, Ruminococcus flavefaciens, Succinomonas amylolytica, Anaerovibrio lipolytica, Selenomonas lactilytica* and *Megasphaera elsdenii.* They produce VFAs compounds like acetate, propionate and butyrate etc. These rumen microbes provide essential nutrients to host like vitamins, proteins, and other metabolites. Grains and feed additives like monensin increases the glucose or propionate level and decreases the fat level in milk [13,14,15,16]. Researchers reported that glucose is the limiting factor for fluconeogenesis. The Pantothenate which is produced by *Flavobacteria, Butyrivibrio and Fibrisolvens,* helps to metabolize diets rich in Sugar and Starch, and maintains the normal pH in host cows that converts the mitochondrial propionyl-coA-carboxylase, methymalonyl-coA mutase and part of TCA cycle to oxaloacetate, which metabolizes from phosphoenolpyruvate carboxykinase to phosphoenolpyruvate (PEP) and end product as glucose whereas improved gluconeogenesis is positively correlated to higher milk yields.

To explore gut microbiota, feces remains the most important source [17] and present day molecular biology techniques and next generation sequencing (NGS) help to identify complex microbial communities. Fecal microbial composition mainly depends upon diet [17], environment, ruminants pH [13,18,19] and health status (13,19). Kim and Wells meta-analysis report showed 10 phyla, 17 classes, 28 orders, 59 families and 110 genera that are present in the fecal microbiome of the cows [17]. The most common phyla reported in several studies are *Firmicutes* for example *Proteobacteria* and *Bacteroidetes* [17].

Scientific community is highly interested dietary interventions using plant extracts that positively influence useful microbial population in the gut. The feed influence microbiota in two ways by providing the direct exposure to microbes and or by manipulating nutritional components to already present bacteria that influence their colony formation [13], that does influence various classes of bacterial and archaeal groups such as methanobacters, fibre and fat digesters etc [13]. Dairy cow depends on microbes for obtaining various products for digestion of proteins, fibres, vitamins and minerals from the diet

[7,13], and the balance of these components are important for overall output in regards of quality and quantity of milk [13]. Saponins, polyphenolic compounds and essential oils have been observed to reduce methane emissions and enhance nitrogen utilization efficiency. Feed contributes to a stable disease-resistant microbiota, as well as an unbalanced microbial community, impairing intestinal barrier function and immunity. To provide evidence-based dietary approaches to prevent infections, researchers must first characterise the effects of dietary components on the host-microbe axis as it relates to gastrointestinal health.

Other than diet, water quality [13], temperature [13,19], altitude, latitude, flora and other factors are influencing host gut microbiota causing multitude of phenotypes. Decline in temperature to about (3^0C) during winter and increase in temperature to (4^0C) during summer other than normal temperatures were observed to negatively impact on microbial proteins up to 30%. Climate change is negatively impacting the dairy industry that needs measures to prevent the losses. Water quality specifically water pH and salt concentrations affect gut microbial composition. Probiotics does positively influence milk yield and their balance in gut microbiota is essential for enhanced milk production. In our present study we evaluate diet and environment effects on gut microbial composition across different ecological zones of Pakistan.

Materials and Methods

Fecal sampling Methodology

The fecal samples were collected from selected dairy animals. The samples of dairy cows were collected in a 50-ml conical centrifuge tubes. After collect in a tubes, labelled it properly, write Tag id on the tubes. After collection of samples, wrapped in sterile polypropylene containers, preserved in -20°C [20]. The samples were pooled for shotgun metagenomic sequencing and delivered ice box spectacular to the Laboratory.

Shotgun Metagenomics

Shotgun metagenomic sequencing is a powerful environmental sequencing approach that provides insight into community biodiversity and function. To keep the reliability of the data, quality control (QC) is performed at each step of the procedure. Library Construction was performed and genomic DNA was randomly sheared into short fragments. The obtained fragments were end repaired, A-tailed and further ligated with Illumina adapter. The fragments with adapters were PCR amplified, size selected, and purified. Latter the library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms, according to effective library concentration and data amount required.

Bioinformatic Analysis

The Raw Data contained a certain percentage of low-quality data after sequencing. To ensure the accuracy and reliability of the subsequent information analysis, quality control and host filtering of the raw data were carried out to obtain effective data (Clean Data); Then metagenome was assembled based on Clean Data after quality control of each sample, and put the unutilized reads of each sample together for mixed assembly to explore the information of low-abundance species of the samples. After making assembly gene prediction was carried out by MetaGeneMark based on the scaftigs which were assembled by single and mixed samples. The predicted genes were pooled together for dereplication to construct gene catalogue. Based on the Clean Data of each sample from the gene catalogue, the abundance information of the gene catalogue for each sample was obtained. In next steps taxonomic annotation was performed. The function of the coding sequence was inferred based on its similarity to sequences in the databases (KEGG, eggNOG, CAZy, etc). Doing this for all metagenomic sequences produced a profile that described the number of distinct types of functions and their relative abundance in the metagenome.

Results and Discussion

Comparative analysis of *Bacteroidetes* in Polluted and Clean zones

Comparative analysis between the two regions revealed the abundance of *Bacteroidetes* in Polluted and Clean. The *Bacteroidetes* which is the first abundant phyla among all. The data indicate that the *Prevotella paludivivens* which the most abundant 69% in Clean region and are 32% in Polluted region. The *Flavobacterium ummariense* which 0.85 present in Polluted and 2% present in Clean region. At the genus level *Prevotella* are more abundantly present in Clean region as compare to the regions of Polluted and *Prevotella* was significantly linked to a high-carbohydrate diet, help in cellulose and starch metabolism, as well as the formation of acetate, butyrate, and propionate, can help cows in the early stages of lactation increase their ruminal fermentation products and milk fat content [21].

Table 1. Comparative analysis of *Bacteroidetes* present in Clean and Polluted region

Phyla (*Bacteroidetes*)	Polluted	Clean
Flavobacterium	0.7%	2.95
Flavobacterium marinum	Absent	0.9%
Prevotella paludivivens	Absent	69%
Bacteroides fragills	0.7%	Absent
Prevotella oryzae	Absent	0.7%

The *Bacteroides* help their host to maintain their digestion and absorption in small intestine. *Bacteroides* play major role in fermentation and produce the short chain fatty acids in the body and it is responsible for degradation of dietary fiber. They can breakdown the complex molecules in to simple one in host and can produce the nutrients which are beneficial for the maintenance of health and prevent from disease. It is also produce VFA in rumens [22].

The rumen bacteria is important for the altering the plant lignans into mammalian lignans. Previous study shows different types of bacteria, especially the *Prevotella* spp. genus involve in the interconversion of the plant lignans into the mammalian lignans. Under most feeding regimens, *Prevotella* species, which are linked to colonic Bacteroides, are the biggest single bacterial group recorded from the rumen of cattle and sheep. *Prevotella* species which are a hereditary and physiologically heterogeneous group of bacteria in the rumen bacterial populations, and are predominate substantially in high-grain as well high-forage rumens [21].

For the production of succinate and fermentation end products, the *Prevotella* species may consume polysaccharides like starches and other polysaccharides which are not cellulosic in nature and the sugars which provide energy in the body. Further, *Prevotella* species exhibiting hemicellulolytic and proteolytic activity in the rumen play important role in digestion of pectin and the cell wall of polysaccharides, as well as protein and peptide metabolism. The relative plenty of *Prevotella* was shown to be positively associated with NH3-N and butyrate concentrations. The higher butyrate content indicates a faster rate of fiber fermentation [23].

Comparative analysis of *Firmicutes* in Polluted and Clean farm

To compare the abundance of the two farm groups of Polluted and Clean, shotgun metagenomics was employed. Differences in particular microorganisms of these two areas can be seen. The *Enterococcus* which are most abundant in Polluted farm (34%) as compare to the Clean farm (0.8%) and *Enterococcus* is the one of the most environmental caustic agent that is responsible for mastitis, can cause different types of diseases in both animal as well as human being that can cause disease because of its high degree of resistance to a variety of antibacterial agents, which is caused by both inherent and acquired mechanisms [24]. In comparison to Clean farm, which has a low abundance of *Enterococcus*, Polluted farm has a higher level of diseases. The *Clostridium* are also present 1% in Polluted as compare to the Clean farms and *Clostridium* group of bacteria produces exotoxins (poisons) that are extremely harmful to mammals. *Firmicutes bacterium* which is 25% in Polluted and 0.85% in Clean. Many bacteria which are unassigned in results are 4% in Polluted and 3% in Clean.

Table 2. Comparative Analysis Between Firmicutes Of Two Regions

Phyla (*Firmicutes*)	**Polluted %**	**Clean %**
Firmicutes bacterium	25%	0.85%
Unassigned Firmicutes	4%	3%
Enterococcus pseudoavium	34%	0.8%
Clostridium beijerinckii	1%	absent
Clostridium tyrobutyricu	Absent	0.9%

Enterococci are common bacteria and are abundantly present and associated with abiotic, biotic factor of ecosystem that have shown intrinsic resistance to a variety of first-line antimicrobials and the capacity to rapidly flourish resistance to antimicrobials such as quinolones, macrolides, tetracycline's, streptogramins, and glycopeptides, among others.

Enterococci perform many functions and prevent from disorders, any type of infection like infection in urinary tract, and any wound infections.

*Enterococcal i*nfection is cure by *Enterococcus faecalis* and *Enterococcus faecium*. *Enterococcal* infections have grown more difficult to treat due to their proclivity for developing resistance to a variety of medicine like antibiotics, most notably vancomycin [25].

Lactobacillus spp. from the vaginal tract have been shown to suppress urogenital pathogenic bacteria's development in a variety of ways, including the generation of organic acids that lower pH, but Mycoplasmas are susceptible to environmental alterations such as pH and osmotic fluctuations because they do not manufacture the cell wall [26].

Lactobacillus and *Bifidobacterium* species have been proven to protect against enteric infection. These beneficial microbes can be used as single or multi-strains and are made up of bacteria and yeast, among other microorganisms [27]. Multi-strain probiotics offer a wide range \of effects against infections caused by a variety of strains, and their synergistic adhesion effect may enhance probiotic therapeutic advantages. Probiotics are frequently used to improve gut health, prevent infectious diseases, and reduce diarrhea and bloating.

Lactobacillus dominated the microbial populations in well-fermented silages. *Lactobacillus* is well-known for its function in silage fermentation, where it produces LA, lowers pH, and decreases harmful bacteria. The amount of Lactobacillus was found to be greatest in F1-treated silage in this study, which might explain why it improved fermentation quality [28]. *Enterococcus* and *Weissella* are both cocci-shaped LABs that can start lactic fermentation early in the ensiling process. After 60 days of ensiling, *Enterococcus* primarily formed in the control silage, which was substantially higher than the *Lactobacillus* species-inoculated silage. *Diaslister sps* 34 percent in Clean region and 4% in Polluted region, according to the results.

Comparative Analysis Between Clean and Polluted bacteria

Comparative analysis between two groups indicates the abundance of bacteria which are present in Polluted and Clean regions. The three dominant phyla including *Bacteroidetes*, *Firmicutes* and *Proteobacteria* respectively are present in two regions. The *Bacteroidetes* which is the first most abundant phyla. In which *Bacteroides xylanisolvens* which is o.9% in Polluted and are absent in Clean. The *Prevotella paludivivens* are absent in Polluted but are abundantly present in Clean 32%. *Streptococcus parauberis* are 2% in Polluted and absent in Clean regions.

The *Firmicutes* are a source for digestion of components, after digestion they play key role in producing VFA in rumens body. The end product, which they produce metabolites citrate, propionate, isocitrate and SCFAs which can absorbed by ruminants in the body and after utilization they play important role in the ruminants including enhance the immunity, help in digestion, and can increase the milk yield in rumens [29].

The *Prevotella sp* play important role for the conversion of plant material into useful material which are beneficial to rumens body and have great impact on rumen digestion. They also can enhance the metabolic process and they are involved in protection from disease. The *Prevotella sp* can generate the lipopolysaccharide and ammonia in the ruminant's body which have positive impact. The *Prevotella sp* also involved in the production of other bacteria which can raise the immunity by increasing the T helper cells [30].

Table 3. Comparative Analysis Between Bacteria of Two Regions

Bacteria	**Polluted %**	**Clean %**
Bacteroides xylanisolvens	0.9%	Absent
Prevotella paludivivens	Absent	32%
Streptococcus parauberis	Absent	3%
Bacteroidales bacterium	2%	Absent
Bacteroides fragills	0.7%	Absent
Actinobacter	Absent	1%

The *Streptococcus* can maintain the homeostasis in ruminant's body and have positive effects on the digestive system, can increase the health and diet efficiency. It can be provide as a supplement which maintain internal body temperature and prevent body from gastrointestinal disease [31].

Conclusion

It is concluded by above set of evidence, that the populations of microbiota found in polluted and clean zones is vary. The comparative analysis, relative abundance and percentage between two zones indicate the presence and absence of microbiota and their functions and impacts in an animal. Results revealed that the plenty percentage of *Prevotella paludivivens* could be the answer to be the microbiota that is performing as a good cop. The relative plenty of *Prevotella* was shown to be positively associated with NH3-N and butyrate concentrations. The higher butyrate content indicates a faster rate of fiber fermentation. This research helps to discover novel and potent prebiotics and probiotics that can be used in feed manipulation to improve the health and productivity of ruminants in industrially polluted areas.

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