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Molecular detection reveals the diversity of gut microbiota among healthy individuals from Haryana villages: A comparative study to understand the role of diet in gut microbiota

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Abstract:

Aim: The aim of the study was to identify the diversity of gut microbiota and the role diet in shaping the gut microbiota of healthy individuals from rural region of Haryana, North India.

Methods: The study was conducted among healthy individuals within the age group 18-55 years from two villages (Kaliawas and Sultanpur) of Haryana. Data pertaining to their diet habits were recorded in predesigned questionnaire, after obtaining informed consent. Subjects were asked to provide stool sample and the samples were processed by both culture dependent and culture independent method to identify both aerobic and anaerobic bacterial isolates.

Results: Stool samples from both the villages were dominated with phylum Firmicutes (*Lactobacillus*), followed by phylum Actinobacteria (*Bifidobacterium*), Bacteroidates *(Prevotella* and *Bacteroides*), *Rhuminicoccus* and Proteobacteria (*E.coli*). Altered flora (*Salmonella* and *Yersinia*) was seen among preserved food consumers. Higher abundance of *Prevotella* and significant *Bacteroides* were found among vegetarians and non vegetarians respectively. Significant difference of *Lactobacillus* was seen among those individuals between both the villages.

Conclusion: To conclude, majority of the individuals from Haryana are dominated by beneficial bacterial flora due high intake of diary products, fermented food along with mixed diet. Present study suggests that individuals from rural region of Haryana might be counted as donar for Fecal microbiota transplant. It also emphasis on that more and more research needs to be conducted to understand the diversity and basic functional attributes of the gut microbiome of Indian population particularly from different regions of Haryana.

Keywords: Gut microbiota, Lactobacillus, Bifidobacterium, Diet, Anaerobic flora, Healthy gut, Fermented food, Dairy products, Preserved food

Introduction:

Gut microbiota plays a significant role within our body, by being involved in the development and growth of immunity and in the regulation of various fundamental metabolic pathways by producing several essential metabolites. Human gut consists of plethora of microbiota, not only bacteria but it contains viruses, fungi and protozoa. Various types of microbes are distributed throughout the gut with different functions depending upon the site. However, majority of microbes are present in distal gut where they contribute in the fermentation of undigested food components. Six microbial phyla contribute in the composition of gut microbiota which includes Bacteroidetes, Firmicutes, Fusobacteria, Actinobacteria, Proteobacteria and Tenericutes. Composition of gut microbiota varies at different age group, Actinobacteria and genus Bifidobacterium are most abundantly present in infant gut as compare to adult [1,2], while in adults Firmicutes and Bacteroidetes may constitute up to 90% of total gut flora.

Several factors like diet, geographical area, environment, lifestyle, frequency of eating, quantity of eating and various other factors plays a significant role in shaping the composition of gut microbiota. India consists of diverse geographical locations with various types of dietary habits, ethnicity and life style. Several studies conducted on diet habits and life style have reported various type of dominant gut microbiota among which presence of Firmicutes have been reported more from healthy individuals [3,4]. Along with Firmicutes, other bacteria like Bacteroidetes, Proteobacteria, Spirobacteria, Veruucomicrobia were also reported from healthy individuals [5,6]. Gut microbes are capable of producing a numerous types of products, the generation of which can be dependent on various factors, including luminal environment, particularly pH and nutrient availability [7] .

Drastic changes in the life style of people have led to alter in gut flora of human being. A lot of factors like poor diet management, lack of exercise, inadequate sleep, irrational use of antibiotics, consumption of preserved or processed food are can lead to the multiplication of transient flora, which could lead to dysbiosis. Diet includes consumption of food, which is expected to be associated with specific bacteria, and thus plays a major role in shaping the gut microbiota [8]. Some studies have been conducted to understand the variations in gut microbiota among the individuals from various geographical regions the factors responsible for diverse flora, however limited information is available about the gut microbiota composition of the individuals from rural region of North India. Present study was conducted to identify the diversity of gut microbiota of healthy individuals from rural region of Haryana, North India to understand the role of diet in shaping gut microbiota composition.

MATERIALS AND METHODS

The study was carried out with permission from the Institutional Ethical Committee (IEC) (ref no. IEC/FMHS/PhD/S/2022-11) for a period of one year from May 2022 to May 2023 at a tertiary care setup hospital in a peri-urban region of Haryana.

Geographical Region: Haryana is located in the northwest India, with an altitude between 700-3600 ft above sea level. The state is spread over an area of 44,212 sq. kms with population of nearly 211.45 lakhs**.** It comprises of various cities, villages and town. Present study was conducted in two villages of Haryana, Kaliawas and Sultanpur, which are rural areas of the State.

Subject Recruitment: Subjects who were willing to participate in the study, were included after obtaining a written consent. Healthy individuals between 18-55 years of both gender (males and females) were recruited with exclusion criteria for the following conditions:

1. Presence of any chronic illness (hypertension/diabetes/kidney disease, liver disease, heart disease or any kind of malignancy
2. Consumption of antibiotics in last 3 months
3. Consuming any other medications including steroids
4. Pregnant ladies
5. Persons having undergone any surgery
6. Presence of any gastrointestinal disorder (IBD/IBS).

A predesigned questionnaire pertaining questions related to sociodemographic factors and diet were recorded and sterile containers were distributed among the study participants and were told to collect their samples in the containers on the next day.

SAMPLE COLLECTION AND PROCESSING

A total of 100 Stool sample from two villages (50 from each village Kaliawas and Sultanpur Village, Haryana) were collected respectively. The samples were immediately transferred to microbiology laboratory for processing. Both macroscopic and microscopic examination of samples were done followed by the identification of aerobic bacteria by conventional aerobic culture technique. For the identification of anaerobic bacteria; microbial genomic DNA was isolated following the standard procedures of the QIAamp DNA mini kit (catalog numbers 51306) with some modifications (Qiagen GmbH, Hilden, Germany). Bacterial DNA was identified by targeting the 16Sr RNA gene amplification by using universal primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-39) and 149R (5'- GGTTACCTTGTTACGACTT-39) (5), followed by the identification of genus *Lactobacillus, Bifidobacterium, Bacteroides, Fusobacterium, Clostridium, Prevotella and Rhuminicoccus* followed by using genus specific primers respectively as shown in table 1 (Biologia Research India Pvt. Ltd).

Each reaction mixture (23µl) contained 3µl of template DNA, 1µl of each primer (pM), 9µl of master mix (Hi-chrom PCR master mix, product code MBT089-100R), and 12µl of nuclease-free water. The PCR reactions were carried out with the following conditions: initial denaturation at 94°C for 1 minute, 37 cycles of 94°C for 30 seconds, 60°C (for *Bifidobacterium*), 55°C (for *Lactobacillus & Prevotella*), 52°C(*Bacteroides*), 53°C (*Fusobacterium* & *Clostridium*), 61°C (*Rhuminicoccus*) for 30 seconds, 72°C for 2 minutes, and a final extension at 72°C for 2 minutes in a T100 Thermal Cycler (BioRad). The PCR products were visualized in a 1.2% agarose gel alongside a 100bp DNA ladder (Hi Media Laboratory Pvt Ltd), and images were captured using the Gel Doc EZ imager (BioRad) as shown in:

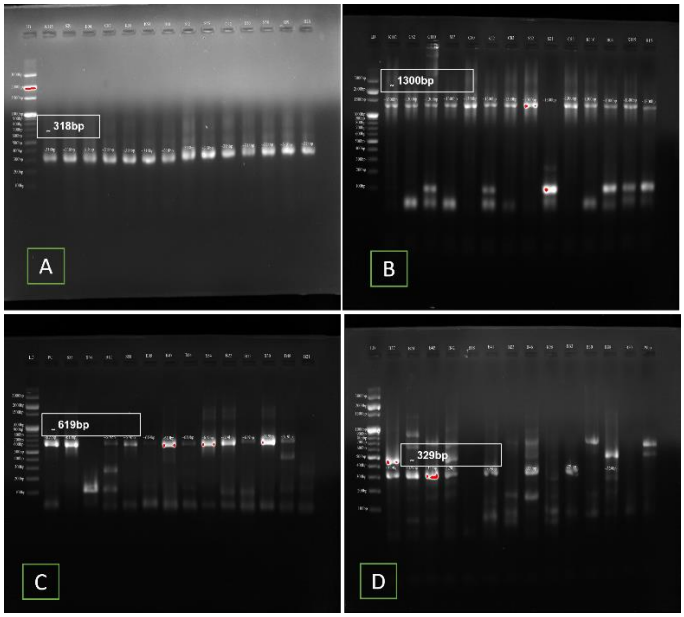


Fig 1: PCR gel images of A. Lactobacillus, B. Bifidobacterium, C. Clostridium, D. Fusobacterium

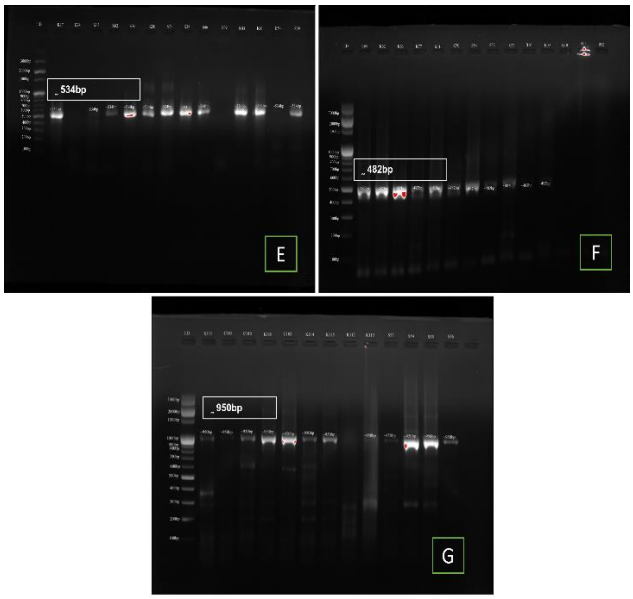


Fig 2:PCR gel images of, E. Prevotella, F. Rhuminicoccus, G.Bacteroides

DATA CALCULATION

All the data of the studied subjects was recorded on MS Excel, and the data graphs were also prepared using MS Excel. The statistical analysis was carried out using SPSS version 29.

Results:

Study population and information about diet: Out of the total 100 individuals (50 individuals from each village Kaliawas and Sultanpur), differences in their diet pattern was observed such as more number of nonvegetarians were found from the village Kaliawas 74% (37/50) as compare to Sultanpur 36% (18/50). Regular milk and milk products consumers were found high from both villages, Kaliawas 94%(47/50) and Sultanpur 96% (48/50). Consumption of regional fermented food was also seen to be higher in both Kaliawas 98%(49/50) and Sultanpur village 96% (48/50). Regular curd consumers were observed moderate in both the villages Kaliawas 48% (24/50) and Sultanpur 42% (21/50). However, high junk food consumers were found from the village Kaliawas 46% (23/50) as compare to Sultanpur 26%(13/50). Preserved

food consumers were found high in Sultanpur 76% (38/50) village as compare to Kaliawas 68% (34/50) The comparison of diet pattern between two villages are mentioned in Table 1:

Type of Diet	Kaliawas N=50		Sultanpur N=50	
	No. of Cases	%	No. of Cases	%
Preserved food	34	68%	38	76%
Vegetarian	13	26%	32	64%
Non vegetarian	37	74%	18	36%
Regular curd intake	24	48%	21	42%
Consumption of Probiotics	15	30%	22	44%
Consumption of milk	47	94%	48	96%
Fermented food	49	98%	48	96%
Frequent left-over / stale food consumer	17	34%	19	38%
Junk food consumer	23	46%	13	26%
Sufficient water intake (2-4L/day)	32	64%	45	90%
Raw Food Consumer (Frequent)	15	30%	14	28%

Table 1: Comparison of diet pattern of the individuals from Kaliawas and Sultanpur Village, Haryana

ota was investigated at the level of genus, by using genus specific primers for anaerobic flora and conventional culture techniques for aerobic flora. By using both culture dependent and independent techniques 16 genus of bacteria was identified from stool samples. Overall the fecal microbiota of both villages were dominated by Firmicutes followed by Bacteroidate.

Overall presence of *Lactobacillus* in both the villages were found to be 93% (93/100), followed by *Bifidobacterium* 89% (89/100), *Prevotella* 83% (83/100), *Rhuminicoccus* 78% (78/100), *Bacteroides* 74% (74/100), *Clostridium* 66% (66/100), *Fusobacterium* and *E.coli* 52% (52/100), *Enterococcus* 51% (51/100), *Streptococcus* 48% (48/100), *Staphylococcus* 28% (28/100), *Candida* 24% (24/100), *Salmonella* 19% (19/100) and lowest presence of *Yersinia* 9% (9/100) was observed in subjects from both the villages.

By comparing the fecal microbiota of both the villages Kaliawas and Sultanpur, it was found that presence of *Lactobacillus, Bifidobacterium, Fusobacterium, Clostridium*, *Bacteroides, Rhuminicoccus, Enterococcus, Streptococcus, E.coli*, *Staphylococcus* and *Candida* were high in Kaliawas as compare to the individuals from Sultanpur Village. Notably, *Klebsiella*, *Salmonella* and *Yersinia* were found relatively high in Sultanpur village. Distribution and comparison of fecal microbiota along with their statistical significance in mentioned in table 2:

Name of Bacteria	Kaliawas N=50		Sultanpur N=50		Phylum	Abundance status in present study and P value
	Total No. of Isolates	%	Total No. of Isolates	%		
Lactobacillus	50	100%	43	86%	Firmicutes	Reduced in Sultanpur subjects

						P value-0.006*
Bifidobacterium	46	92%	43	86%	Actinobacteria	Reduced in Sultanpur subjects P value-0.337
Prevotella	41	82%	42	84%	Bacteroidetes	Reduced in Kaliawas subjects p value-0.791
Fusobacterium	28	56%	24	48%	Fusobacteriota	Reduced in Sultanpur subjects P value-0.424
Clostridium	36	72%	30	60%	Firmicutes	Reduced in Sultanpur subjects P value-0.206
Bacteroides	42	84%	32	64%	Bacteroidetes	Reduced in Sultanpur subjects **P value-0.023***
Rhuminicoccus	46	92%	32	64%	Actinobacteria	Reduced in Sultanpur subjects **P value-0.001***
Enterococcus	*29*	58%	*22*	44%	Firmicutes	Reduced in Sultanpur subjects P value-0.689
Streptococcus	*24*	48%	*24*	48%	Firmicutes	Reduced in Sultanpur subjects P value-0.68
E.coli	*38*	76%	*32*	64%	Proteobacteria	Reduced in Sultanpur subjects P value 0.23
Staphylococcus	*18*	36%	*10*	20%	Firmicutes	Reduced in Sultanpur subjects P value-0.134

Bacillus	*14*	28%	*16*	32%	Bacillota	Raised in Sultanpur subjects P value-0.603
Klebsiella	*2*	4%	*6*	12%	Pseudomonadota	Reduced in Kaliawas subjects P value-0.24
Candida	*13*	26%	*11*	22%	Ascomycota	Reduced in Sultanpur subjects P value-0.603
Salmonella	*7*	14%	*12*	24%	Pseudomonadota	Reduced in Kaliawas subjects P value-0.137
Yersinia	*0*	0%	*9*	18%	Pseudomonadota	Absent in Kaliawas Subjects **P value-0.006***

Table 2: Comparison of isolates of bacteria from the stool samples of the individuals, from Kaliawas and Sultanpur Village, Haryana

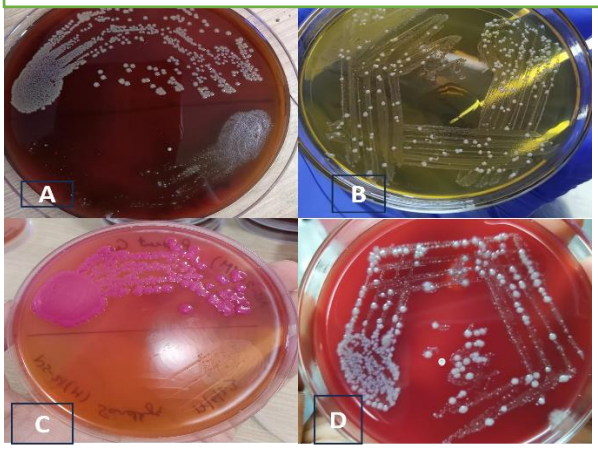


Fig 3: Aerobic culture growth A. Growth of bacteria on Blood agar, B. Growth of Lactobacillus on MRS agar, C. Growth of E.coli on MacConkey, D. Growth of mixed isolates on Blood agar.

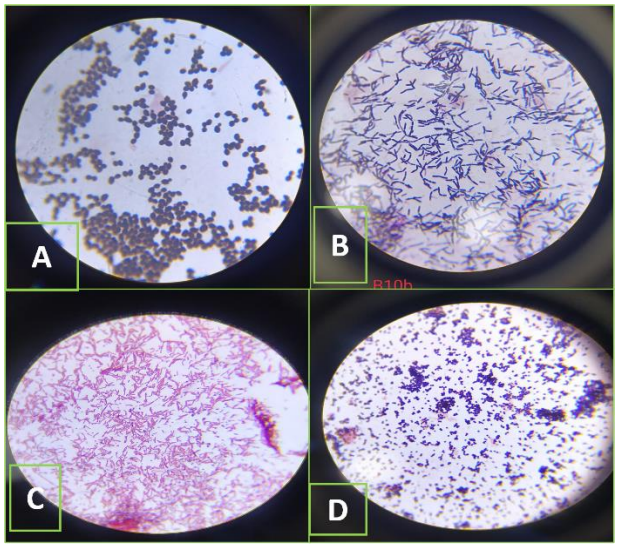
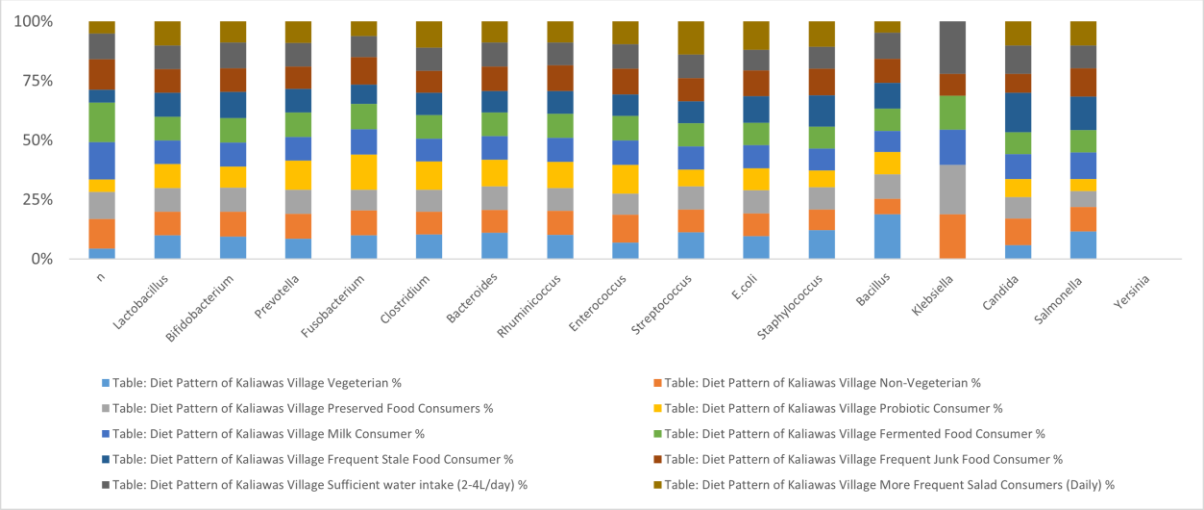
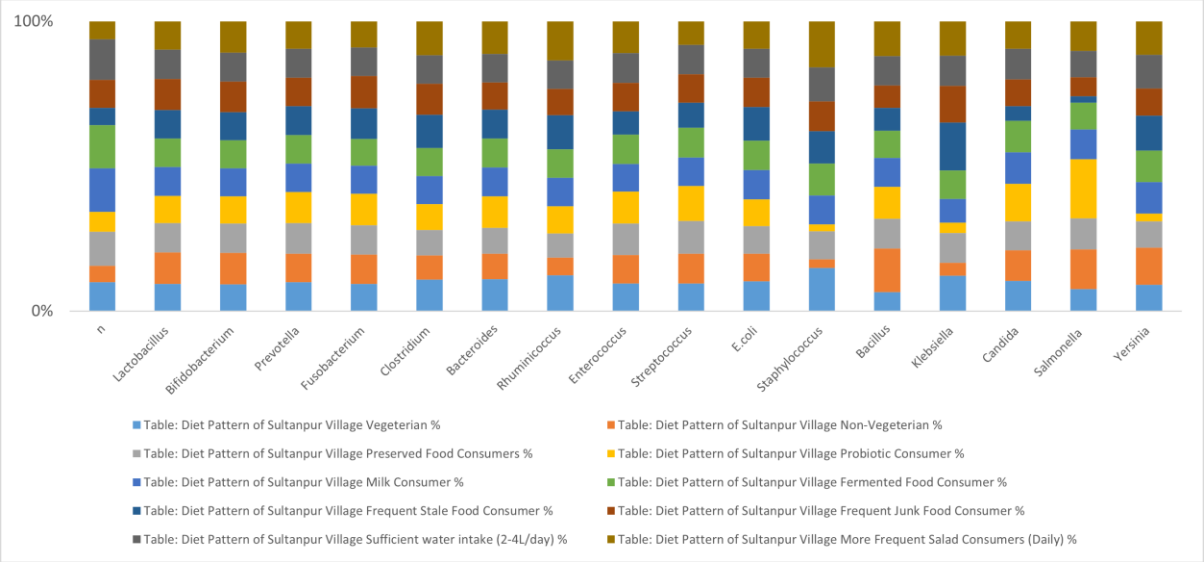


Fig 4: Microscopic identification of Bacteria by Gram stain, A. Microscopic view of Candida, B. Gram positive bacillus, C. Gram negative rods, D. Gram positive cocci

Association of diet and fecal microbiota: Different diet pattern contribute a lot in shaping the composition of the gut microbiota, for this purpose the association between food type and abundance of bacteria were analysed. Highest percentage of mixed diet eaters which includes both vegetarian food and non-vegetarian food were observed among the individuals belong from the village Kaliawas, whereas in Suntanpur, individuals were observed to consume more vegetarian based diet. Subjects from both the villages used to consume milk, curd and fermented food almost on regular basis, therefore it was observed that *Lactobacillus* (100%) and *Rhuminicoccus* (92%) were found highest in both vegetarians and non-vegetarians. Furthermore, in vegetarian subjects *Bacteroides (*92.3%) were in high abundance. We found that *Enterococcus*, *E.coli* were found to be high among stale food eaters, preserved food eaters and junk food eaters, whereas *Klebsiella and Salmonella* showed a higher prevalence in non-vegetarian individuals. The detailed distribution of bacteria in comparison with different food type is mentioned in Graph 1 and Graph 2.



Graph 1: Abundance of bacterial genus of individuals from Kaliawas Village (Haryana) on the basis of diet



Graph 2*:* Abundance of bacterial genus of individuals from Sultanpur Village (Haryana) on the basis of diet

Lactobacillus, Bifidobacterium and *Prevotella* were found to be present in high abundance amongst the people used to consume milk, curd, probiotics and fermented food on regular basis. Among the frequent stale food eaters *Clostridium*, *Bacteroides, Rhuminicoccus* and *E.coli* were found to be present in high abundance compared to other bacteria. In preserved food eaters *Fusobacterium* , *Clostridium* , *Enterococcus* and *Staphylococcus* were found in less abundance compare to others.

Comparison of overall diet and fecal microbiota in two villages of Haryana:

Individuals from kaliawas village used to consume more non vegetarian diet, junk food, milk, curd and comparatively less consumption of stale food, preserved food and probiotics as compare to Sultanpur village subjects. On the other side subjects from Sultanpur village used to consume more preserved food, stale food, alcohol and probiotics as compare to Kaliawas village. In terms of fecal microbiota, overall high count of *Lactobacillus, Bifidobacterium, Fusobacterium, Clostridium, Bacteroids and Rhuminicoccus* were isolated from the fecal samples of Kaliawas village residents, in contrast *Staphylococcus, Salmonella, Yersinia and Klebsiella* were isolated more from the fecal sample of the subjects belong to Sultanpur.

Discussion:

Human gut contains a diverse group of microbiota. Distal portion of gut is considered to be one of the most metabolically active organ in human body, which plays a vital role to process the dietary fibres by utilizing the metabolites produced by the gut microbiota [9] Gut microbiota also play an important role in metabolism and immune functions of host [10].

Lot of factors are responsible in influencing the composition of gut microbiota, people having different diet have got different composition of microbiota, similarly gut microbiota also varies at different stage of age group, geographical area, life style pattern, chronic illness, still a very limited data is available about the composition of gut microbiota from different geographical region of India. In our study among both the villages, we found overall highest abundance of *Lactobacillus* 93% (Phylum Firmicutes) over any other genus. Genus *Lactobacillus* was seen significantly high in the population of Kaliawas village 100% as compare to Sultanpur village 86%, which is similar finding by Arumugam et al, in which they have also stated high

abundance of Firmicutes over Bacteroidetes[11]. However in few studies it have reported that the gut microbiota of the Indians are dominated by the phylum *Bacteroidates* [12,13,14]. Additionally it was also observed in our study that transient flora like *Salmonella*, *Yersinia*, *Klebsiella* were seen comparatively less in Kaliawas village as compare to Sultanpur village. One of the possible reason could be that *Lactobacillus* protects the intestinal barrier from infection by alter the luminal pH by producing lactic acid and also it produces certain other barrier related proteins like short chain fatty acids (SCFA), hydrogen peroxide, and bacteriocins which in further inhibit the growth of transient flora [15]. Overall in both the villages, we observed the second highest genus were *Bifidobacterium* 89%, followed by *Prevotella* 83%, which was concordant result finding by Parijat Hazarika et.al where they have stated high abundance of *Bifidobacterium* and *Prevetolla* from the tribal population of Arunachal Pradesh [16]. Similarly in a study conducted by Sekene K. et al, Rowland I. et al, Rafter J. et have stated the potential effect of Bifidobacterium along with prebiotics in reducing the potency of carcinogen in mice model [17,18,19,20].

In Sultanpur village *Prevotella* was comparatively high i.e 84% than Kaliawas 82%. One of the possible reasons could be individuals from Sultanour village used to consume more plant based(vegetarian) diet than Kaliawas. This finding supports the observation of Wu GD et al. who have also mentioned that *Prevotella* species are more prevalent in non-Western populations likely due to its association with high fibre and low fat diets [21,22]. A metagenomic study from India has also revealed the association of *Prevotella* species with plant based diet [23,24]. Similarly study conducted on western population like US migrant individuals and Spain have revealed low abundance of *Prevotella* species [25,26]. *Prevotella* spp. have also been found to predominate over *Bacteroides* spp. in the microbiota of rural communities including hunter gatherers [27,28] due to more intake of fat rich diet, which is similar to the present study.

In several studies it has been mentioned that *Bacteroides* have been positively correlated among non vegetarians due to consumption of long term diet rich in animal protein., which may be due to their ability to tolerate bile which is common in the gut environment of non vegetarians [29,30]. In the present study similar findings were obtained in which *Bacteroides* were seen significantly high among the individuals from Kaliawas village 84% as compare to Sultanpur village 64%.

In this study we have also found a significant high abundance of Rhuminicoccus in Kaliawas village 92% as compare to Sultanpur village. Probable reason could be individuals of Kaliawas village used to consume mixed diet which contains both plant based (vegetarians) and animal rich proteins (non vegetarians), whereas majority subjects from Sultanpur village only used to consume vegetarian diet. Similar findings was stated in a review study by Aleksendra T et. Al in 2019 [31]. A significant high number of *Yersinia* isolates were identified from Sultanpur village (14%), whereas in Kaliawas not a single isolate of *Yersinia* were found, one of the probable reason could be frequent consumption of stale food and junk food. Study conducted by Guillame LB et at 2018 have stated the presence of Yersinia in Crohns disease patients [32], It has been mentioned in previous study that *Yersinia* is psychotropic in nature and thus can survive in cold temperature, therefore even in refrigerated food they could be viable. E.coli was seen high in abundance among the people from Kaliawas as compare to Sultanpur. Although their pathogenic potential and antibiotic resistant genes were not identified in this study. In

present study, 20% and 16% presence of *Candida* was reported in this study from the village Kaliawas and Sultanpur respectively. In several studies it has been reported that presence of *Candida* in gut microbiota is quite normal, only if it is present in less number. Overgrowth of candida can result to cause several gastrointestinal disorders.

Salmonella and Klebsiella were highly present in amongst non vegetarians and preserved food eaters, in the present study both the *Salmonella* and *Klebsiella* were found high abundance amongst Sultanpur population as compare to Kaliawas. In a report from CDC, it has been stated that *Salmonella* can be found in a variety of foods, including chicken, beef, pork, eggs, fruits, vegetables, and even processed foods [33].

Conclusion:

To conclude, subjects from Haryana were observed to be consumed more milk products, curd, regional fermented food, probiotics as well as diet containing both plant based and animal based protein, due to which higher abundance of Lactobacillus, Bifidobacterium were found. Gut microbiota of vegetarians were also dominated with Prevotella as compared to nonvegetarians where dominance of Bacteroides and Clostridium were found. Which indicates gut microbiota of subjects from rural region is enriched with good bacteria, due to following a healthy diet pattern. Although few number of transient flora like Salmonella and Klebsiella were identified from non vegetarians, preserved food consumers and isolates of Yersinia was identified from frequent stale food consumers. Present study could be included as a reference study, where the diversity of gut microbiota of different disease conditions (obesity, hypertension, diabetes, Inflammatory Bowel syndrome, Crohns disease, colitis) can be compared. However, this study suggests that individuals from rural region of Haryana might be counted as donar for Fecal microbiota transplant. It also emphasis on that more and more research need to be conducted to understand the diversity and basic functional attributes of the gut microbiome of Indian population particularly from different regions of Haryana to get more significant prevalent gut microbiota.

DATA AVAILABILITY:

Supplementary data will be made available.

ETHICAL APPROVAL:

The study was carried out with permission from the Institutional Ethical Committee (IEC)-number- IEC/FMHS/PhD/S/2022-11

CRediT authorship contribution statement:

Suchandra Gupta: Conceptualization, Data curation, Writing-original draft, Writing- review and editing, Visualization, Investigation, Validation, Formal analysis, Methodology.

Manisha Khandait: Conceptualization, Writing-original draft, Writing- review and editing, Visualization, Formal analysis, Methodology

Sandhya: Conceptualization, Data curation, Writing-original draft, Writing- review and editing, Visualization, Investigation, Validation, Formal analysis, Methodology.

Rakesh Sehgal: Formal analysis, Supervision, Writing- review and editing

Sunil Chamola: Formal analysis, Validation

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CONFLICT OF INTEREST

The authors declares that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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