

# Metagenomic analysis of total microbial diversity and antibiotic resistance of culturable microorganisms in raw chicken meat and mung sprouts (*Phaseolus aureus*) sold in retail markets of Mumbai, India

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**Raw chicken meat and ready-to-eat sprouts are potential sources of food-borne infections. Development and spread of antibiotic resistance (AR) in microflora associated with food is a major health concern. In this study, we employed culturable and non-culturable methods to characterize microflora associated with chicken meat and mung. Pathogens belonging to Enterobacteriaceae were dominant in the culturable set. Rare species like *Citrobacter amalonaticus*, *Kluyvera georgiana*, *Kurthia gibsonii* and *Staphylococcus hominis* were isolated and metagenomic study revealed overall good species richness in both food types, *Firmicutes* and *Gammaproteobacteria* were dominant phyla in chicken meat and sprouts respectively. Common food-borne and opportunistic pathogens like *Campylobacter*, *C. perfringens*, *Streptococcus*, *Shewanella*, *Pseudomonas*, *Aeromonas hydrophila*, *Staphylococcus*, *E. coli*, *Acinetobacter*, *Enterobacter*, *Klebsiella* were detected and 18% of the genera were common to both food types. We observed high AR bacterial count (5 to 9 log CFU/g) in the microflora. Fifty AR isolates per food type were identified with high multiple AR index of 0.3–0.9.**

**Keywords:** Chicken meat, food-borne pathogens, metagenomics, multiple antibiotic resistance, mung sprouts.

DEVELOPING countries lose billions of dollars in terms of medical costs on illnesses and death caused by food-borne pathogens<sup>1</sup>. Chicken, consumed worldwide, is also a major source of food-borne pathogenic outbreaks like Salmonellosis<sup>2</sup>. Bulk of the poultry products consumed in India comes from small unorganized open air retail shops with poor sanitation and personal hygiene. Overcrowded cages and drinking water contaminated with faecal matter are responsible for the spread of contamination. Trans-

mission of pathogens via under-cooked food and cross-contamination<sup>2</sup> pose a risk to human health.

Other than chicken, produce like fruits (e.g. melons, tomatoes, mangoes, strawberries) and mung sprouts are also responsible for major food-borne outbreaks<sup>3,4</sup>. There are many reported outbreaks caused by *Salmonella*, *Yersinia enterocolitica*, *Aeromonas hydrophila*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli* O157:H7 and non-O-157 *E. coli* related to consumption of alfalfa and mung bean sprouts confirming their role as carrier of pathogens<sup>5,6</sup>. In urbanized cities like Mumbai, many roadside eateries displaying raw salads, cut fruits and sprouts are very popular. Lack of basic hygiene in handling, usage of contaminated water in washing of food and utensils and menace of flies in such open areas<sup>7</sup> may lead to high microbial contamination. Often, such food is eaten raw and hence there is a high probability of infection with potential food pathogens.

While study of food-associated pathogens is clinically relevant, it is also important to study the composition of the total microbial flora. Culturable methods give an aerobic count of microbial load carried by food items indicating the shelf life and quality of food consumed. It can also give insight into the nature of microbes present, their pathogenicity, biofilm forming ability and antibiotic resistance (AR) pattern<sup>8,9</sup>. However, culturable organisms constitute only 1% of total flora while the rest comprises diverse species including poorly characterized and ignored anaerobes which might carry AR traits. As commensal microorganisms outnumber pathogens in food microbiota, they act as a reservoir of AR genes that can be potentially transmitted to pathogens by means of horizontal gene transfer (HGT) events<sup>10,11</sup>. Such non-culturable bacteria are studied by high throughput next generation sequencing (HT-NGS) providing in-depth analysis and larger coverage, enabling detailed and complex analysis of environmental communities<sup>12</sup>.

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In India, there has been a significant rise in the use of antibiotics in clinical practice and agricultural settings including crop, animal and fish farming<sup>13,14</sup>. About 80% of the antibiotics used in animal farming are applied to poultry industry<sup>15</sup> which may become a major source for emergence of AR bacteria. A major concern would be dissemination of AR traits across countries via travel and migration; a good example being worldwide spread of organisms producing New Delhi Metallo- $\beta$ -lactamase-1<sup>16</sup>.

This study was undertaken to estimate the microbial load, the total microbial profile and distribution of AR in the microbial population for two of the most commonly consumed food varieties. There is a high probability of cross-contamination between food from different sources especially chicken and sprouts<sup>5</sup>. Application of animal manure to crops, waste water run-off from animal farms into fields, use of same equipment and utensils for chicken and vegetables, unhygienic handling are all probable sources of cross-contamination<sup>5,8,17,18</sup>. Hence it is important to study these two food sources together for dissemination of pathogens and their resistant traits.

## Materials and methods

### Sample collection

The overall experimental work conducted is represented in [Supplementary Figure S1](#). A total of 14 chicken meat and 13 ready-to-eat (RTE) sprouted mung bean samples were procured from different retail poultry shops and local street vendors respectively from Mumbai ([Supplementary Table S1](#)). The colour, pH and texture of both sample types are mentioned in [Supplementary Table S2](#). To avoid variations in day temperatures and post-slaughter timings, chicken breast and leg samples were collected between 12 pm and 2 pm within 2–3 h of bird slaughter. Since microbial flora proliferates favourably during 72 h of sprouting stage owing to moisture content and optimum temperature of 21°C to 25°C (ref. 5), 72 h sprouted mung beans were taken. Meat and sprout samples were transported to lab in autoclaved glass bottles kept in ice within 90 min of procurement and processed immediately.

### Sample processing, estimation of total microbial load and antibiotic resistant bacterial count

Samples were thoroughly washed to remove surface micro-organisms and 12.5 g of sample was weighed, macerated in 112.5 ml of 0.85% (w/v) NaCl and kept for agitation at 200 rpm, 37°C for 2 h under aseptic conditions. Appropriate dilutions of the homogenate were spread on plate count agar for determination of total bacterial count. Lower dilutions of the supernatants ( $10^3$ -fold and  $10^5$ -fold for chicken and sprout respectively) were

spread on antibiotic plates (bacitracin, ciprofloxacin, doxycycline, erythromycin and trimethoprim) to determine total AR bacteria.

### Antibiotic susceptibility test

Multiple antibiotic resistance (MAR) is defined as resistance to a minimum of 4 antibiotics tested<sup>19</sup> while MAR index is calculated as the ratio of number of antibiotics to which organism is resistant to total number of antibiotics to which organism is exposed. To analyse MAR patterns in food microflora, 50 AR colonies per food type were isolated, purified and antibiotic susceptibility test was carried out thrice following Clinical and Laboratory Standards Institute (CLSI) guidelines by using commercial antibiotics ( $\mu$ g or units/disc) (Himedia Laboratories, Mumbai, India), viz. bacitracin (10 units), ciprofloxacin (5), chloramphenicol (30), cefotaxime (30), doxycycline (30), erythromycin (15), gentamicin (50), kanamycin (5), neomycin (30), penicillin G (10 units), rifampicin (30), streptomycin (25) and trimethoprim (10)<sup>20,21</sup>. Also, the minimum inhibitory concentration (MIC) of the above antibiotics for 18 pathogenic micro-organisms representing different genera was determined by broth micro-dilution method according to CLSI guidelines. For quality control *E. coli* ATCC 25922 (MIC and disc diffusion) and *S. aureus* ATCC 29213 (MIC) were used. Resistance was assessed according to clinical breakpoint tables of European Committee on Antimicrobial Susceptibility Testing (EUCAST)<sup>22</sup>.

### 16S rRNA sequencing

Crude DNA was isolated from all the 100 AR isolates by boiling method as described earlier<sup>23</sup>. Forty microlitre reaction mix was set up using 20  $\mu$ l 2x Taq Master Mix (Himedia Laboratories, Mumbai, India), 1.5  $\mu$ l each of 16 S forward and reverse primers at 10 pico mole concentration, 1.5  $\mu$ l of crude DNA as template and volume made up with sterile distilled water. The cycling condition used was: initial denaturation at 94°C for 10 min followed by 35 cycles of amplification with denaturation at 94°C for 20 s, annealing at 50°C for 30 s, extension at 72°C for 40 s and final extension at 72°C for 5 min. The expected product size was 457 bp. The primer sequence used was 16S rRNA F-Primer: 5'-GGAGGCAGCAGTAAGGAAT-3' and R-Primer: 5'-CTACCGGGGTATCTAATCC-3'. The primers covered V3 and V4 regions of 16S rRNA gene<sup>24</sup>. PCR products were sequenced at Xcelris Labs Pvt Ltd (Ahmedabad, India). Sequence identity was determined using the BLASTN ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)). Sequences were deposited in the GenBank database using the web-based data submission tool, BankIt (<http://www.ncbi.nlm.nih.gov/BankIt/>). The accession numbers for bacterial isolates from chicken meat

samples are KX355642–KX355691 and for isolates from mung sprouts are KX355692–KX355741.

### *Metagenomic DNA extraction, 16S rRNA metagenomic sequencing and analysis*

The food homogenate was filtered through sterile muslin cloth and a portion of filtrate was stored at  $-80^{\circ}\text{C}$  till DNA extraction. For total DNA extraction, filtrates of samples belonging to each food type were mixed in equal proportion and DNA was extracted from the pooled filtrate using the PowerFood<sup>®</sup> microbial DNA isolation kit (MOBIO, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was subsequently used for performing 16S rRNA amplicon sequencing on the Illumina's MiSeq<sup>®</sup> sequencing system (Illumina, San Diego, CA, USA) at SciGenom Labs Private Limited.

Using forward and reverse primers designed with overhang adapters, V3 region of the 16S rRNA gene from the metagenomic DNA was amplified using the above mentioned cycling conditions except for annealing at  $55^{\circ}\text{C}$  for 30 s with an amplicon size of  $\sim 459$  bp. The forward and reverse primer sequence used was 5'-TCGTCGGCAGC-GTCAGATGTGTATAAGAGACAG-3' and 5'-GTCTC-GTGGGCTCGGAGATGTGTATAAGAGACAG-3' respectively. After trimming the unwanted sequences and applying various filters to get high quality consensus V3 sequences, a total of 728,848 and 695,417 consensus V3 region reads were obtained for chicken meat and mung sprout respectively which were further processed to give pure 333,413 and 467,853 reads respectively. These reads were pooled and clustered into operational taxonomic units (OTUs) based on their sequence similarity using Uclust program with similarity cut-off of 0.97 to generate a total of 5497 and 1534 OTUs for chicken and mung sprout respectively<sup>25</sup> ([Supplementary Table S3](#)). QIIME program was used for the entire downstream analysis<sup>26</sup>. Representative sequence identified for each OTU was aligned against Greengenes core set of sequences using PyNAST program<sup>27,28</sup> and taxonomically classified from phylum to species level using Ribosomal Database Project classifier and Greengenes OTUs database with a confidence threshold of 80%.

## Results

### *Microbial load in food*

The study showed high viable bacterial count in the range of 5 to 9 log CFU/g in both chicken meat and sprout samples ([Supplementary Table S4](#)).

### *Potential antibiotic resistant culturable pathogens*

Prevalence of culturable pathogens (50 isolates for each food type) is shown in Table 1. In chicken meat and

mung sprouts, 60% and 88% of identified AR pathogens respectively belonged to Enterobacteriaceae family. Out of a total of 100 AR isolates characterized, common human pathogens were: *Enterobacter* sp. (23%), *E. coli* (22%), *K. pneumonia* (20%), *K. oxytoca* (1%), *Staphylococcus* sp. (8%) and *Acinetobacter* sp. (5%).

### *Antibiotic resistances of culturable pathogens*

The prevalence of AR in the above isolated pathogens was further analysed. Bacteria showed high resistance against macrolide,  $\beta$ -lactam, polypeptide, neomycin, rifampin, quinolone, trimethoprim and tetracycline class of antibiotics. Although food type-specific AR among most isolates was observed (e.g. high tetracycline and quinolone resistance in chicken), cefotaxime resistance was higher in isolates from mung sprouts. The complete AR profile of isolated pathogens is represented in [Supplementary Table S5](#). Co-resistance pattern to multiple antibiotics was observed for most isolates (Table 2). MAR index value of  $>0.2$  indicates high risk of contamination where antibiotics are often used<sup>29</sup>. Ninety five percent of isolates had MAR index in the range of 0.3 to 0.9.

MIC profile of antibiotic resistant pathogens is presented in [Supplementary Table S6](#). Most of the isolates showed MIC much above the breakpoint values. Maximum resistance (4- to 128-fold) was observed for erythromycin. Many Enterobacteriaceae and Staphylococcaceae isolates showed multi-fold increase in MIC against doxycycline, aminoglycosides and trimethoprim.

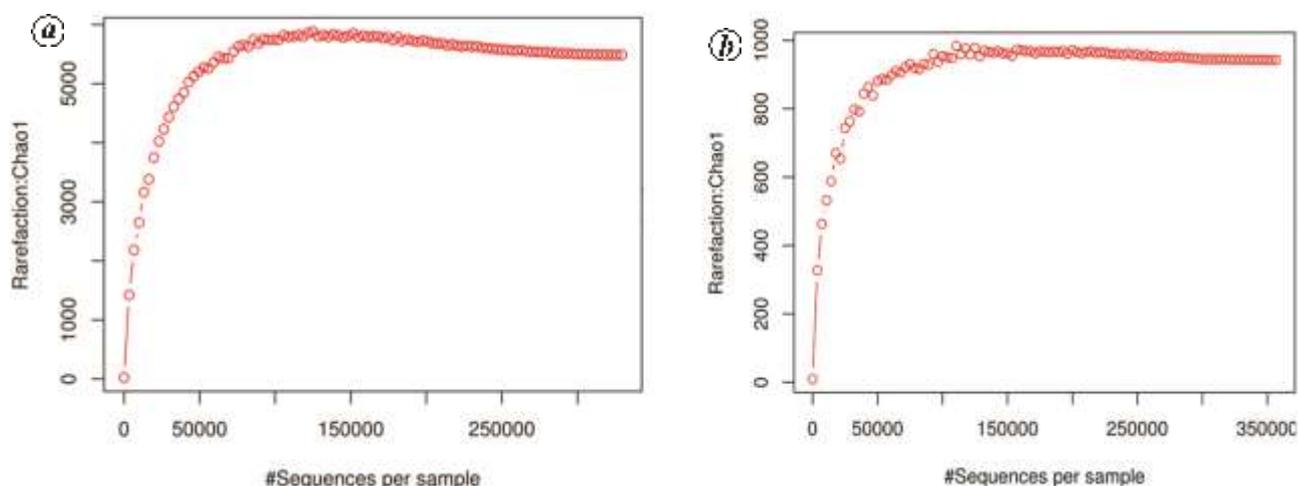
### *Metagenomic analysis of microbial diversity*

Using HT-NGS platform about 93.4% and 99.8% of total sequence reads were obtained for chicken meat and mung sprout respectively. The total diversity from phylum to species level is given in [Supplementary Information S2](#). Rarefaction curve with a plateau represents good microbial diversity up to 100,000 reads (Figure 1). This suggests sufficient sampling with total microbial flora being represented by identified phyla and genera. Similar curves have been reported earlier for alfalfa sprout and chicken caeca microflora<sup>30,31</sup>. Chicken had higher bacterial diversity and species richness than mung sprout. The observed sequence similarity cut-off of  $\leq 97\%$  suggests identification of potentially new species<sup>31</sup>. In chicken and mung samples, 17 and 7 bacterial phyla were identified respectively. *Firmicutes* (44.2%) and *Proteobacteria* (88.9%) were dominant phyla in chicken and sprout respectively. *Clostridia* (34.2%) and *Bacteroidia* (9.7%) were prominent classes while *Clostridiales* (32.4%) and *Bacteroidales* (9.7%) were dominant orders detected in chicken. *Gammaproteobacteria* (88%) was the single dominant class in mung sprout microflora while *Enterobacteriales* (71.7%) was the predominant order. Taxonomic

**Table 1.** Antibiotic-resistant pathogens identified in both culturable and metagenomic microflora

Microorganisms	Family	Chicken meat		Mung sprout	
		No. of AR culturable pathogens <i>n</i> (%)	No. of OTUs <i>n</i> (%) <sup>a</sup>	No. of AR culturable pathogens <i>n</i> (%)	No. of OTUs <i>n</i> (%) <sup>b</sup>
<i>Escherichia coli</i>	<i>Enterobacteriaceae</i>	21 (42)	5 (0.09)	1 (2)	1 (0.06)
<i>Klebsiella</i> sp.		3 (6)	2 (0.03)	18 (36)	24 (1.6)
<i>Enterobacter</i> sp.		1 (2)	4 (0.07)	15 (30)	29 (1.9)
<i>E. cloacae</i>		1 (2)	ND	6 (12)	1 (0.06)
<i>Cronobacter sakazakii</i>		ND	ND	1 (2)	1 (0.06)
<i>Citrobacter</i> sp.			2 (0.03)	ND	1 (0.06)
<i>C. amalonaticus</i>		1 (2)	ND	ND	ND
<i>C. freundii</i>		2 (4)	ND	ND	ND
<i>Kluyvera</i> sp. <sup>c</sup>		ND	ND	1 (2)	ND
<i>K. georgiana</i>			ND	2 (4)	ND
<i>Proteus</i> sp.		1 (2)	1 (0.01)	ND	ND
<i>P. vulgaris</i>			ND	ND	ND
<i>Enterococcus</i> sp.	<i>Enterococcaceae</i>	1 (2)	4 (0.07)	ND	ND
<i>E. faecalis</i>			ND	ND	ND
<i>Aeromonas</i> sp.	<i>Aeromonadaceae</i>		5 (0.09)	ND	ND
<i>A. nosocomialis</i>		1 (2)	ND	ND	ND
<i>A. veronii</i>		1 (2)	ND	ND	ND
<i>Acinetobacter</i> sp.	<i>Moraxellaceae</i>	1 (2)	47 (0.8)	4 (8)	64 (4.2)
<i>Staphylococcus</i> sp.	<i>Staphylococcaceae</i>		6 (0.11)	ND	2 (0.13)
<i>S. sciuri</i>		4 (8)	ND	ND	ND
<i>S. epidermidis</i>		1 (2)	ND	1 (2)	ND
<i>S. hominis</i>		1 (2)	ND	1 (2)	ND
<i>S. pasteurii</i>		0	ND	ND	ND
<i>Macrococcus</i> sp.		4 (8)	2 (0.03)	ND	ND
<i>M. caseolyticus</i>				ND	ND
<i>Lactococcus</i> sp.	<i>Streptococcaceae</i>		3 (0.05)	ND	11 (0.7)
<i>L. garvieae</i>		2 (4)	6 (0.11)	ND	ND
<i>L. lactis</i>		2 (4)	ND	ND	ND
<i>Kurthia gibsonii</i> <sup>c</sup>	<i>Planococcaceae</i>	1 (2)	ND	ND	ND
<i>Corynebacterium</i> sp.	<i>Corynebacteriaceae</i>	1 (2)	8 (0.14)	ND	ND
Total		50	95	50	134

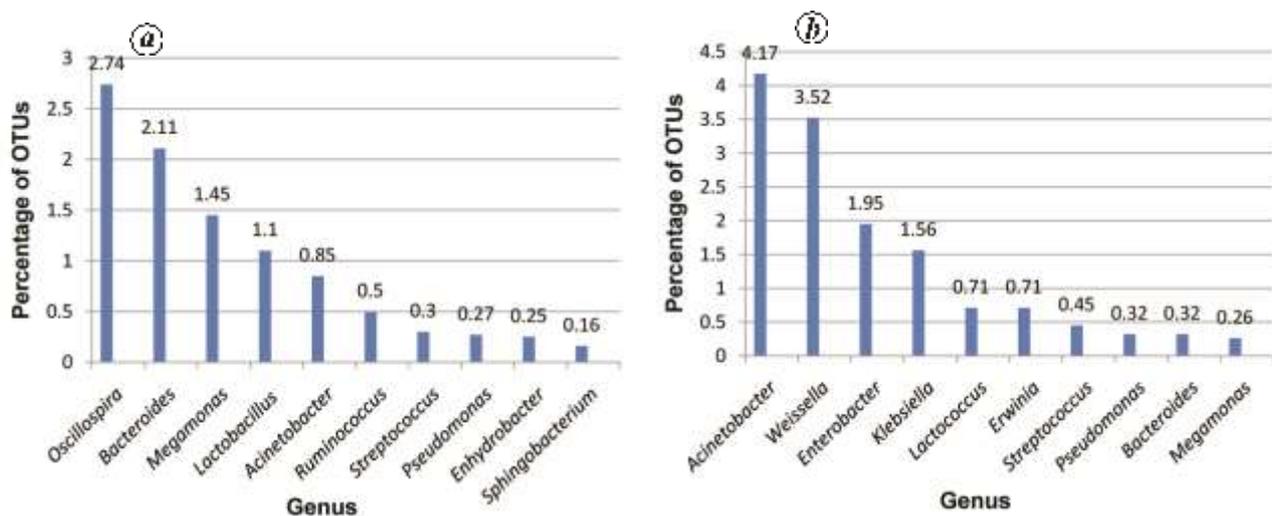
ND, Not detected. Numbers in parentheses represent percentage. <sup>a</sup>Percentage of total 5497 OTUs from chicken meat metagenome. <sup>b</sup>Percentage of total 1534 OTUs from mung sprout metagenome. <sup>c</sup>Species not detected in metagenomic microflora.



**Figure 1.** Rarefaction curves of the OTUs clustered at 97% sequence similarity for (a) chicken meat and (b) mung sprout. OTUs are represented on Y-axis and number of sequence reads on X-axis. Alpha diversity was computed using Chao1 metrics with rarefied OTU table size of 100. The metric calculation was performed using QIIME software. #: Number of high quality pre-processed consensus V3 region reads.

**Table 2.** Pathogenic organisms with more than five multiple antibiotic resistance pattern

Genus/species	Dominant MAR pattern	MAR index range	Percent prevalence
<i>E. coli</i>	DOX-ERY-KAN-NEO-PEN-RIF-TRM-CIP-CEF	0.7–0.9	31.8
<i>Klebsiella</i> sp.		0.8	9.5
<i>E. coli</i>	DOX-ERY-KAN-NEO-PEN-RIF-TRM-CIP	0.7–0.9	40.9
<i>Klebsiella</i> sp.		0.7–0.8	23.8
<i>Staphylococcus</i> sp.	DOX-ERY-KAN-NEO-TRM-CIP	0.5	50
<i>Citrobacter</i> sp.	DOX-ERY-KAN-PEN-RIF-TRM	0.5–0.7	66.6
<i>Enterobacter</i> sp.	ERY-KAN-NEO-PEN-RIF-CEF	0.5–0.8	34.7
<i>Acinetobacter</i> sp.	NEO-PEN-TRM-CIP-CEF	0.4–0.6	60

**Figure 2.** Microbial genus distribution of top 10 genera based on per cent OTU representation for (a) chicken meat metagenome and (b) mung sprout metagenome. The values show percentage of OTUs represented by each genus out of total identified OTUs for each sample.

classification identified 91 families, 123 genera and 66 species from chicken. From mung sprout 30 families, 30 genera and 21 species were identified. Figure 2 represents 10 most prevalent genera identified for both food types. Different food-borne and opportunistic pathogens were also identified (Table 3) accounting for more than 50% of identified genera. Animal origin microorganisms like *Oscillospira*, *Bacteroides*, *Megamonas*, *Ruminococcus*, *Helicobacter*, *Shewanella*, *Alistipes*, *Faecalibacterium*, *Cloacibacterium* and *Myroides* were identified in chicken while those of plant origin like *Weissella*, *Erwinia*, *Ewingella*, *Hafnia*, *Trabulsiella* were specifically present in mung sprout.

#### Common microflora in culturable and nonculturable population

*Kluyvera* sp. and *Kurthia gibsonii* were isolated and identified in culturable population. However, these two species were not detected by metagenomics (Table 1). All other micro-organisms detected by culturable method were also detected by metagenomic analysis with similar food type specific distribution pattern.

#### Discussion

High microbial load was observed in both chicken meat and sprouts samples. In a tropical country like India, warm and humid conditions facilitate rapid proliferation of bacteria in food<sup>32</sup>. Moreover, undercooked food and unhygienic handling practices also contribute to high bacterial load in food samples<sup>5,7,18,33</sup>.

We observed high incidence of AR bacteria against most of the antibiotics used in the study. In the present study, five different antibiotics were selected based on their clinical significance. Total AR bacterial count was highest for erythromycin; this may be due to the long history of exposure. Low bacterial count for ciprofloxacin was observed ([Supplementary Table S4](#)) as it is comparatively a new generation antibiotic<sup>19</sup>.

High Enterobacteriaceae counts (Table 1) in food can be attributed to contamination during food handling and downstream processing steps<sup>8,15,34,35</sup>. Prevalence of major human pathogens like *Klebsiella*, *Enterobacter*, *P. vulgaris*, *Staphylococcus* in retail food indicates human source of food contamination. *E. coli*, *Enterobacter*, *Citrobacter* and *Klebsiella* suggest faecal contamination

## RESEARCH ARTICLES

**Table 3.** Major opportunistic and food-borne pathogens detected in chicken meat and mung sprout metagenome

Opportunist/ food borne pathogens	Chicken meat	Mung sprout
	OTUs	OTUs
<i>Bacteroides</i>	116	5
<i>Acinetobacter</i> sp.	37	62
<i>A. johnsonii</i>	5	ND
<i>A. schindleri</i>	3	ND
<i>A. lwoffii</i>	1	ND
<i>A. guillouiae</i>	1	2
<i>Enterobacter</i> sp.	2	21
<i>E. ludwigii</i>	1	7
<i>E. aerogenes</i>	1	ND
<i>E. cloacae</i>	ND	1
<i>E. cowanii</i>	ND	1
<i>Klebsiella</i> sp.	2	24
<i>Streptococcus</i> sp.	17	7
<i>Pseudomonas</i> sp.	15	5
<i>Lactococcus</i> sp.	3	11
<i>L. garvieae</i>	6	ND
<i>Sphingobacterium</i> sp.	3	ND
<i>S. mizutaii</i>	1	
<i>S. multivorum</i>	4	
<i>S. faecium</i>	1	
<i>Corynebacterium</i> sp.	7	ND
<i>C. stationis</i>	1	
<i>Shewanella</i> sp.	6	ND
<i>S. algae</i>	2	
<i>Flavobacterium</i>	8	ND
<i>Helicobacter</i>	7	ND
<i>Chryseobacterium</i>	7	1
<i>Staphylococcus</i> sp.	2	1
<i>S. sciuri</i>	1	1
<i>S. succinus</i>	2	ND
<i>S. haemolyticus</i>	1	ND
<i>Bacillus</i> sp.	5	ND
<i>B. cereus</i>	1	
<i>Anaerotruncus</i>	6	ND
<i>Bilophila</i>	6	ND
<i>Butyrivimonas</i>	6	ND
<i>Prevotella</i>	6	ND
<i>Escherichia coli</i>	5	1
<i>Aeromonas</i> sp.	3	ND
<i>A. hydrophila</i>	2	
<i>Parabacteroides</i> sp.	3	ND
<i>P. distasonis</i>	2	
<i>Enterococcus</i> sp.	3	ND
<i>E. cecorum</i>	1	
<i>Clostridium</i> sp.	1	ND
<i>C. piliforme</i>	1	
<i>C. perfringens</i>	1	
<i>Campylobacter</i>	3	ND
<i>Comamonas</i>	3	ND
<i>Sphaerochaeta</i>	3	1
<i>Citrobacter</i>	2	1
<i>Trabulsiella</i>	1	3
<i>T. farmeri</i>	ND	1
<i>Stenotrophomonas</i>	1	1
<i>Photobacterium damsela</i>	1	1
<i>Hafnia alvei</i>	ND	3
<i>Cronobacter sakazakii</i>	ND	1

ND, Not detected.

during slaughter process<sup>15</sup> and use of poorly composted animal manures in fields<sup>18</sup>. Enteric bacteria in mung can have serious health concern as sprouts are mostly consumed raw. To our knowledge, this study is the first to isolate multiple AR pathogens like *Kurthia gibsonii*, *A. nosocomialis*, *C. amalonaticus*, *L. garvieae*, *S. hominis* from chicken samples and *S. hominis*, *Kluyvera georgiana* from mung sprouts.

High resistance against macrolides, penicillin and newer generation doxycycline, ciprofloxacin and cefotaxime is attributed to its rampant use as veterinary therapy in livestock industry and organized farms<sup>15,19,36,37</sup>. This is a serious concern as most of them are clinically significant antibiotics. Higher resistance of identified pathogens to older generation antibiotics like bacitracin, erythromycin, kanamycin, neomycin, penicillin is because of its long history of applications and exposure volume over decades<sup>19,38</sup>. Findings of other studies also indicate that extensive usage of antibiotics has led to spread of AR bacteria in the environment (food samples). Unchecked use of sub-lethal doses of bacitracin as growth promoters<sup>32</sup> explains observed high incidence of bacitracin-resistant pathogens. High tetracycline and quinolone resistance in chicken is attributed to their use in animal farms. Resistance patterns for the remaining antibiotics suggest probable cross-contamination as well as inter-ecosystem spread of AR pathogens in these two food types (e.g. Trimethoprim resistance even in absence of its usage in poultry or plant farming and high cefotaxime resistance in sprouts even though it is not used in plant agriculture)<sup>39</sup>. The MAR index clearly suggests high resistance to multiple antibiotics in most bacterial species identified. More than 10 pathogenic species were resistant to 5–12 antibiotics belonging to different classes. The MAR index and co-resistance pattern can be attributed to R-plasmids or integrons harbouring multiple AR gene cassettes<sup>37,40</sup>.

Almost all the isolates showed multi-fold increase in MIC values suggesting the presence of resistant traits. Increased erythromycin resistance is consistent with AST data. *E. coli*, *E. cloacae*, *P. vulgaris* and *Staphylococcus* sp. exhibiting high MICs against cefotaxime, doxycycline, aminoglycosides and trimethoprim suggest presence of over expressing  $\beta$ -lactamase, *tet*, aminoglycoside modifying and *dfr* genes respectively<sup>8,33,40,41</sup>. *S. epidermidis* and *S. hominis* showed 4- and 8-fold increase in MIC against rifampicin; antibiotic used in treating serious *Staphylococcal* and tuberculosis infections. The high MIC of *A. veronii* may be associated with resistance plasmids from polluted waters<sup>42</sup>.

To understand the complete microbial community of retail food, HT-NGS technique was used. There are very few reports on microbiological diversity of different food samples studied by HT-NGS. Moreover, the available reports on metagenomics have identified limited microbial diversity in alfalfa sprouts, chicken caeca, gut<sup>30,43,44</sup>

without much emphasis on pathogenic nature. V3 region was targeted and the total microbial diversity characterized up to species level. Presence of *Firmicutes* in chicken could be due to cross-contamination with other parts of chicken during meat processing as high incidence of *Firmicutes* in chicken gut, ilea and caeca have been reported<sup>31,44</sup>. Phylum composition of mung is similar to other metagenomic studies of fresh fruits and vegetable<sup>45</sup>. High prevalence of *Clostridiales* in chicken suggests possible faecal contamination as faecal indicators like *Ruminococcus* and *Megamonas* belong to this order. In mung, bacterial distribution was uneven with microflora like *Proteobacteria*, *Gammaproteobacteria*, *Enterobacteriales* and *Enterobacteriaceae* being most dominant, represented by >71% OTUs. High prevalence of *Enterobacteriaceae* in mung is due to moist humid conditions during sprouting which favours proliferation of bacteria<sup>30</sup>.

Chicken harbouring *Enterobacteriaceae*, *Moraxellaceae*, *Pseudomonadaceae*, *Aeromonadaceae*, *Staphylococcaceae*, *Clostridiaceae*, *Campylobacteraceae*, *Vibrionaceae* could be a source of spread of food-borne pathogens ([Supplementary Table S7](#)). Abundance of opportunistic pathogens like *Acinetobacter*, *Enterobacter*, *Klebsiella*, *H. alvei* in mung sprout and *Escherichia*, *Acinetobacter*, *Enterococcus*, *Serratia*, *Providencia* in chicken could be a serious health issue. Identification of major food-borne pathogens like *Streptococcus*, *Pseudomonas*, *Shewanella*, *Helicobacter*, *C. perfringens*, *Campylobacter*, *Staphylococcus*, *A. hydrophila*, *E. coli*, etc. confirms the poor quality of retail food consumed. *Campylobacter* is an enteric pathogen predominant in processed poultry meat<sup>46</sup>. *Pseudomonas* contamination is a major reason for spoilage of poultry products. *C. perfringens* inhabits animal gastrointestinal tracts<sup>47</sup> and is a cause of diarrhoea worldwide. Presence of anaerobic pathogens *Prevotella* and *Veillonella* in chicken meat is interesting since these genera are commonly present in oral microflora of humans<sup>48,49</sup>. This suggests human source of food contamination. *Alistipes* and *Faecalibacterium* are rare genera not widely reported earlier in retail chicken meat. Species like *L. garvieae*, *E. coli*, *A. johnsonii* detected in chicken and *E. ludwigii* in sprout are opportunistic pathogens causing serious life threatening infections in immune compromised or aged patients. *Ruminococcus* and *Megamonas* are faecal indicators suggesting faecal contamination. Similarly, faecal indicators like *Bacteroides plebeius* and *B. coprophilus* in sprouts suggests contaminated water run-off from nearby animal pastures or animal manure used as fertilizers. Most of the identified non-culturable micro-organisms belong to potential pathogens (including anaerobic) which may go un-noticed during normal food screening, highlighting the importance of metagenomic analysis for food quality evaluation. Such detailed metagenomic analysis of pathogenic bacteria distribution in chicken meat and mung sprout is not reported elsewhere.

*Acinetobacter guillouiae* was detected in mung sprout. It is a clinical pathogen known to carry transferable AR traits<sup>50</sup>. *Ewingella americana* detected in mung is MAR opportunistic pathogen and is implicated in many human infections<sup>51</sup>. These may act as ready reservoirs of AR traits easily transferrable to human pathogens. Both culturable and metagenomic prevalence of *L. garvieae* and *Aeromonas* in chicken while *E. cloacae* and *Photobacterium damsela* in sprouts can be attributed to inter-ecosystem cross-contamination, since *E. cloacae* is predominant in chicken while the rest are frequently found in aquatic ecosystems. Most of the mung microflora was similar to that found in wastewater treatment plants and manure treated agro ecosystems<sup>52,53</sup>. This suggests raw manure-treated soil or contaminated irrigation water as potential source of pathogens in mung microflora.

About 18% of the total genera identified in our metagenomic analysis are common for both food samples studied. This could be due to two reasons. One is the ubiquitous nature of some bacteria in environment and ecosystems which contaminate food source<sup>54</sup>. The other is cross-contamination of these pathogens prior to harvest, during slaughter stages and subsequent food transporting, processing, storage and marketing by handlers<sup>17</sup>.

Many of the identified culturable pathogens were also reflected in metagenomic diversity. Both approaches showed high dominance of *Enterobacteriaceae* (88% culturable; 71.7% OTUs) followed by *Moraxellaceae* (8%; 4.5%) in mung thus corroborating reports of sprout associated pathogenic outbreaks across the world<sup>5</sup>. However *Kluyvera* sp. and *K. gibsonii* detected by culturable method were not represented in metagenomic analysis. This may be because of differences in the primers used to amplify 16S rRNA genes in the two methods. While for culturable isolates primers belonging to both V3 and V4 regions of 16S rRNA gene were employed, for metagenomic analysis only V3 region was targeted.

Finally, high microbial diversity and AR suggests that micro-organisms within their respective hosts efficiently adapt to various stresses associated with many anthropogenic impacts and can spread across the community.

## Conclusion

To the best of our knowledge, our study is the first to extensively characterize both pathogenic and commensal diversity of retail chicken meat and RTE mung sprouts by HT-NGS in the Indian sub-continent. The study also gives a snapshot of the AR culturable micro-organisms. Cross-contamination has been implicated as a possible mode of dissemination. Possibility of human impact on the production, processing and marketing practices of both food types under investigation is also highlighted. The overall microbiological quality of retail food investigated was very poor as we found high load of potential

pathogens by both culturable and HT-NGS methods. Large proportion of nonculturable potential pathogens detected underscores the need for sophisticated nonculturable approaches for characterization and prevention of spread of food-borne pathogens. AR profiling revealed the presence of many multiple AR pathogens and faecal indicators which could be attributed to the anthropogenic activities. Presence of AR organisms, like *E. coli*, which readily disseminate AR traits, indicates the rapid mobility of resistance factors within the food associated microbial community. Analysis of other potential contaminating sources like poultry litter, animal farms, wastewater runoff, sewage water, cutting equipment and utensils used in retail shops, water used for washing sprouts and salads and manure used as conventional fertilizers will provide a more complete picture of possible modes of dissemination of both micro-organisms and AR.

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