Assessment of Bacterial Density, Diversity, and Antibiotic Resistance-Dissemination from Multidrug-Resistant *Escherichia coli* to Rat's Gut Microbiota in Presence and Absence of Antibiotic Treatment: a Useful Animal Model for Future Investigations

Imran Khan[1,2]
Muhammad Yasir[2]
Taha Kumosani[3]
Aymn T. Abbas[2,4]
Elie K. Barbour[5]*
Asif Ahmad Jiman-Fatani[6]
Esam I. Azhar[2,7]

[1]Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, 21452, Saudi Arabia.

[2]Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, 21452, Saudi Arabia

[3]Biochemistry Department, Faculty of Science; Production of Bioproducts for Industrial Applications Research Group, and Experimental Biochemistry Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

[4]Biotechnology Research Laboratories, Gastroenterology Surgery Center, Mansoura University, Mansoura, 35511, Egypt

[5]Faculty of Agriculture, American University of Beirut, Beirut, Lebanon; Adjunct to Biochemistry Department, and Production of Bioproducts for Industrial Applications Research Group, King Abdulaziz University, Jeddah, 21452, Saudi Arabia

[6]Department of Medical Microbiology and Parasitology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia

[7]Medical Laboratory Technology Department, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, 21452, Saudi Arabia

*Corresponding Author: Elie K. Barbour, Department of Agriculture, Faculty of Agricultural and Food Sciences, American University of Beirut, Beirut, Lebanon, Fax: 00-961-1744460, Tel: 00-961-1-350000, E-mail: eb01@aub.edu.lb

KEY WORDS: Animal Model, Antimicrobial Resistance, Gut Microbiota, *Escherichia coli*, Multi-Drug Resistance

ABSTRACT

Aim

The increasing prevalence of multi-drug resistant (MDR) *Escherichia coli* is one of the intractable, economic veterinary and public health obstacle of the 21st century. As a component of the gut microbiota (GM), it is aimed in this study to establish a rat model to examine the role of *E. coli* in contributing to the increasing antimicrobial resistance of GM.

Methods and Results

Ten rats were divided into two equal groups (RG–1 and RG–2), and their GM was characterized before and after an amoxicillin treatment. The first treatment was applied on all rats, administering to each an equal count of Multiple Drug Resistant *E. coli* (MDR E. coli). The second treatment was restricted to rats of the RG-2 group, treating them with amoxicillin, effective 48 hrs following the MDR *E. coli* administration, to examine the persistence of MDR *E. coli* and the post-treatment profile of the GM resistome. Stool samples, collected at different times, were aerobically cultured at 37°C, and the bacterial cultures were tested against ten antibiotics from different classes. The bacterial isolates were analysed by matrix-assisted laser desorption ionisation time-of-flight mass spectrophotometry (MALDI-TOF MS) and some by 16S RNA sequencing. In four phyla, 12 genera and 16 species were identified by culturing 8020 fecal colonies. The rat GM was dominantly inhabited by the genus *Enterococcus*, encoding resistance to amoxicillin, D-cycloserin, gentamicin, carbenicillin and kanamycin. The GM of rats in the two groups had significantly greater antimicrobial resistant colony count ($p<0.01$) after administration of exogenous MDR *E. coli* compared to that before treatment. The amoxicillin treatment in the second group was efficient in reduction of the bacterial density, associated with enhanced resistance diversity. The Bacteriodetes emerged as a new resistant phylum after the amoxicillin treatment.

Conclusions

In conclusion, the administration of MDR *E. coli* caused a change in the resistome of the GM, and the additional treatment with amoxicillin increased the drug resistant-colony forming units, and led to the isolation of new antimicrobial resistant species.

Significance and Impact of Study

This study proves the significance of a rat model in studying the role of ingestion of MDR microorganism, in absence and presence of antimicrobial treatment, on the drug resistome of the GM. The impact of this pioneer study on future control of the problem of drug resistance in GM, due to ingestion of MDR microorganisms by animals and humans, in absence and presence of antimicrobial treatment, is in accord with recent influx of documentations in this research scope.

INTRODUCTION

Escherichia coli resides mainly in the mammalian gut, with a rare presence in the gut of reptiles, avians and fish. This bacterium is quite diversified and can contaminate the ecosystems of animals and humans, including water, soil, plants and feed and food[1]. The Gut Microbiota (GM) can produce vitamins (B12 and K) for the host, in exchange of seeking shelter and nutrients for their growth. The association of *E. coli* with its host is not a self-to-self association, since some of its strains are antigenic, causing serious illnesses such as urinary tract infections (UTIs), abdominal sepsis, meningitis, septicaemia, haemolytic-uremic syndrome and diarrhoea[1–6]. Furthermore, the acquisition of various β-lactamases genes in this bacteria has currently worsened the severity of the resulting diseases, thereby increasing the duration of morbidity and causing unproductive exposure to antimicrobials used in treatment[7]. Certain *E. coli* strains, originating from animals, such as *E. coli* O157:H7, E. coli–bearing bla_{CTX-M} gene and *E. coli* encoding blaAmpC genes, can cause serious human infections, in addition to their

potential in dissemination of antimicrobial resistance[1,8,9]. In poultry, the O1, O2, O35, and O78 are serotypes that are known to cause economic losses in broilers, associated with multiple resistance to drugs[10].

Recently, multi-drug resistant *E. coli* (MDR E. coli) strains have been isolated from meat, water, dairy products, fermented products and probiotics[11,12]. Extended spectrum beta lactamase (ESBL)–producing *E. coli* is ubiquitously, existing in the Indian sub-continent, in which around 87% of tourists to India develop colonisation with ESBL E. coli[3]. Around 75,000 cases are affected annually by food illness caused by contamination with *E. coli* O157:H7 that originated from animals[2]. Every year, billions of dollars are spent worldwide on the treatment of *E. coli* infections in animals and humans[1,14–16]. In the United States alone, around 405 million dollars are spent on infection control[1,17]. The increasing prevalence and emergence of MDR *E. coli* is one of the most intractable veterinary and public health obstacles of the 21st century[18–20], which has led to complications in infection treatments and dissemination of antimicrobial resistance to other bacteria[13].

Besides resulting in infections, the pervasive presence of MDR *E. coli* in the environment and food continuously modulates animal and human gut microbiota (GM)[17,18], affecting the general health. The GM is an expanding functional reservoir of antibiotic-resistant genes that is most probably associated with ingesting feed of animals or food of humans, and water contaminated with antibiotic-resistant bacteria[21–24]. The resistant genes can be shared, directly or indirectly, among the bacterial species in GM[26], due to the high conjugation ratio within the genus[24]. In addition, inter-species and inter-family DNA sharing has been recently reported GM[25]. The ability of these organisms to acquire and share antimicrobial resistance, and being a natural inhabitant of GM, led to the hypothesis that *E. coli* could potentially contribute to an increase in antibiotic resistance of the GM[13,25,26].

This is the first study that presents detailed data on culturomics and resistome of rat GM, aiming at using this rat model to determine the diversity, density and resistome of the cultured microbiota of the rat. An antimicrobial resistance dissemination was evaluated by introducing to the rat's gut an exogenous MDR *E. coli* strain, in presence and absence of amoxicillin treatment. Rats were orally administered MDR *E. coli* and their fecal colonies were counted, purified and identified using matrix-assisted laser desorption ionisation time-of-flight mass spectrophotometry (MALDI-TOF MS). Isolates that failed to be identified by MALDI-TOF MS were subjected to16S RNA sequencing for completing their identification.

MATERIALS AND METHODS

Experimental Design

Ten sexually mature female Sprague–Dawley rats, eight weeks old, and each weighing between 210–250 g, were obtained from the animal breeding unit at King Fahd Medical Research Center, King Abdulaziz University Jeddah, Saudi Arabia. The rats were reared under standardised laboratory conditions[27]. The study protocol was approved by the Research Ethics Committee of the Faculty of Medicine at King Abdulaziz University, and the animal experiments were carried out in accordance with the approved study guidelines (HA-02-J-008). A MDR *E. coli* strain resistant to tetracycline, oxy-tetracycline, D-cycloserin and carbenicillin was provided by the Microbiology laboratory at King Abdulaziz University Hospital, Jeddah, Saudi Arabia. The resistance profile of the strain was confirmed using VITEK-2 (Biomerieux-Vitek, Inc., Hazelwood, MO, USA).

Before the administration of MDR *E. coli*, all rats' GMs were screened to confirm the absence of colonisation by MDR *E. coli*. The GM colonisation was disrupted for three days by administration of a cocktail of antibiotics containing azithromycin (45 mg/kg/day), amoxicillin (50 mg/kg/day) and cefaclor (67.5 mg/kg/day) to facilitate *E. coli* colonization[28,29]. The rats were then divided into two equal groups namely, RG-1

and RG-2. At first day of the trial, before the administration of MDR *E. coli* or antimicrobial, their collected stool samples served as controls (C-D0). Each of the 10 rats were then orally inoculated with MDR *E. coli* (1×10^5 colony forming units) to study the potential dissemination of antimicrobial resistance to rats's GM. The rats of the RG-1group were sampled on the 2nd (Ec-D2), 7th (Ec-D7) and 14th (Ec-D14) days following the MDR *E. coli* administration. Rats of the RG-2 group were additionally treated with amoxicillin (50 mg/kg/day) for five days, effective 48 hrs following the administration of the MDR *E. coli* inoculation, aiming at the study of the impact of antibiotic administration on *E. coli* persistence and shift in GM resistome post treatment. The rats of the RG-2 group were sampled before MDR *E. coli* inoculation (C-D0), 2 days after the MDR *E. coli* administration (Ec-D2), and 2 days (Ec-Amx-2D) and 9 days (Ec-Amx-9D) after the amoxicillin treatment.

GM culturing for enumeration and drug resistome identification

The agar medium (Table 3) was developed to improve the recovery of diversified bacteria cultured from different fecal samples of the gut. Ten antimicrobials were selected from four known classes, and individually supplemented into the medium for studying the GM resistance. The gentamicin, tetracycline, oxy-tetracycline and kanamycin were selected from the class of 30S inhibitors, the chloramphenicol from class of 50S inhibitors, the amoxicillin, ampicillin-G, carbenicillin and D-cycloserine from the class of cell-wall inhibitors, and ciprofloxacin from the class of DNA synthesis inhibitors. The same concentration (20 µg/mL) of the individually selected antimicrobials was supplemented in the medium throughout the experiment. Each of the collected stool samples (1 g) was serially diluted, and the dilutions were each individually plated in 0.1 ml/plate in triplicate, as described previously[30]. The plates were incubated at 37°C for 48 hours. The counts in colony forming unit (CFU) were recorded after 48 hours. Colonies were sub-cultured for purification.

MALDI-TOF MS based identification of GM colonies

The identity of the purified isolates was determined by MALDI-TOF MS (Bruker Daltonics, Billerica, Mass., U.S.A.)[31]. Each isolate was smeared on MALDI-TOF target plate and then covered with 1 µL matrix solution. The matrix solution was prepared by mixing 475 µL HPLC grade water in 500 µL acetonitrile and 25 µL trifluoro acetic acid, followed by the addition of 5 mg of α-cyano-4- hydroxycinnamic acid, followed by vortexing.

Each spot was targeted with laser, and the spectra were mechanically collected through flexControl 3.0 software and analyzed by MALDI-Biotyper 2.0 software. The colonies were screened in triplicate, and threshold scores for identification were set near 2.0 (>1.931)[32]. Strains that could not be identified by MALDI-TOF MS were subjected to16S RNA sequencing[33].

16S rRNA gene sequencing

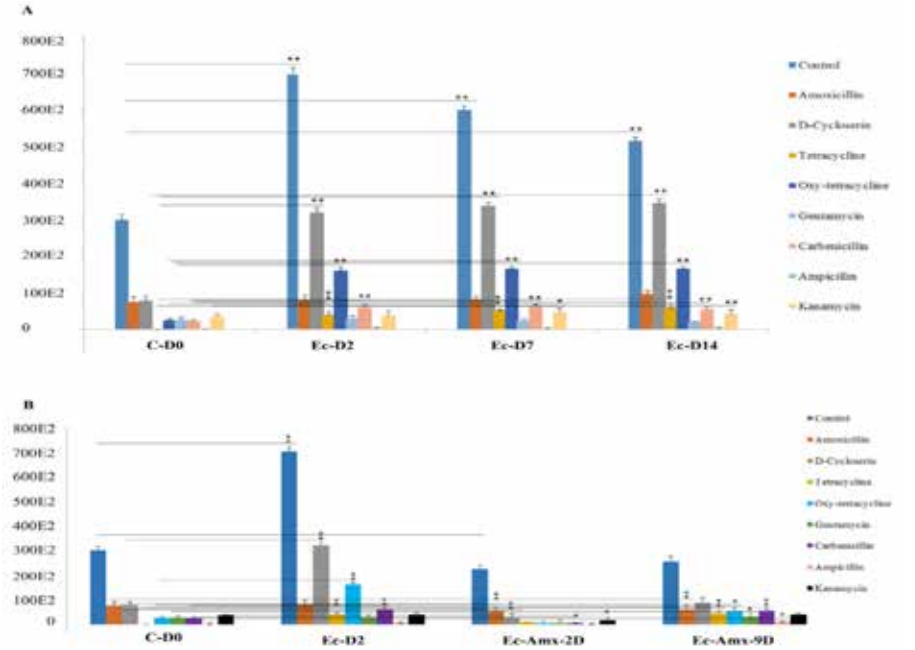
Genomic DNA was extracted from the fresh colonies of isolates using 5% Chelex-100 and boiled for 20 min. The supernatant was used as template, and the PCR amplification of 16S rRNA gene was performed using universal 27F and 1492R primer pairs as described previously[34]. After an agarose banding of the ampolicons, the purified PCR products from the gel were sequenced through Sanger sequencing technology, using ABI prism sequencer 3730 (Applied Biosystems, USA), and following the manufacturer's protocol. The sequencing result was blasted using EzTaxon server (http://www.ezbiocloud.net/eztaxon) to identify the closely related genome of the examined strains.

RESULTS

Rat's cultured microbiota and their resistome

The isolated 8020 colonies from all collected fecal samples were identified and screened for antimicrobial resistance (Fig. 1). A total of 3850 and 4170 colonies were pro-

Figure 1



cessed from RG-1 and RG-2 groups of rats, respectively. There was an apparent shift in resistance pattern by time, and following the administration of MDR *E. coli* and MDR *E. coli*/amoxicillin in RG-1 and RG-2 groups, respectively (Fig 1, A and B).

Sixteen different species were identified from the following four phyla namely, Firmicutes (67.9% of isolates), Bacteriodetes (3.7% of isolates), Proteobacteria (19.6% of isolates) and Actinobacteria (8.6% of isolates) (Fig. 2). The total number of detected genera was 12, with the highest number of species isolated from the genus *Enterococcus* (59.2%). The other most predominant genera were the *Microbacterium* (8.8%), *Escherichia* (7.9%), *Acinetobacter* (6.8%), *Streptococcus* (5.6%) and *Elizabethkingia* (3.76%). The genera *Staphylococcus* (1.08%), *Bacillus* (1.66%), *Corynebacterium* (0.6%), *Pseudomonas* (1.8%), *Lactobacillus*(1.5%) and *Klebsiella* (1%) constituted a minor detected category. Approximately a 65% of the total isolates were resistant to one of the tested antibiotics (Tables 1 and 2). Most of the resistant isolates (~49%)

Figure 2

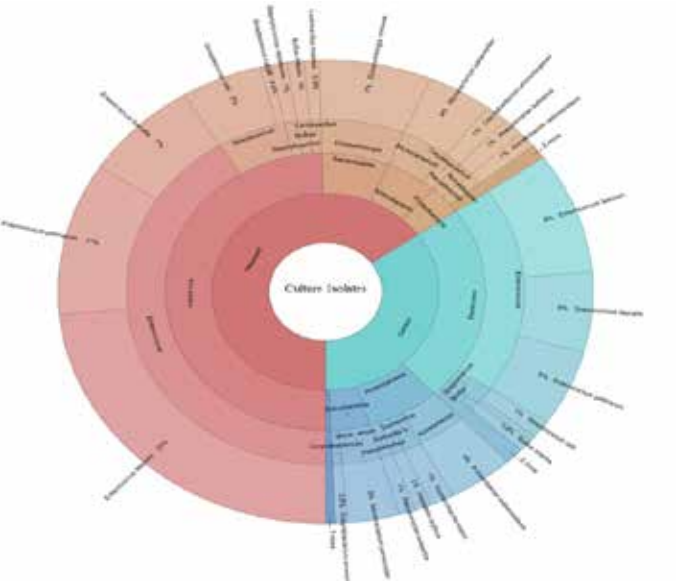


Table 1. *The Mean percent resistance of identified RG-1 fecal species to 8 antimicrobials before and after the MDR E. coli administration*

Sample	Species Identified	Control	Amoxicillin	D-cycloserin	Gentamycin	Carbenicillin	Kanamycin	Tetracycline	Oxy-tetracycline	Ampicillin
C-D0	*Enterococcus gallinarum*	17 ±1.6	0	8±2.5	6±1.62	8 ±1.6	0	0	0	0
	Microbacterium paraoxydan	10±2	0	0	3±1.3	0	0	0	0	0
	Enterococcus faecium	30±2.2	7 ±1.9	8±2.3	7±1.7	5±1.02	6±1.69	0	0	0
	Enterococcus faecalis	24 ±2.3	0	0	0	0	0	0	0	0
	Acinetobacter radioresistens	15 ±1	0	0	0	0	5 ±1	0	0	0
EC-D2	*Enterococcus gallinarum*	10 ±2.5	0	9 ±1.9	10 ±1.7	10 ±0.9	0	0	0	0
	Microbacterium paraoxydan	8 ±2.1	0		8 ±1.92		0	0	0	0
	Enterococcus faecium	21 ±3.5	9 ±2.08	10 ±2.7	0	6 ±1.35	7±1.82	0	0	0
	Enterococcus faecalis	13±3.6	0	9 ±2.5	0	0	0	0	0	0
	Acinetobacter radioresisten	10 ±2.5	0	0	0	0	7±1.3	0	0	0
	Escherichia coli	14 ±3.2	0	11 ±1.55	11±1.52	6 ±2.1	0	6 ±1.8	11 ±1.55	0
EC-D7	*Enterococcus faecium*	9 ±2.7	6±2.15	12 ±2.31	6 ±2.01	5 ±1.91	7 ±1.6	0	0	0
	Escherichia hermanni	8 ±2.81	0	0	0	0	0	8 ± 1.45*	0	0
	Klebsiella oxytoca	8 ±2.36	0	0	0	0	0	0	8 ± 2*	0
	Escherichia coli	8 ±3.2	0	6 ±2.1	0	2 ±2.1	0	3 ±2.1	3±2.1	0
	Enterococcus gallinarum	11 ± 2	11± 2*	11 ±1.26	6 ±1.74	8 ±1.58	0	0	0	0
	Acinetobacter radioresistens	6 ±1.9	0	0	0	0	6 ±1.1	0	0	0
	Microbacterium paraoxydan	6±1.08	8 ± 1.2*	4 ±1.08	12±2.03	0	5 ± .04*	0	8 ±2.2*	0
EC-D14	Enterococcus faecium	19 ±4.2	7 ±2.36	8 ±2.6	9 ±2.1	9 ±2.14	9 ±2.11	0	10 ±1.2	5 ±2.05
	Escherichia hermannii	7 ±2.08	0	0	0	0	0	0	0	0
	Microbacterium paraoxydan	4±1	5±2	6±2	6±1	0	6 ±1	0	0	0
	Lactobacillus murinus	9 ±4.2	0	0	0	0	8 ± 1.22	7 ±3.32	0	0
	Enterococcus gallinarum	7 ±2.74	5 ±2.7	7 ±1	9 ±2.1	7±3.3	5 ± 2	7 ±2.1	0	0
	Acinetobacter radioresisten	7 ±1	0	0	0	0	7±1	0	0	0

The stearic (*) sign represents new resistant bacteria with respect to the previous sample. Control, bacteria isolated from media without antimicrobial; CD-0, control sample collected at day 0; Ec-D2 and Ec-D7are samples collected 2 and 7 days after E. coli administration respectively.

belonged to phylum Firmicutes. The highest resistance was recorded against D-cycloserin (21.5%), followed, in decreasing order, by gentamicin (14.6%), amoxicillin (13.6%), kanamycin (12.9%), oxy-tetracycline (12.8%), carbenicillin (11.5%), tetracycline (10%) and ampicillin (2.9%).

RG-1 culturomics

The colonies of the control fecal culture of RG-1 group of rats (C-D0) stool samples, collected before the administration of MDR *E. coli*, were screened for native MDR *E. coli* and found negative. An average bacterial density of 30×10^3 CFU were observed in these control stool samples. This control sampling showed that the rat's randomly selected colonies of their normal GM possess resistance to D-cycloserin ($78\pm13 \times 10^2$CFU), amoxicillin ($76\pm13 \times 10^2$CFU), kanamycin ($36\pm6 \times 10^2$CFU) and carbenicillin ($24\pm24 \times 10^2$CFU) (Fig 1A). Most of the control sample (C-D0) isolates were from the phyla Firmicutes (79.24%), Proteobacteria (12.57%) and Actinobacteria (8.17%) (Fig 2).The isolates of genus *Enterococcus* (79.2%) were the most resistant, including the following most resistant species namely, *Enterococcus faecium* (39.6% of the isolates), *Enterococcus gallinarum* (24.5%) and *Enterococcus faecalis* (15%) (Table 1).The majority of the *E. faecium* recovered from the C-D0 were resistant to kanamycin (6±1.69 isolates), carbenicillin (5±1.02 isolates), gentamicin (7±1.7 isolates), D-cycloserin (8±2.3 isolates) and amoxicillin (7±1.9 isolates) (Table 1). The *Enterococcus gallinarum* was the second most resistant species, possessing resistance against carbenicillin (8±1.6 isolates), gentamicin (6±1.6 isolates) and D-cycloserin (8±2.5 isolates).The *Microbacterium paraoxydans*, recovered from the C-D0, and identified by16S RNA sequencing, was resistant to gentamicin (3±1.3 isolates). In addition, the *Acinetobacter radioresistens* was resistant to kanamycin (5±1 isolates) (Table 1).

In the Ec-D2 fecal samples, collected from the RG-1 group of rats, at two days following the administration of the of the MDR *E. coli*, the bacterial density was significantly ($p=0.01$) increased on the plates supplemented with either the D-cycloserin ($320\pm11 \times 10^2$CFU), tetracycline ($40\pm8 \times 10^2$CFU), oxy-tetracycline ($160\pm9 \times 10^2$CFU) or carbenicillin ($60\pm7 \times 10^2$CFU) in comparison to the count obtained in the control C-D0 fecal samples of the same rats belonging to RG-1 group (Fig. 1). The administered MDR *E. coli* isolates were recovered from the feces with a similar resistance profile to the initial one. However, the *E. coli* count was lower at different sampling times following the administration of the MDR *E. coli* namely, an average of 240 colonies recovered from Ec-D2 samples, 110 colonies from the Ec-D7, and an absence of E.coli colonies in the Ec-D14.

There was an increase in resistant colonies recovered from the Ec-D7 samples, in particular, a significant increase in colonies ($p=0.04$) resistant to kanamycin. In addition, the Ec-D14 samples had a further significant increase in colonies resistant to kanamycin compared to that of the C-D0 samples ($P<0.01$) (Fig 1A). The diversity in the resistant species was greater in the Ec-D7 samples as compared to the C-D0 samples. For instance, new resistant species were identified in the Ec-D7 samples, namely tetracycline-resistant *Escherichia hermannii* (8±1.45 isolates); oxy-tetracycline-resistant *Klebsiella oxytoca* (8±2 isolates); amoxicillin-resistant *E. gallinarum* (11±2 isolates), and *M. Paraoxydans* showing resistance to kanamycin (5±0.4 isolates), amoxicillin (8±1.2) and oxy-tetracycline (8±2.2).

Species isolated from Ec-D14 fecal samples showed resistance to eight different antibiotics (Table 1). The *E. faecium* was the most resistant species (24.31%) in the Ec-D14 samples followed by *M. Paraoxydans* (26.6%) and *E. gallinarum* (17.88%). The emergence of resistance to antibiotics continued, detecting two new kanamycin-resistant species, namely *E. gallinarum* (5±2 isolates) and *Lactobacillus murinus* (8±1.22 isolates). A higher resistance to kanamycin was detected in 5 species recovered from

Table 2. *The Mean percent resistance of identified RG-2 fecal species to 8 antimicrobials before and after the MDR E. coli and the amoxicillin administrations*

Sample	Species Identified	Control	Amoxicillin	D-cycloserin
C-D0	*Enterococcus gallinarum*	17 ±1.6	0	8±2.5
	Microbacterium paraoxydan	10±2	0	0
	Enterococcus faecium	30±2.2	7 ±1.9	8±2.3
	Enterococcus faecalis	24 ±2.3	0	0
	Acinetobacter radioresistens	15 ±1	0	0
EC-D2	*Enterococcus gallinarum*	10 ±2.5	0	9 ±1.9
	Microbacterium paraoxydan	8 ±2.1	0	
	Enterococcus faecium	21 ±3.5	9 ±2.08	10 ±2.7
	Enterococcus faecalis	13±3.6	0	9 ±2.5
	Acinetobacter radioresisten	10 ±2.5	0	0
	Escherichia coli	14 ±3.2	0	11 ±1.55
Ec-Amx-2D	*Enterococcus faecium*	12 ±2.06	7 ±1.23	7 ±2.1
	Enterococcus gallinarum	12 ±2.5	7 ± 1.4*	7 ±2.1
	Pseudomonas balearica	10 ±2.13	0	0
	Enterococcus faecalis	8 ±3.3	0	5±2
	Corynebacterium ammoniagenes	12 ±2	0	0
	Streptococcus caballi	7 ±2.1	0	0
	Bcillus infantis	6 ±1.12	0	0
	Streptococcus ratti	9 ±2.4	0	0
	Microbacteriumparaoxydan	6 ±1.2	0	0
	Acinetobacter radioresistens	6 ±1.12	0	0
Ec-Amx-9D	*Elizabethkingia miricola*	9±2.36	9 ± 2.1*	8± 2.3*
	Streptococcus ratti	10 ±1.99	9 ± 1.35*	8 ± 1.76*
	Staphylococcus nepalensis	9±1.12	8 ± 1.55*	
	Enterococcus faecalis	8 ±1.3	7 ± 2.2*	7 ±1.1
	Enterococcus faecium	5 ±1.2	7 ±1.32	0
	Enterococcus gallinarum	4 ±0.97	7 ±1.22	7 ±1.4
	Microbacteriumparaoxydan	4±0.7	0	0
	Pseudomonas balearica	6 ±1.01	0	0
	Bcillus infantis	7 ±2.1	0	0
	Acinetobacter radioresistens	6 ±1.14	0	0
	Corynebacterium ammoniagenes	10± 0.6	0	0
	Streptococcus caballi	6± 0.6	0	0

***Table 2 cont**. The Mean percent resistance of identified RG-2 fecal species to 8 antimicrobials before and after the MDR E. coli and the amoxicillin administrations*

Gentamycin	Carbenicillin	Kanamycin	Tetracycline	Oxy-tetracycline	Ampicillin
6±1.62	8 ±1.6	0	0	0	0
3±1.3	0	0	0	0	0
7±1.7	5±1.02	6±1.69	0	0	0
0	0	0	0	0	0
0	0	5 ±1	0	0	0
10 ±1.7	10 ±0.9	0	0	0	0
8 ±1.92		0	0	0	0
0	6 ±1.35	7±1.82	0	0	0
0	0	0	0	0	0
0	0	7±1.3	0	0	0
11±1.52	6 ±2.1	0	6 ±1.8	11 ±1.55	0
0	0	4 ±1.2	7 ± 1.5*	0	0
0	0	0	0	5 ± 3*	0
0	0	0	6 ± 2.6*	0	0
0	0	0	6± 2.3*	4 ± 2.5*	6±2.1
0	0	0	0	5 ± 1*	0
0	0	0	0	4 ± 2.6*	0
0	0	0	0	4 ± 1.2*	0
0	0	5 ± 0.87*	4 ± 2.1*	± 1.3*	0
0	0	0	0	0	0
0	0	0	0	0	0
7 ±1.5	7 ± 1.5*	6± 2*	9 ± 2.3*	4 ± 1.2*	0
0	0	5 ±2.22	6 ±0.7	8 ±1.4	0
0	0	0	0	0	0
0	6± 2.2*		10 ± 2.4*	0	9 ±2.35
0	0	1 4±.042	7 ±1.2	7 ±1.2	0
0	0	0	0	8 ±1.1	0
0	0	0	0	0	0
0	0	0	7±1.1	0	0
0	0	0	0	6±0.9	0
0	0	0	0	0	0
0	0	0	0	8±1	0
0	0	0	0	6± 0.6*	0

The stearic (*) sign represents new resistant bacteria with respect to the previous sample. Control, bacteria isolated from media without antimicrobial; CD-0, control sample collected at day 0; Ec-D2 and Ec-D7 samples collected at 2 and 7 days after E. coli administration, respectively; Ec-Amx-2D and Ec-Amx-9D samples collected at 2 and 9 days after amoxicillin administration.

Table 3. *List of nutrients used in media for growing different antibiotic resistant isolates.*

Ingredients	**Grams per Litre**
Acid Hydrolysate of Casein	0.24
Yeast Extract	0.24
Dextrose	0.87
Soluble Starch	0.24
Dipotassium Phosphate	1.42
Magnesium Sulfate Heptahydrate	0.024
Sodium Pyruvate	0.14
Calf brain	30.76
Beef heart	38.4
Proteose peptone	2.16
Sodium chloride	1.58
Disodium phosphate	0.43
Pancreatic digest of casein	1.7
Papaic digest of soyabean meal	0.3
Agar	13.0

the Ec-D14 samples compared to only two species in the control C-D0 samples (Table 1). In addition, *L.murinus* was detected for the first time in Ec-D14 samples, exhibiting resistance to kanamycin (8±1.22 isolates) and tetracycline (7±3.32 isolates) (Table 1).

RG-2 culturomics

The amoxicillin treatment in RG-2 group of rats reduced substantially the bacterial density and declined the resistant colonies to amoxicillin (p=0.01), oxy-tetracycline (p=0.01), kanamycin (p=0.05) and carbenicillin (p=0.05) in comparison to the samples collected before the antibiotic administration (Fig 1B).The amoxicillin treatment was effective against the MDR *E. coli*; however, an increasing diversity was noted after the antibiotic administration. A total of four different phyla were identified in samples Ec-Amx-2D (fecal samples collected after 2 days of antibiotic administration) and Ec-Amx-9D (samples collected after 9 days of antibiotic administration). Among the identified phyla was the Bacteriodetes (23% of the isolates), a newly emerged resistant phylum that was absent in the control C-D0 samples. A total of seven resistant species were detected after the amoxicillin therapy compared to three resistant species identified in the control C-D0 samples (Table 2). Furthermore, there was an increase in the number of resistant isolates from the phyla Firmicutes (775), Actinobacteria (40) and Proteobacteria (35) compared to that of the C-D0 that were Firmicutes (275), Actinobacteria (15) and Proteobacteria (25). The *Enterococcus* (51.59% of the isolates) was the most dominant genus in samples Ec-Amx-2D, followed by genera *Streptococcus* (19.1%), *Corynebacterium* (9%), *Pseudomonas* (8.5%), *Bacillus* (5.3%), *Acinetobacter* (3.1%) and *Microbacterium* (3.1%). The *E. faecium* (19.6% isolates) was dominant in the Ec-Amx-2D samples, encoding resistance against kanamycin (4±1.2 isolates), tetracycline (7±1.5 isolates), D-cycloserin (7±2.1 isolates) and amoxicillin (7±1.2 isolates) (Table 2). The ampicillin-resistant *E. faecalis* (6±2.1 isolates), tetracycline-resistant *Pseudomonas balearica* (6±2.1 isolates) and the three oxytetracycline-resistant species namely, *Streptococcus caballi* (4±2.6

isolates), *Bacillus infantis* (4±1.2 isolates) and *Corynebacterium ammoniagenes* (5±1), were recovered for the first time in this study from the Ec-Amx-2D samples. In addition, the *Streptococcus ratti* was newly detected, encoding resistance against tetracycline (4±2.1 isolates) and oxy-tetracycline (7±2.1 isolates).

There was greater diversity of resistant species in the Ec-Amx-9D samples compared to the control samples (Table 2). The *Elizabethkingia* (total 295 isolates) emerged as newly recovered genus with resistance to amoxicillin, gentamicin, kanamycin, D-cycloserin, tetracycline, oxy-tetracycline and carbenicillin. It is worth noting that six amoxicillin-resistant species, including also the *Staphylococcus nepalensis* (8±1.5 of the isolates), were detected in the Ec-Amx-2D samples compared to only one in the control C-D0.

DISCUSSION

The rat is a common experimental model, extensively used in many segments of science, including its recent sporadic use in microbiome research, to determine the underlying drug resistance mechanisms, and possible solutions for animal and human diseases[35–37]. In this study, rats were orally inoculated with MDR *E. coli* and anothers subjected to an additional treatment with amoxicillin, and stool samples were collected before and after these treatments, to determine the shift in GM-antibiotic resistance, fecal bacterial density and diversity. The culturomics approach was adopted since its usefulness has been recently emphasised for profiling GM diversity and their antibiotics susceptibility[38]. A total of 8020 isolated colonies were screened for antibiotics resistance against ten different antibiotics and the species were identified by MALDI-TOF and some by 16S sequencing. Sixteen species were identified in our modified culture conditions, which were assigned to twelve genera and four phyla. The isolates belonged to Firmicutes, Proteobacteria and Actinobacteria. These phyla are also predominantly abundant in human gut[39]. To our knowledge, this is the first study that presents detailed data on culturomics and resistome of GM using such a rat model.

Overall, the *Enterococcus* was the most dominant genus in culturomics, accounting for 59.2% of the total identified isolates; however, in our metagenomic data (unpublished data), targeting the rat GM, using Illumina MisSiq, *Enterococcus* density in GM was only 0.099%. Additionally, *E. faecium* and *E. gallinarum* were not detected through the metagenomic approach (unpublished data). The fact that some GM species exist in low density, this leads to a higher error in missing the identification through metagenomics[30,40]. Additionally, DNA extraction errors could also be attributed to reporting in literature of lower *Enterococcus*s spp. density. It has been observed that most of the bacteria do not grow in co-cultures[41]. In our previous study of human GM culturomics, it was observed that the genus *Enterococcus* ubiquitously possessed the ability of producing antimicrobial agents (unpublished data). However, in human GM the majority of bacteriocins encoding genes have been reported in *Lactobacillus* and *Streptococcus*[42]. The proven dominancy of the genus *Enterococcus* in this rat model could be due to their antimicrobial-producing ability. Moreover, a genus and species were totally absent in metagenomics data namely, *Microbacterium, A. radioresistens, P. balearica, C. ammoniagenes, Streptococcus caballi, Bacillus infantis, S. ratti, Elizabethkingia micricola* and *S. Nepalensis* (unpublished data).

The detected normal intestinal rat microbiota in this model carried resistance to amoxicillin, D-cycloserin, gentamicin, carbenicillin and kanamycin (Fig 1A, B; and Tables 1 and 2). This is in agreement with other documents in literature[43,44]. The *Enterococcus* was the most resistant genus, including in it the *E. faecium, E. gallinarum* and *E. faecalis*. *Enterococcus* species are commonly present in the gastrointestinal tract of various hosts, and have been recently identified as emerging nosocomial MDR pathogens[45,46]. The majority of resistant genes identified in

animal and human GM are identified in the family *Enterobacteriaceae*[26,47]. Moreover, several health problems in animals and humans have been associated with *Enterococcus* species, such as abdominal abscesses, urinary tract infections, peritonitis, bacteraemia and endocarditis[45,48]. The other resistant dominant genera include *Microbacterium, Escherichia, Acinetobacter, Streptococcus* and *Elizabethkingia*. The genus *Escherichia* was represented by *E. coli* and *E. hermannii*; however; the isolated *E. coli* is most likely attributed to the inoculated MDR *E. coli*, since the drug resistance profile of the strain, before inoculation and after recovery from fecal samples, was similar.

The fecal antimicrobial-resistant colonies increased after MDR *E. coli* administration (Fig. 1 A and B). Higher resistant colony counts were detected against D-cycloserin, tetracycline, oxy-tetracycline and carbenicillin; this could be attributed to the resistant genes present in the inoculated MDR *E.coli*. The MDR *E. coli* strain was retrieved from the stool up to the 7th day post inoculation; however, its shedding disappeared by the 14th day. It has been reported that the normal GM possesses a colonisation resistance phenomenon against foreign bacteria[49]. Despite the known perturbing colonisation resistance, the persistence of *E. coli* shedding existed for a certain period. In addition, the amoxicillin-administration suppressed successfully the *E. coli* in the gut, with an associated decrease in bacterial density and an increase in the diversity of isolated species (Fig. 1 B). Antibiotics not only target pathogens but also reduce the overall bacterial population[50,51]. The increasing diversity may be attributed to an acquired drug resistance or a decrease in the bacteria that synthesise antimicrobial peptides. It is noteworthy that the total bacterial count was maintained back again at nine days post the amoxicillin treatment (Fig. 1B).

Moreover, seven days after the amoxicillin administration, the D-cycloserine, oxytetracycline, tetracycline, gentamicin, ampicillin, amoxicillin and carbenicillin-resistant species were isolated (Fig. 1B and Table 2). The emergence of new species may be attributed to a decrease in the *Enterococcus* spp. or due to acquired resistance. These results are in agreement with previously published research[43] who reported an increasing resistance in oral microbiota after amoxicillin treatment. It is important to mention that antibiotics dissemination from MDR strain depends on strain mutation rate, ability for colonisation and horizontal gene transformation[52]. Antibiotics-induced resistance in bacteria is known to persist for a long time; however, the exact period is controversial. Previous research workers[53] concluded that antibiotics-induced resistance reverted to baseline after 90 days of therapy. Anothers[50] claimed that bacteria lost its antibiotic resistance a few weeks after withdrawal of the drug[54]; however, a previous document[55] proved that the increasing resistance against clarithromycin persisted for one year after the administration of the drug. Future investigations will be directed at evaluating the GM resistome under multiple culture conditions, over a longer period, and in the period following the withdrawal of the drug.

CONCLUSION

The pioneer model used in this study was useful in identification of baseline data on rat GM, which will be a prerequisite for future investigations related to control of enteric bacterial infections and chemotherapy. The GM in this model was predominantly inhabited by the phylum Firmicutes, followed in decreasing order by Proteobacteria, Actinobacteria and Bacteroidetes. The rat GM contains genes encoding resistance to antimicrobial agents. The inoculation of MDR *E. coli* modulated the rat GM resistome pattern. The genus *Enterococcus* was the most resistant to drugs. Both the MDR *E. coli* and amoxicillin intervention led to a shift in antibiotic resistance of the GM and bacterial density. Culturomics-associated to this rat model seems like a heuristic approach for evaluation of antimicrobial resistance diversity; a future upgrading with multiple culturing conditions could further

widen the identification of more bacterial diversity in this animal model.

ACKNOWLEDGEMENT

This project was supported by the NSTIP strategic technologies program in the Kingdom of Saudi Arabia - Project No (12-MED3108-03). The authors also acknowledge with thanks the Science and Technology Unit, King Abdulaziz University for their technical support.

COMPETING INTEREST

The authors declare the absence of any competing financial interest related to this concluded project

REFERENCES

1. Blount, Z.D: The unexhausted potential of E. coli. *Elife*, 4:1–12. 2015
2. Hessain, A.M., Al-Arfaj, A.A, Zakri, A.M, El-Jakee, J.K., Al-Zogibi, O.G., Hemeg, H.A., Ibrahim I.M: Molecular characterization of *Escherichia coli* O157:H7 recovered from meat and meat products relevant to human health in Riyadh, Saudi Arabia. *Saudi J Biol Sci*, 22:725–729. 2015
3. Barbieri, N.L., Oliveira, A.L., de Tejkowski, T.M., Pavanelo, D.B., Matter, L.B., Pinheiro, S.R.S., Vaz T.M.I., Nolan, L.K., Logue, C.M., Brito, B.G. de: Molecular characterization and clonal relationships among *Escherichia coli* strains isolated from broiler chickens with colisepticemia. *Foodborne Pathog Dis*, 12:74–83. 2015
4. Ewers, C., Janßen, T., Kießling, S., Philipp, H.C., Wieler, L.H: Molecular epidemiology of avian pathogenic *Escherichia coli* (APEC) isolated from colisepticemia in poultry. *Vet Microbiol*, 104:91–101. 2004
5. Mellata, M: Human and avian extraintestinal pathogenic Escherichia coli: infections, zoonotic risks, and antibiotic resistance trends. *Foodborne Pathog Dis*, 10:916–32. 2013
6. Yu L.C.H., Shih, Y.A., Wu, L.L., Lin, Y.D., Kuo, W.T., Peng, W.H., Lu, K.S., Wei, S.C., Ni Y.H: Enteric dysbiosis promotes antibiotic-resistant bacterial infection: systemic dissemination of resistant and commensal bacteria through epithelial transcytosis. *Am J Physiol Gastrointest Liver Physiol* 307:824–835. 2014
7. Pitout, J.D: Recent changes in the epidemiology and management of extended-spectrum beta-lactamase-producing Enterobacteriaceae. F1000 *Med Rep*, 1. 2009
8. Pascual, V., Ortiz, G., Simó, M., Alonso, N., Garcia, M.C., Xercavins, M., Rivera, A., Morera, M.A., Miró, E., Espejo, E., Navarro, F., Gurguí, M., Pérez, J., Rodríguez-Carballeira, M., Garau, J., Calbo, E: Epidemiology and risk factors for infections due to AmpC B-lactamase-producing *Escherichia Coli*. *J Antimicrob Chemother*, 70:899–904. 2015
9. Sodha, S. V., Heiman, K., Gould, L.H., Bishop, R., Iwamoto, M., Swerdlow, D.L., Griffin ,P.M: National patterns of *Escherichia coli* O157 infections, USA, 1996-2011. *Epidemiol Infect*, 143:1–7. 2014
10. Lutful, Kabir, S.M: Avian Colibacillosis and Salmonellosis: A Closer Look at Epidemiology, Pathogenesis, Diagnosis, Control and Public Health Concerns. *Int J Environ Res Public Health*, 7:89–114. 2010
11. Alwan, N., Saleh, I., Beydoun, E., Barbour, E., Ghosn, N., Harakeh, S: Resistance of Brucella abortus isolated from Lebanese dairy-based food products against commonly used antimicrobials. *Dairy Sci Technol*, 90:579–588. 2010
12. Iyer, A., Barbour, E., Azhar, E., Salabi, A.A. E.l., Hassan, H.M., Qadri, I., Chaudhary, A., Abuzenadah, A., Kumosani, T., Damanhouri, G., Alawi, M., Nawas, T., Nour, A.MA., Harakeh, S: Transposable elements in *Escherichia coli* antimicrobial resistance. *Adv Biosci Biotechnol*, 4:415–423. 2013
13. Sidjabat H.E., Paterson D.L: Multidrug-resistant *Escherichia coli* in Asia: epidemiology and management. *Expert Rev Anti Infect Ther*, 13:575–591. 2015
14. Kirk, M.D., Pires, S.M., Black, R.E., Caipo, M., Crump, J.A., Devleesschauwer, B., Döpfer, D., Fazil A., Fischer-Walker, C.L., Hald T: World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral diseases, 2010: A Data Synthesis. *PLoS Med*, 12:e1001921. 2015
15. Grace, D: Zoonoses of Poverty: Measuring and Managing the Multiple Burdens of Zoonoses and Poverty. In *Zoonoses-Infections Affecting Humans and Animals*. Springer,1127–1137. 2015
16. Lecomte, V., Kaakoush, N.O., Maloney, C.A., Raipuria, M., Huinao, K.D., Mitchell, H.M., Morris, M.J: Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. *PLoS One,* 10:e0126931. 2015
17. Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R. V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M: Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis*, 17:7–15. 2011
18. Skurnik, D., Clermont, O., Guillard, T., Launay, A., Danilchanka, O., Pons, S., Diancourt,.L, Lebreton, F., Kadlec, K., Roux, D: Emergence of Antimicrobial-Resistant *Escherichia coli* of Animal Origin Spreading in Humans. *Mol Biol Evol*:msv280. 2015
19. Wichmann, F., Udikovic-Koli, N., Andrew, S., Handelsman, J: Diverse antibiotic resistance genes in dairy cow manure. *MBio*, 5. 2014
20. Clemente, J.C., Pehrsson, E.C., Blaser, M.J., Sandhu, K., Gao Z., Wang, B., Magris, M., Hidalgo, G., Contreras, M., Noya-Alarcon O., Lander, O., McDonald, J., Cox, M., Walter, J., Oh, P.L., Ruiz, J.F., Rodriguez, S., Shen, N., Song, S.J., Metcalf, J., Knight, R., Dantas, G., Dominguez-Bello MG: The microbiome of uncontacted Amerindians. *Sci Adv*, 1:e1500183–e1500183. 2015
21. Ballard, D.P., Peterson, E.A., Nadler J.L: Antibiotic Use in Animal Feed and its Impact on Antibiotic

Resistance in Human Pathogens. *Food Microbiol Hum Heal Dis*:137. 2015

22. Dai, M., Lu, J., Wang, Y., Liu, Z., Yuan, Z: In vitro development and transfer of resistance to chlortetracycline in Bacillus subtilis. *J Microbiol*, 50:807–812. 2012
23. Fouhy, F., Ross, R.P., Fitzgerald, G.F., Stanton, C., Cotter, P.D: A degenerate PCR-based strategy as a means of identifying homologues of aminoglycoside and β-lactam resistance genes in the gut microbiota. *BMC Microbiol*, 14:25. 2014
24. Lester, C.H., Frimodt-Møller, N., Sørensen, T.L., Monnet, D.L., Hammerum, A.M: In vivo transfer of the vanA resistance gene from an *Enterococcus* faecium isolate of animal origin to an E. faecium isolate of human origin in the intestines of human volunteers. *Antimicrob Agents Chemother*, 50:596–599. 2006
25. Moore AM, Ahmadi S, Patel S, Gibson MK, Wang B, Ndao MI, Deych E, Shannon W, Tarr PI, Warner BB, Dantas G: Gut resistome development in healthy twin pairs in the first year of life. *Microbiome*, 3:27. 2015
26. Coyne MJ, Zitomersky NL, McGuire AM, Earl AM, Comstock LE: Evidence of extensive DNA transfer between bacteroidales species within the human gut. *MBio*, 5. 2014
27. Frye, J.G., Jackson, C.R: Genetic mechanisms of antimicrobial resistance identified in Salmonella enterica, Escherichia coli, and Enteroccocus spp. isolated from U.S. food animals. *Front Microbiol*, 4(MAY):1–123. 2013
28. Kumar, Hidau, M., Singh, Y., Shahi, S., Mounika,.P, Kumar., Singh, S: LC-MS/MS Assay for Quantification of a Novel Antitubercular Molecule S006-830: Pharmacokinetic and Plasma Protein Binding Studies in Rats. *Curr Pharm Anal*, 11:35–42. 2015
29. Ghosh, M: Fundamentals of experimental pharmacology. *Indian J Pharmacol*, 39:100–101. 2007
30. Gottberg, B., Berne., J., Quinonez, B., Solorzano, E: Prenatal effects by exposing to amoxicillin on dental enamel in wistar rats. *Med Oral Patol Oral Cir Bucal*, 19:e38. 2014
31. Dubourg, G., Lagier, J.C., Robert, C., Armougom, F., Hugon, P., Metidji, S., Dione, N., Dangui, N.P.M., Pfleiderer, A. Abrahao, J., Musso, D., Papazian, L., Brouqui, P., Bibi, F., Yasir, M., Vialettes ,B., Raoult, D: Culturomics and pyrosequencing evidence of the reduction in gut microbiota diversity in patients with broad-spectrum antibiotics. *Int J Antimicrob Agents*, 44:117–124. 2014
32. Angelakis, E., Yasir, M., Azhar, E.I., Papadioti, A., Bibi F., Aburizaiza, A.S., Metidji, S., Memish, Z.A., Ashshi, A.M., Hassan, A.M., Harakeh, S., Gautret, P., Raoult, D: MALDI-TOF mass spectrometry and identification of new bacteria species in air samples from Makkah, Saudi Arabia. *BMC Res Notes*, 7:892. 2014
33. Radosevich, J.L., Wilson, W.J., Shinn, J.H., DeSantis, T.Z., Andersen, G.L: Development of a high-volume aerosol collection system for the identification of air-borne micro-organisms. *Lett Appl Microbiol*, 34:162–167. 2002
34. Nitecki, S.S., Teape, N., Carney, B.F., Slater, J.W., Bruck, W.M: A duplex qPCR for the simultaneous detection of *Escherichia coli* O157:H7 and Listeria monocytogenes using LNA probes. *Lett Appl Microbiol*, 61:20–27. 2015
35. Yasir, M., Aslam, Z., Kim, S.W., Lee, S.W., Jeon, C.O., Chung, Y.R: Bacterial community composition and chitinase gene diversity of vermicompost with antifungal activity. *Bioresour Technol*, 100:4396–403. 2009
36. Prajapati, B., Rajput, P., Jena, P.K., Seshadri, S: Investigation of chitosan for prevention of diabetic progression through gut microbiota alteration in sugar rich diet induced diabetic rats. *Curr Pharm Biotechnol*, 17:1–12. 2016
37. Round, J.L.J., Mazmanian, S.S.K: The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol*, 9:313–23. 2009
38. Song, H, Han, W., Yan, F., Xu, D., Chu, Q., Zheng, X: Dietary Phaseolus vulgaris extract alleviated diet-induced obesity, insulin resistance and hepatic steatosis and alters gut microbiota composition in mice. *J Funct Foods*, 20:236–244. 2016
39. Lagier, J.C., Hugon, P., Khelaifia, S., Fournier, P.E., La Scola, B., Raoult, D: The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. *Clin Microbiol Rev*, 28:237–264. 2015
40. Koren, O., Goodrich, J.K., Cullender, T.C., Spor, A., Laitinen, K., Bäckhed, H.K., Gonzalez, A., Werner, J.J., Angenent, L.T., Knight, R., Bäckhed, F., Isolauri, E,. Salminen, S., Ley, R.E: Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell*, 150:470–80. 2012
41. Lagier, J.C., Edouard, S., Pagnier, I., Mediannikov, O., Drancourt, M., Raoult, D: Current and past strategies for bacterial culture in clinical microbiology. *Clin Microbiol Rev*, 28:208–236. 2015
42. Rettedal, E.A., Gumpert, H., Sommer, M.O.A: Cultivation-based multiplex phenotyping of human gut microbiota allows targeted recovery of previously uncultured bacteria. *Nat Commun*, 5:4714. 2014
43. Drissi, F., Buffet, S., Raoult, D., Merhej, V: Common occurrence of antibacterial agents in human intestinal microbiota. *Front Microbiol*, 6: 1-6. 2015
44. Feres, M., Haffajee, A.D., Allard, K., Som, S., Goodson, J.M., Socransky, S.S: Antibiotic resistance of subgingival species during and after antibiotic therapy. *J Clin Periodontol*, 29:724–735. 2002
45. Sommer, M.O., Dantas, G., Church, G.M: Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* (80-), 325:1128–1131. 2009
46. Lebreton, F., Gilmore, M.S: *Enterococcus* Diversity, , Origins in Nature , and Gut Colonization. In: Gilmore MS, Clewell DB, Ike Y, et al., editors. Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. Boston: Massachusetts *Eye and Ear Infirmary*; 2014;:1–123. 2014
47. Schaik, W. Van: The human gut resistome. R Soc Publ, 370:1–9. 2015

48. Hleba L: Antibiotic resistance of Enterobacteriaceae strains isolated from different animals gastrointestinal tracts. *Sci Pap Anim Sci Biotechnol,* 48:128–131. 2015

49. Thumu, S.C.R., Halami, P.M: Phenotypic expression, molecular characterization and transferability of erythromycin resistance genes in *Enterococcus* spp. isolated from naturally fermented food. *J Appl Microbiol*, 116:689–699. 2014

50. Lawley, T.D., Walker, A.W: Intestinal colonization resistance. *Immunology*, 138:1–11. 2013

51. Jernberg, C., Lo, S., Edlund, C., Jansson, J.K: Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology*, 156:3216–3223. 2010

52. McFarland, L.V: Epidemiology, Risk Factors and Treatments for Antibiotic-Associated Diarrhea. *Dig Dis*, 16:292–307. 1998

53. Andersson, D.I., Hughes, D: Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol*, 8:260–71. 2010

54. Jakobsson, H.E., Jernberg, C., Andersson, A.F., Sjolund-Karlsson, M., Jansson, JK., Engstrand L: Short-term antibiotic treatment has differing long-term Impacts on the Human Throat and Gut Microbiome. *PLoS One*, 5:1–12. 2010

55. Sjölund, M., Tano, E., Blaser, M.J., Andersson, D.I., Engstrand, L: Persistence of resistant Staphylococcus epidermidis after single course of clarithromycin. *Emerg Infect Dis*, 11:1389–1393. 2005