ARB and ARGs survived from the extremely acidity posing a risk on intestinal bacteria in an in vitro digestion model by horizontal gene transfer

Qiujie Cai
Guangdong University of Technology - University Town Campus: Guangdong University of Technology

Yanbin Xu (✉ hopeybxu@163.com)
Guangdong University of Technology - University Town Campus: Guangdong University of Technology
https://orcid.org/0000-0002-8874-069X

Min Zhou
Guangdong University of Technology - University Town Campus: Guangdong University of Technology

Ling Yu
Guangdong University of Technology - University Town Campus: Guangdong University of Technology

Pengqian Ouyang
Guangdong University of Technology - University Town Campus: Guangdong University of Technology

Li Zheng
Guangdong University of Technology - University Town Campus: Guangdong University of Technology

Research

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Abstract

Background: Antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) have been considered as emerging contaminants, which even might be closely related to human health.

Methods: To investigate the disease-producing risk of ARB and the horizontal gene transfer (HGT) risks of both extracellular ARGs (eARGs) and intracellular ARGs (iARGs), an in vitro digestion model was established to simulate the process of ARB and ARGs going through digestive tract. CTC/DAPI-FCM assay was used to study the survival of ARB during digestion, and the changes of genes (including tetA, tetG, tetM, sul1, sul2, bla_EBC, blaFOX, intI1 and 16S rRNA) were determined by QPCR.

Results: The results showed that ARB were mostly affected by pH of gastric juice. About 99% ARB (total population of $2.45 \times 10^9 – 2.54 \times 10^9$) were killed by the gastric juice of pH 2.0 for the severely damage of bacterial cell membrane, but more than 80% ARB (total population of $2.71 \times 10^9 – 3.90 \times 10^9$) were still alive with intact cell membrane when the pH of gastric juice increased to 3.0 and above. ARGs, intI1 and 16S rRNA could be detectable even at extreme pH when most bacteria died. The eARGs (accounting for 0.03% – 24.56% of total genes) were less than iARGs obviously. The eARGs showed greater HGT potential than that of iARGs, suggesting transformation occurs more easily than conjugation. The transfer potential followed the order as: tet (100%) > sul (75%) > bla (58%), related to the high correlation of intI1 with tetA and sul2 ($p < 0.01$). Moreover, gastric juice of pH 1.0 could decrease the transfer frequency of ARGs by 2–3 order of magnitude compared to the control, but still threatening human health.

Conclusions: Under the treatment of digestive juice, ARGs still have high gene horizontal transfer potential, suggesting that food-borne ARB pose a risk of ARGs horizontal transfer to intestinal bacteria.

Highlights

1. ARB and ARGs passing through digestive tract were simulated with an in vitro model
2. The pH of gastric juice determines the viability of bacteria to a great extent
3. eARGs and iARGs survive at extreme pH value and iARGs were more abundant than eARGs
4. Food-borne ARB pose a risk of ARGs horizontal transfer to intestinal bacteria
5. The horizontal transfer potential of ARGs followed the order as: tet > sul > bla

Background

As the major breakthroughs of modern medicine, antibiotics have made an outstanding contribution to kill or inhibit the bacteria hazardous to human and animal health, as well as to promote the growth of animals in aquaculture, livestock and poultry farms. According to a surveillance study from 2000 to 2015 in 76 countries, antibiotic consumption increased to 34.8 billion defined daily doses (DDDs) from 21.1 billion DDDs with a growth rates of 65% [1]. A huge consumption of antibiotics resulted in a large amount of antibiotics releasing into environment due to its poor absorption in the gut of human and
animals [2]. In recent decades, antibiotics were detected in manure, soil, surface water and food (meat and milk) [3–5].

Therefore, the antibiotics became a new class of contaminants because of the emerging concern for antibiotic resistance. More and more alarming evidences suggested that the increasing level of antibiotic resistance results from rampant antibiotic use [6–9]. Antibiotic can not only induce the appearance of antibiotic resistance genes (ARGs) through gene mutations, but also accelerate the spread of ARGs with the mechanism of horizontal gene transfer (HGT) [10, 11]. Bacteria can acquire ARGs more easily through HGT than through gene mutations. The mechanisms of HGT include conjugation, transformation and transduction and is mediated by the mobile genetic elements (MGEs) including plasmids, transposons, integrons and bacteriophage [12]. HGT is playing an important role in helping bacteria share ARGs across species [13]. Overall, the overuse and misuse of antibiotics exacerbate the spread of the ARGs on a global scale by increasing the tempo of HGT and bacterial evolution for many traits.

Since livestock and poultry farms, aquaculture site and wastewater treatment plants (WWTPs) are ‘hotspots’ of antibiotic overuse, discharge or centralized processing, ARGs are frequently detected in raw wastewater and effluent of WWTPs [14, 15], animal manure [16] and aquaculture wastewater [17]. Nowadays, ARGs haven’t been treated with effective processes as a novel contaminant, still surviving the existing solid waste and wastewater treatment processes [14, 18–20]. Thus, those ARGs will further spread to their nearby environments through waste application on farm or water cycle [21], risking water and food safety [19, 22].

ARGs in foods are rarely studied compared to that in environmental media, and its potential risk to human beings is unknown but does need more concern. ARGs have been found in fermented food [23, 24], aquatic products [25], vegetables [19], etc., greatly increasing the risk of ARGs on human or animal bodies, especially on their intestines. There is no direct evidence showing that ARGs in food can transfer to the indigenous bacteria in intestines of human and animal. However, evidence has shown that ARGs can be shared between environmental bacteria and human pathogens [26, 27].

In order to reveal the effect of foreign ARB and ARGs on the indigenous bacteria after passing through mouth, stomach and reaching small intestine, an in vitro digestion model was established to simulate the process of ARB and ARGs passing through digestive tract, ARGs and the live ARB in different stage of digestion were quantified by QPCR and flow cytometry (FCM), respectively. Strain Enterbacter hormaechei was used as ARB (ARGs carrier) in this study, which was isolated from a fish-duck pond in our previous research and was conformed to be with multiple antibiotics resistance [17]. As a very common pathogenic bacterium for animal diarrhea in farm, Enterbacter hormaechei with antibiotics resistance just can represent a risk of ARGs from farm to human being. The results of the present research would give some guides on health risk evaluation of ARGs and ARB.

Methods
Bacterial source and culture preparation

The strain *E. hormaechei* (Fig. S1), a multidrug-resistant bacterium from a fish-duck pond, was used as the carrier of foreign ARGs to study the variation of ARGs along the digestive tract and the HGT potential of foreign ARGs in the intestine tract. PCR assays showed the strain *E. hormaechei* carried multiple resistance genes including eight tetracycline resistance genes (*tetA*, *tetC*, *tetG*, *tetL*, *tetM*, *tetO*, *tetS* and *tetX*), three sulfonamide resistance genes (*sul1*, *sul2* and *sul3*), two beta-lactamase genes (*blaFOX* and *blaEBC*) and two integron genes (*intI1* and *intI2*), resulting in resistance to tetracycline, cefotaxime sodium and sulfonamide which also be proved by drug resistance detection. The minimum inhibitory concentration (MIC) for *E. hormaechei* was 0.5 mg/L of cefotaxime sodium, 16 mg/L of tetracycline and 256 mg/L of sulfonamide, respectively.

The HGT potential of foreign ARGs was evaluated by the filter mating with the donor strain of *E. hormaechei* treated by stimulated digestive juices and the receptor strain of *E. coli* BL21(DE3) (Fig. S1) hosting pET28a-EGFP plasmid with kanamycin resistance. Both the donor strain and receptor strain used in the experiment was prepared by innoculating the strain of logarithmic growth phase in fresh Luria-Bertani (LB) broth and culturing in a shaker at 37°C overnight. The cells were collected in 50 mL tubes by centrifugation at 3000rpm for 10 mins and washed twice with phosphate buffered saline (PBS) buffer to remove the medium and metabolites, and finally resuspended in PBS buffer. The OD$_{600nm}$ of the bacterial suspensions were adjusted to around 1.0 and stored at 4°C before used. All experiments were performed within 24 hours after cells collection.

Simulation of ARB and ARGs passing through digestive tract

The *in vitro* digestion model used in the present study is a whole digestive tract including mouth, stomach and small intestine, which was first built by Oomen et al., [28] and then developed by Versantvoort et al., [29] based on human physiology. In this study, we used the model and recipes of digestion juices. The digestive juices were prepared according to the reference [29] as shown in Table S2 and ARB and ARGs simulated to passing through the digestive tract by following the schematic diagram shown in Fig. 1. Group C simulated *in vitro* stage, group S simulated mouth stage, group G (G1, G2, G3, G4) simulated stomach stage at different pH conditions, and group D (D1, D2, D3, D4) simulated small intestine stage. Briefly, the digestion was started by addition of 3.0 mL saliva to a 50-mL tube containing bacteria cells. After resuspension with brief vortex, the mixture was shaken at 55 rpm at 37°C. Five minutes later, 6 mL gastric juice (with pH of 1.0, 2.0, 3.0 or 4.0) was added and shaken at 37°C. After 2 hours, 10 mL intestinal juice (6 mL duodenal juice, 3 mL bile juice and 1 mL NaHCO$_3$) was added. This mixture was shaken at 37°C for 2 hours. Each mixture at different stage was filled up to 19 mL with PBS buffer, making all the mixture to a same volume. After vortex, 5 mL of the prepared mixture was used for DNA extraction, and the rest of them were detected by the flow cytometry (FCM, Bruker) and scanning electron microscope (SEM, Hitach 800, Japan).
Detection and enumeration of active bacteria by CTC/DAPI-FCM

In the present research, a modified method of CTC/DAPI double staining combined with flow cytometry (CTC/DAPI-FCM) was used to detect the populations of live cell. As two common intracellular fluorescent probes, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) have been widely used to quantify active bacteria (live bacteria) and total bacteria, respectively [30–32]. CTC can be reduced to a red fluorescent CTC-formazan product (CTF) by live cells with the aid of the electron transport chain, and DAPI can release strong blue fluorescence when combined with DNA as shown in Fig. S2.

The procedure of CTC/DAPI-FCM was described as below. Cells from 200 µL suspension was washed twice by PBS buffer and was added to a 1.5-mL tube to mix with 40 µL of 25 mM CTC solution. Then, the mixture was incubated at 37°C for 2 h in the dark, and the reduction reaction was ended with 200 µL of a 4% formaldehyde solution. After a 10 min's fixation with ice bath, cells were washed twice with PBS buffer. Counterstaining of total cells was done with 4.4 µL 1 mg/mL DAPI solution at 37°C for 5 min in the dark. A Flow Sight® Imaging Flow Cytometer equipped with 405 nm, 488 nm, 642 nm and 785 nm lasers was used in this study. Samples were either analyzed immediately or frozen in liquid nitrogen after fixation. Sample acquisition and data analyses were processed with INSPIRE® software and IDEAS® software, respectively.

DNA extraction and genes analysis

Each sample was centrifuged at 4°C and filtrated with 0.22 µm filter to separate sediment and cell free supernatant. Cell free supernatant was used to obtain extracellular DNA (eDNA), sediment was used to harvest intracellular DNA (iDNA). DNA concentration and purification were performed by using regents of Soil DNA kit (Omega Bio-Tek, USA). The obtained DNA were stored at -20°C. Polymerase chain reaction (PCR) detecting system (Eppendorf, Germany) was used to determine ARGs in the samples. The PCR assay was performed as described by [33]. PCR products were dyed by 4S Green Plus Nucleic Acid Stain and analyzed by the gel electrophoresis using 1.0% (W/V) agarose in a tris-borate-EDTA (TBE) buffer. 100 bp DNA ladder was used in the gel electrophoresis and the bands were visualized by UV transillumination.

The abundance of ARGs, intI1 and the 16S rRNA were analyzed by quantitative polymerase chain reaction (QPCR). The QPCR reactions were performed in 96-well plates with 20 mL mixtures, which included 7 µL ddH₂O, 10 µL 2 × iTaq™ universal SYBR® Green supermix (BIO-RAD, USA), 1µL of each primer (10 µM) and 1 µL template DNA. All the primers used in this study were shown in Table S3. The temperature program was started with denaturation at 95°C for 3 min, followed by 40 cycles of 10 s at 95°C, then 30 s at the annealing temperature, with a final extension for 5 min at 65°C to 95°C for 5 s. Each reaction was run in triplicates on BIO-RAD CFX connect system (BIO-RAD, USA).

HTG assays of eARGs and iARGs
A small amount of ARGs and intI1 could be detected in cell-free supernatant and the majority was within cells (Fig. 4), after ARB were treated by different digestive juices. In this study, receptor strain may acquire ARGs by transformation of extracellular ARGs (eARGs) and conjugation of intracellular ARGs (iARGs). In order to explore the transformation potential of eARGs and conjugative transfer potential of iARGs, the cell-free supernatant and donor cells were used to prepare the HTG assays of ARGs, respectively. After treated with different digestive juice, 1/3 mixture containing donor cells were used to prepare HTG assay. The experimental conditions for obtaining transconjugants or transformants were shown in Fig. S3. The cell-free supernatants and donor cells were obtained by centrifugation at 8000 rpm for 5 min and filtration with 0.22 µm pore size filter. Both twice volume LB broth of supernatant and 1 mL receptor was added in the cell-free supernatant and incubated at 37°C overnight in a shaker for ARGs transfer assay. At the same time, the donor cells were mixed intensively with 1 mL receptor cells and PBS buffer. After that, the mixture was load on 0.22 µm filter membranes by filtration, during which cells were washed by PBS buffer to remove the residual digestive uid. Finally, the filter membranes were placed on the LB agar and incubated at 37°C overnight for conjugative transfer of ARGs. Then, the filter membranes were pick out and cut into small pieces before being put into the tubes containing 25 mL of PBS and 0.5 g sterile glass beads. The bacteria were detached from the filter membranes by vortex shaking for 2 min. The bacterial suspension was serial diluted with PBS and sampled 100 µL aliquots to spread on the selective LB agar media containing 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG), 50 mg/L Kanamycin and 16 mg/L tetracycline (or 0.5 mg/L cefotaxime sodium or 256 mg/L sulfanilamide). All plates were put in an incubator at 37°C for 24–48 h. Only the ARGs received fluorescent receptor by HTG could grow on the plates holding selective media and showed fluorescence under UV light. And then the cell dilution of 100 µL aliquots was plated onto the selective LB agar media containing 0.5 mM IPTG and 50 mg/L kanamycin to count the receptors.

Statistical analysis

Data were processed with Microsoft Excel 2017, and diagramming and correlation analysis were performed using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) and Origin Pro 8.1 (OriginLab Co., MA, USA).

Results And Discussion

Variation of ARB during digestive process

In this study, cells treated with different digestive juice (Table S1) were washed by PBS buffer and then stained with CTC/DAPI. Finally, live cells and dead cells can be differentiated and counted by FCM. As Fig. 2 shown, compared to in vitro simulation (group C), the number of live cells was barely changed in mouth simulation (group S) as the proportions of live cell were 88.00% and 87% (total cell were both about 3.5 × 10^9), respectively. In the simulated stomach (group G), the pH of gastric juice has great influence on cells. When gastric juice pH was more than 2.0, almost all the ARB were killed and only no more than 0.2% (total population of 2.45 × 10^9–2.54 × 10^9) ARB survived. However, when gastric juice pH was over 3.0, more than 80% ARB (total population of 2.71 × 10^9–3.90 × 10^9) survived after a 2-hour
treatment. In the simulated small intestine (group D), living cell populations in group D1, D2, D3 and D4 were $1.35 \times 10^7$, $1.30 \times 10^7$, $1.31 \times 10^9$ and $1.68 \times 10^9$, and the percentages of living cells were 6.12%, 1.04%, 51.80% and 42.60%, respectively. Therefore, the pH of gastric juice might be the most important reason affecting the survival of ARB when they passed through the digestive tract. Moreover, except the survival ARB seriously damaged by stomach acid could not recover for the reason of the digestive enzyme pancreatin and lipases, others adapted, grew and reproduced in the intestinal juice.

According to the SEM images shown in Fig. 3, the cells in the control group are intact and well-structured, while the cells in the treatment groups show various morphology. The lower pH was, the more seriously the cells were damaged, the fewer the number of cells is. Compared to the control (Fig. 3e), greater damages can be found in the cells treated by the gastric juice with pH 1.0 (Fig. 3a) and pH 2.0 (Fig. 3b), showing obvious ruptures and particles on cell surface. However, bacteria cells in the gastric juice with pH 3.0 (Fig. 3c) and pH 4.0 (Fig. 3d) have similar morphology to the control and their ability of reproduction can be proved by cell division. Most bacteria can optimally grow at pH ranging from 6.7 to 7.5 but only a few can survive at extreme pH [34, 35], and ARB used here are no exception. Bacteria could adapt to low pH environments by reducing membrane lipid methyl moieties [36]. The FCM result and SEM images provided strong evidence that most of ARB died at extremely low pH ($\text{pH} \leq 2.0$) with cell membrane greatly damaged, but survived with intact cell if the pH of gastric juice is no less than 3.0. Those surviving ARB even could recover and reproduce in suitable environment such as intestine tract, which might threaten human health.

**Variation of eARGs and iARGs during digestive process**

Both eARGs (Extracellular antibiotics resistance genes) and iARGs (intracellular antibiotics resistance genes) in ARB cultures treated with different digestion juices were detected by QPCR, and the results were showed in Fig. 4. In general, the abundance of iARGs was higher than that of eARGs, differing by 0–5 orders of magnitude. DNA is generally present inside cells and plays a role in the storage and transmission of genetic information as the main material foundation of biogenetics. DNA is released only when cells are damaged or lyzed by self-induced lysis or harsh conditions such as ultrasound, extreme pH, and high temperatures [37, 38]. Notably, eARGs accounted for 0.03–24.56% of total genes in the control (group C) only treated with PBS buffer. It was probably because *Hormaechei* could actively excrete DNA just like *Acinetobacter, Bacillus, Flavobacterium, Micrococcus, Pseudomonas* and *Streptococcus* [39–41]. Among eARGs, the average abundances of tetA, 16S rDNA, intI1 and sul2 were at high level (order of magnitude, i.e. 7–9). In the same way, among iARGs, the average abundances of sul2, intI1, 16S rDNA and tetA were also at high level (order of magnitude, i.e. 11–13). The boxplots of Fig. S5 obviously showed that the abundances of tetA, 16S rDNA, intI1 and sul2 are high in both eARGs and iARGs. 16S rDNA is the DNA sequence coding 16S rRNA, which exists in all bacterial chromosome genes. The abundances of tetA and sul2 higher than other ARGs, which is consistent with some other reports [42, 43]. The Pearson correlation coefficients (Fig. S4) of intI1 with tetA and sul2 are significantly high ($r = 0.801-0.937$, $p < 0.01$), suggesting that intI1 contains a site for integrating with tetA and sul2.
Along the simulated digestive tract, first in mouth, saliva had very little impact on the abundance of eARGs and iARGs only with slight fluctuations, which was consistent with the effect of saliva on the proportion of live ARB cells. Mercer [44] found that adding pure acids into human saliva could not lead DNA to degrade immediately and even persist for hours. Then to stomach, ARB were treated with gastric juices at different pH (i.e. 1.0, 2.0, 3.0, 4.0). Although the pH values of gastric juice increased owing to the inflow of saliva (Table S1), the acidic environment in stomach made both eARGs and iARGs decrease. The greatest downturn of ARGs recorded at the gastric juice pH of 1.0, especially the extracellular \textit{tetA}, \textit{sul2}, \textit{intI1}, \textit{bla}_{FOX} and intracellular \textit{tetA}, \textit{sul2}, \textit{intI1}, \textit{bla}_{FOX}, \textit{bla}_{EBC}, 16S rRNA, which all decreased by more than 91% owing to the cell injury (Fig. 3) and the bacteria activity (Fig. 2). However, both eARGs and iARGs decreased slightly when gastric juice pH ≥ 2.0, nearly in the same order of magnitude. Ferrini et al. [45] used the fresh collected human gastric juice to treat the maize Bt176 DNA carrying target traits of \textit{bla}_{TEMl}, cry/A(b) and sub-cry/A(b) and found that cry/A(b) was completely degraded when gastric juice pH = 1.59, and the fragments of the three genes can be detected when gastric juice pH ≥ 2.0, which was similar to our results. When ARB entered from stomach with gastric acid of pH 1.0, 2.0, 3.0 and 4.0 to small intestine with neutral intestinal juice, their habitat pH returned to pH 8.03, 8.45, 9.33 and 9.66, respectively (Table S1). Even though, each gene of eARGs or iARGs was different from group to group. It was found that smaller genes could survive gastric juice better [45]. However, the boxplots of Fig. S6 show the downtrend of eARGs, the uptrend of iARGs and downtrend of total genes, from group D1 to group D4. Those may result from many reasons. The stronger rupture of ARB caused by gastric juice probably lead to more genes released from cells and the reduction of genes may due to pH value or certain substances in intestinal juice.

Combined with the change of living ARB percentage along the digestive tract, it is not difficult to find: pH of gastric juice was the key reason affecting the activity of ARB and the abundance of ARGs reaching intestinal tract. The lower the pH of gastric juice was, the more obvious the destructive effects of gastric juice on ARB and ARGs were. Therefore, the food-borne ARB carrying ARGs may successfully reach the small intestine when gastric acid secretion was insufficient for some reasons such as drugs, illness and so on. Subsequently, HGT might trigger between the immigratory ARB or ARGs and the local intestinal bacteria, posing a risk of human health.

**The HGT potential of eARGs and iARGs**

In order to investigate the potential of immigratory ARGs horizontal transferring to intestinal bacteria, cell free supernatant (containing eDNA) and sediment (containing iDNA) were separated and used to performed ARGs horizontal transfer assays.

As shown in Fig. 5, the transfer frequency of eARGs is generally higher than that of iARGs, although the abundance of iARGs is higher than that of eARGs (Fig. 4). Transformation does allow for the uptake of plasmid DNA and chromosomal DNA and transformation with chromosomal DNA fragments is usually more efficient than with plasmids [46]. Moreover, DNA uptake by competent bacteria occurs rapidly \textit{in vitro} [47, 48]. Therefore, transformation occurs more easily than conjugation, which depends on the
mating-pair formation apparatus. Transconjugants and transformants peaked in selective plate with tetracycline with highest detection of 100%, followed in plate with sulfanilamide with detection of 75% and declined to the lowest in plate with cefotaxime sodium with detection of 58%. Many reports have showed that mobile integrin \textit{intI1} played an important role in horizontal transfer of ARGs [7, 26, 49].

Based on the previous result (Fig. S4), the correlations of \textit{intI1} with \textit{tetA} and \textit{sul2} are very significant ($r = 0.801–0.937$, $p < 0.01$). Under \textit{in vitro} condition (group C), ARGs from \textit{Hormaechei} also had horizontal transfer potential, with transfer frequency ranging from $5.50 \times 10^{-3}$ to $1.30 \times 10^{-1}$. In natural environment, there are 0–100% bacteria that can develop time-limited competence in response to specific environmental conditions such as altered growth conditions, nutrient access, cell density (by quorum sensing) or starvation [11]. Soda et al. also found the high transfer frequency (transfer frequencies were $5.0 \times 10^{-2}$ to $7.5 \times 10^{-1}$/donors and $4.6 \times 10^{-3}$ to $1.3 \times 10^{-2}$/potential recipient) can be detected under natural condition [50]. Saliva barely affected on the horizontal transfer potential of ARGs since the transfer frequencies of eARGs and iARGs are similar with that under \textit{in vitro} condition. Those slight changes possibly resulted from the short-time treatment of saliva.

However, there were significant differences in the transfer frequencies of eARGs and iARGs are significantly different in group G and group D. Generally, the transfer frequencies of eARGs and iARGs in group G4 are higher than those in group G1. The ARGs horizontal transfer cover three mechanisms: conjugation, transformation and transduction. Conjugation includes two preconditions: (1) cell-to-cell junctions; (2) the presence of a pore through which ARGs can pass. Transformation includes two preconditions: (1) the release and persistence of eARGs; (2) the presence of competent bacterial cells. The precondition of transduction is the presence of bacteriophage [10, 11]. Based on it, the mechanisms for the horizontal transfer of eARGs and iARGs are transformation and conjugation, respectively. Therefore, the transfer frequency of eARGs probably related to the pH value of supernatant and the abundance of eARGs, while the transfer frequency of iARGs probably related to the activity of donors and the density of receptors and donors.

**ARGs in transformants and transconjugants**

The relative abundance of tetracycline resistance genes (\textit{tetA}, \textit{tetG}, \textit{tetM}), sulfonamide resistance genes (\textit{sul1}, \textit{sul2}) and cephalosporin resistance genes (\textit{bla}_{FOX}, \textit{bla}_{EBC}) in transformants and transconjugants is shown in Fig. 6a. In general, the relative abundance of tetracycline resistance genes (\textit{tet}) was the highest among the three resistance genes, followed by sulfonamide resistance genes (\textit{sul}) and cephalosporin resistance genes (\textit{bla}).

Among the transformants, there was no significant difference in the relative abundance of \textit{tet} between group C (control group), group S, group G4 and group D1, all of which were about $5.0 \times 10^{-2}$. The relative abundance of \textit{tet} in group G1 and group G4 was significantly higher than those in other groups ($1.39 \times 10^{-1}$ and $1.98 \times 10^{-1}$, respectively). The relative abundance of \textit{sul} in each group was significantly different. The relative abundances of \textit{sul} in group S and group G4 were lower than that in group C (control group). The relative abundance of \textit{sul} in group G1 and group D4 was significantly higher than that in
other groups (3.7 × 10^{-2} and 3.46 × 10^{-2}, respectively). Bla gene was lower than that in D1 group. Except for the control group, the transformants in the other groups were higher than those in the C group (control group), and the gene abundance was about 2.09 × 10^{-3}.

In the transconjugants, the relative abundance of tet in group D4 was significantly higher than that in other groups (tet relative abundance was 1.8 × 10^{-1}), and the relative abundance of tet in other groups was about 1.2 × 10^{-1}; the relative abundance of sul in each group was not significant, the relative abundance of sul in group G4 was slightly higher than that in other groups; the relative abundances of bla in group C, S, G1 were similar, all of which were about 1.5 × 10^{-3}–2.0 × 10^{-3}. The relative abundances of bla in group D1 and group D4 transconjugants were both about 1.0 × 10^{-3}.

The proportions of three types of ARGs (tet, sul and bla) were similar among the groups of transconjugants and transformants (Fig. 6b). tet, sul and bla were 78–87%, 11–21%, 1–2% in transformants, 77–88%, 12–22% and 0–1% in transconjugants, respectively.

The relative abundance of ARGs in transformants or transconjugants varied a lot among groups, but the proportions of tet, sul and bla were quite constant. The relative abundance of ARGs is related to the copy number of ARGs in cells. When ARGs integrated into chromosomal DNA, ARGs replicate along with chromosomal DNA, which only occurs when cells divide. When ARGs integrated into stringent plasmid, the stringent plasmid replicate at the same time as chromosomal DNA. However, when ARGs integrated into relaxed plasmid, ARGs can replicate along with relaxed plasmid during the whole cell cycle [51].

**Conclusion**

A series of physicochemical properties have been studied during simulation of ARB passing through *in vitro* digestive tract. ARB mostly affected by pH value of gastric juice. Under extreme pH (gastric juice pH ≤ 2.0), most bacteria were seriously damaged with cell wall broken and more than 99% bacteria were killed. However, the surviving bacteria can still grow and reproduce in the neutralization of small intestinal juice. eARGs can not only exist in PBS buffer and saliva, but also survive in gastric juice (pH 1.0–4.0) and intestinal fluid. Generally, iARGs have higher abundance than eARGs and the lowest abundance of each gene occurred upon gastric juice at pH 1.0. Although the abundance of iARGs was higher than that of eARGs, the transfer frequency of eARGs is generally higher than that of iARGs. Even treated with digestive juice, ARGs still have high gene horizontal transfer potential, suggesting that food-borne ARB pose a risk of ARGs horizontal transfer to intestinal bacteria.

**Declarations**

**Ethics approval and consent to participate**

N/A

**Consent for publication**
Availability of data and material

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

Consent form

N/A

CRediT authorship contribution statement

Qiujie Cai: Investigation, Conceptualization, Formal analysis, Writing - original draft, Data curation, Visualization, Writing - review & editing. Yanbin Xu: Funding acquisition, Project administration, Supervision, Writing - review & editing. Min Zhou: Validation, Visualization. Ling Yu: Acquisition, Analysis and Interpretation of Data; Pengqian Ouyang: Validation, Investigation. Li Zheng: Validation.

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Abbreviations

ARB: Antibiotic resistance bacteria

ARGs: antibiotic resistance genes

eARGs: extracellular ARGs

iARGs: intracellular ARGs

HGT: horizontal gene transfer

MGEs: mobile genetic elements
**DDD**s: defined daily doses

**WWTPs:** wastewater treatment plants

**FCM:** flow cytometry

**MIC:** minimum inhibitory concentration

**PBS:** phosphate buffered saline

**SEM:** scanning electron microscope

**CTC:** 5-cyano-2,3-ditolyl tetrazolium chloride

**DAPI:** 4',6-diamidino-2-phenylindole dihydrochloride

**CTF:** CTC-formazan product

**eDNA:** extracellular DNA

**iDNA:** intracellular DNA

**TBE:** tris-borate-EDTA

**IPTG:** β-D-thiogalactopyranoside

**QPCR:** quantitative polymerase chain reaction

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Figures

Figure 1

Schematic diagram of the in vitro digestion procedure
Figure 2

Live cells and dead cells analyzed by FCM. The symbols of C, S, G1 G2 G3 G4 D1 D2 D3 and D4 are treatment of PBS buffer, Saliva, Saliva + Gastric (pH 1.0), Saliva + Gastric (pH 2.0), Saliva + Gastric (pH 3.0), Saliva + Gastric (pH 4.0), Saliva + Gastric (pH 1.0) Intestinal fluid, Saliva + Gastric (pH 2.0) Intestinal fluid, Saliva + Gastric (pH 3.0) Intestinal fluid and Saliva + Gastric (pH Intestinal fluid, respectively.)
Figure 3

SEM photos of Hormaechei cells treated with gastric juice of different pH (a) In gastric juice of pH 1.0. (b) In gastric juice of pH 2.0. (c) In gastric juice of pH 3.0. (d) In gastric juice of pH 4.0. (e) The control.
Figure 4

ARGs abundance in supernatant and sediment. The symbols of C, S, G1 G2 G3 G4 D1 D2 D3 and D4 are treatment of PBS buffer, Saliva, Saliva + Gastric (pH 1.0), Saliva + Gastric (pH 2.0), Saliva + Gastric (pH 3.0), Saliva + Gastric (pH 4.0), Saliva + Gastric (pH 1.0) Intestinal fluid, Saliva + Gastric (pH 2.0) Intestinal fluid, Saliva + Gastric (pH 3.0) Intestinal fluid and Saliva + Gastric (pH 4.0) Intestinal fluid, respectively.
Figure 5

Transfer frequency of eARGs and iARGs after exposure to different digestion juice. (The symbols of C, S, G1–G4, D1 and D4 are the treatment groups of PBS buffer, Saliva, Saliva + Gastric (pH 1.0), Saliva + Gastric (pH 4.0), Saliva + Gastric (pH 1.0) + Intestinal fluid, and Saliva + Gastric (pH 4.0) + Intestinal fluid, respectively.)
Figure 6

ARGs relative abundance (a) and proportion (b) of transformants and transconjugants. The symbols of C, S, G1 G4 D1 and D4 are the treatment group of PBS buffer, Saliva, Saliva Gastric (pH 1.0), Saliva + Gastric (pH 4.0), Saliva + Gastric (pH 1.0) Intestinal fluid, and Saliva + Gastric (pH 4.0) Intestinal fluid, respectively.

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