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Domestic refrigerators: An overlooked breeding ground of antibiotic resistance genes and pathogens

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ABSTRACT

Domestic refrigerator is a widely used appliance to keep food fresh and retard food spoilage in household. However, our understanding of microbial health risk associated with food under such circumstance still remains very poor. Here, typical types of food (vegetable, fish, and pork) were kept in a domestic refrigerator at 4 °C for 3–30 days. Temporal dynamics of antibiotic resistome, pathogens, bacterial and fungal communities during this period were investigated via high-throughput quantification and Illumina sequencing technologies. Results showed that a large number (21–134) of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) were detected across the three food types, including 10.06 % of high-risk ARGs classified by their risk ranks. Moreover, four bacterial pathogens (i.e., Bacillus cereus, Cronobacter spp., Klebsiella pneumoniae and Staphylococcus aureus) targeted by marker genes including the pathogen-specific genes or virulence factor genes, and some potential fungal pathogens (e.g., Fusarium, Candida, and Aspergillus) were detected, indicating the occurrence of microbial risk even at the normally regarded safe storage temperature. Among all food types, the total bacterial density and ARG abundances in fish rapidly increased after only 3 days, much faster than vegetable and pork after 10 days. In addition, fish samples contained the highest ARG and pathogen abundances, indicating its potentially higher health risk than other food types. Finally, the shifts of ARG pattern were mainly contributed by bacterial communities and MGEs. This study highlights that food preserved in refrigerator at 4 °C could still be an unneglected microbial risk, and raises awareness of improving food safety in domestic environment.

1. Introduction

Global warming and the consequent increase of temperature, rainstorm, and other extreme weather events, pose a great challenge to food supply and safety (Misiou and Koutsoumanis 2021). Food safety is a worldwide problem that human beings are facing due to the frequently occurred food borne illnesses. According to the statistics from World Health Organization (WHO), about 600 million people in the world fall ill after eating contaminated food, and around 420,000 people die from food borne illness each year (WHO, 2020). Notably, food borne illnesses occurred in private homes is more than three times higher than in public food service, mainly due to inappropriate storage, inadequate cooking, and cross-contamination (Macias-Rodriguez et al., 2013; Roccato et al., 2017). Cold storage is a commonly efficient way to keep food fresh and retard microbiological spoilage by controlling the rate of chemical and enzymatic reactions as well as the rate of microbial growth (Iquo and Itodo, 2017). In household, refrigerator is a most widely used application to store food products in a desirable low temperature (4–5 °C). Based on the regulations of food safety and inspection service launched by the U.S. department of agriculture (USDA), the storage times of refrigerated foods are different for various food types and can range from several days (1–2 days) to weeks (2–3 weeks) (USDA, 2015). In China, the residents often store the food in refrigerator for several days in daily life, and the elderly individuals living alone in some rural areas may cook more food at a time and then store the leftovers in refrigerator for several weeks. So, refrigerator may be a potential breeding ground for microbial cross-contamination. Microbes from unwashed raw foods or unclean packages can be transferred to other stored food and the internal surfaces of refrigerators. Moreover, food products that constitute rich nutrient source and high moisture could facilitate

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microbiological growth and food spoilage (Bautista, 2014; Odeyemi et al., 2020), causing subsequently long-term contaminations and bringing health risks. Therefore, understanding of microbial contamination of food stored in domestic refrigerators is of great importance for food safety and human health.

Presently, studies have showed that microbes including many pathogenic bacteria are capable of surviving at low temperature. Pathogenic bacteria such as Staphylococcus aureus, Escherichia coli, Shigella spp., Salmonella typhi, and Klebsiella pneumonia were frequently detected in refrigerators (Macias-Rodriguez et al., 2013; Iquo and Itodo, 2017), and they could directly infect consumers with the aid of virulence factor (e.g., invasion protein, adherence factors, et al.) or secrete toxins, thus causing serious diseases (Hein et al., 2006; Lee et al., 2006). It was estimated that 241, 000 S. aureus food poisoning cases occurred in the United States each year (Stewart, 2017). In particular, the notorious bacteria Listeria, which is known as "refrigerator killer" and reported to cause the third-highest number of foodborne illnesses death, can survive in −20 ℃ for even a whole year (Brown et al., 2018). In addition, fungal microbes or "molds" can grow and even produce mycotoxins under adverse conditions such as the cold refrigerator during food spoilage (Hassan et al., 2016). Therefore, food security problems such as microbial cross-contamination and food poisoning could still occur under the domestic refrigerator conditions.

Moreover, microorganisms exhibiting antibiotic resistance are frequently detected in various types of food as antibiotics are widely used or overused in livestock breeding (Qian et al., 2018), agricultural (McDermott et al., 2002) and aquacultural production (Suyamud et al., 2021). Previous studies showed that >200 antibiotic resistance genes (ARGs) subtypes (Li et al., 2021b) and 149 multidrug-resistant isolates (Makinde et al., 2021) were detected in ready to eat food. More seriously, ARGs can quickly spread within pathogens among people, animal, environment through horizontal gene transfer (HGT) in food supply, health care facilities, and various environments (e.g., soil, water, air) (Che et al., 2021). WHO had recognized antimicrobial resistance as the global health threat in 21st century and called for One Health approach to combat it (WHO, 2014). In terms of cold storage in refrigerators, that is one important segment of food supply, microorganisms on food surface would easily enter competency state stimulated by cold temperature or metal ion in food matrix (Hasegawa et al., 2018), thereby facilitating gene transfer (Mc Mahon et al., 2007). For example, multi-drug resistant Bacillus cereus resistant to tetracycline, rifampin, and clindamycin were identified during cold chain (Park et al., 2020), raising nonnegligible threat of antibiotic resistant pathogens to human beings under cold conditions.

Systematic research on ARG profiles and pathogens in foods during cold storage in domestic refrigerator were seldom reported. Moreover, previous studies on foods targeted only a few types of antibiotic resistant bacteria or genes by using traditional culture-dependent method and molecular-based PCR/qPCR approach (Ye et al., 2019; Jeong et al., 2020; Zhao et al., 2020). In this study, a high throughput qPCR technology, which enables 5184 PCR reactions to quantify 296 ARGs or 72 human pathogenic markers in one run (An et al., 2020), was applied. Moreover, Illumina sequencing was also used to determine both bacterial and fungal communities. Three typical food types were stored in refrigerator to simulate the real scenario and we aimed to: (1) temporally identify and quantify ARGs and bacterial pathogens during cold storage and dependence on food type; (2) characterize both the bacterial and fungal communities; (3) identify the role of microbial communities and MGEs on the variation of ARGs profiles under cold conditions. Finally, some suggestions on household refrigerator management and good domestic hygienic practices were provided.

2. Material and methods

2.1. Experimental design and sample collection

Three typical Chinese foods (fried green vegetables, steamed fish, and braised pork in brown sauce) were cooked in canteens of Institute of Urban Environment, Chinese Academy of Sciences (Xiamen, China), and then immediately took to the laboratory. After cooling, each type of food was divided into 15 pieces in a sterile plastic box with lids to prevent other impurity to fall on it, and then kept them in refrigerator chamber at a constant temperature (4 °C) for 3–30 days (Fig. S1). Food samples were collected with three parallels after an interval storage (3, 5, 10, 20 and 30 days), and stored at −20 °C for further usage. To better describe the spoilage process, two stages were defined: early (3 and 5 days) and late (10, 20 and 30 days). Notably, food samples stored at room temperature (maximum 28–29 °C) in summer for 1 day were set as control.

2.2. Physicochemical properties analysis

In order to determine the pH value, 5 g food samples were homogenized for 2 min in 45 mL 8.5 g/L of KCl with a stirring machine (QSJ-B02X5, Bear Electric Appliance Co. ltd, Shunde, China), and then measured by an Is126 pH meter (Shanghai Insmark Instrument Technology Co. ltd, Shanghai, China) (Cauchie et al., 2019). In addition, plate count agar (PCA) was applied to enumerate the total bacterial number. Briefly, 25 g of food samples were diluted with 225 mL sterile saline (0.85 % NaCl) and homogenized for 2 min (Hou et al., 2021). The suspension was collected, and aliquots of 0.1 mL of the appropriate dilutions were spread on PCA for incubation at 37 °C for 48 h (Ercolini et al., 2006). Results were calculated as the means of three replicates. Other physiochemical factors of food matrix at initial time (0 h) including the total organic matter (TOM), total carbon (TC), total nitrogen (TN), total phosphorus content (TP), and moisture content were measured based on our previous study (Lin et al., 2022). The C/N is the ratio of TC to TN. The data were shown in Fig. S2.

2.3. DNA extraction and Illumina sequencing

All food samples were homogenized as described above; the supernatant was set for 1 min to remove the large deposit; and then transferred to a new sterile tube to concentrate by centrifugation at 8000 rpm for 20 min (Ercolini et al., 2006; Doster et al., 2020). The resulting supernatant was discarded, and the pellets was used for DNA extraction with a FastDNA SPIN kit (MP Biomedicals, Santa Ana, California, USA) according to the protocol described by the manufacturer (Wu et al., 2018). Finally, the DNA concentrations were measured using a Qubit Fluorometer (Invitrogen, Ghent, Belgium) and then detected with 1 % agarose gel electrophoresis.

Primer sets 515F (5′-GTGCCAGCMGCCGCGG-3′)/907R (5′-CCGTCAATTCMTTTRAGTTT-3′) targeting the V4-V5 region of bacterial 16S rRNA gene, and ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′)/ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) targeting the fungal ITS1 region, were used to conduct PCR amplification, respectively. The PCR reaction system and condition were followed a previous study (Ding et al., 2020). The tag-encoded high-throughput sequencing was performed by Illumina Hiseq 2500 platform (Majorbio, Shanghai, China).

The sequencing data were analyzed using QIIME software based on the online instructions. Briefly, the primer sequences were removed and low-quality sequences was filtered to obtain clean reads. Then, the operational taxonomic units (OTUs) were clustered by a 97 % of similarity using the UPARSE software and the representative sequences were assigned in taxonomic status by RDP Classifier 2.2 (Chen et al., 2020). The alpha diversity i.e., Shannon diversity was calculated from relative abundance of OTUs, and the NMDs analysis was used to present the microbial community distribution according to the Bray-Curtis distance.

2.4. Detection ARGs and pathogens by high-throughput quantitative PCR

The characterization of ARGs (including MGEs) and human pathogens were quantified using HT qPCR technology on a SmartChip Real-time PCR system (Wafergen Inc., Fremont, CA). A total of 384 primer sets that targeted 326 ARGs and 57 MGEs (12 transposases, 24 insertions, 7 integrases and 14 plasmids), and one 16S rRNA gene were used (Stedtfeld et al., 2018). The HT qPCR reaction systems and amplification conditions were based on previous studies (Zheng et al., 2018). Each sample was detected in triplicate and the DNA concentration was adjusted to 25 ng/μL. Amplification was confirmed only when three replicates were positive. The abundances of ARGs and MGEs were calculated based on the formula as described in a previous research (Chen et al., 2019), and the threshold cycle (31) was determined as the detection limit. According to the ARG risk rank framework developed by previous studies (Zhang et al., 2021; Zhang et al., 2022), the detected ARGs were divided into four risk ranks based on the rank of risk index (RI) that was calculated as the product of four indicators: human accessibility (HA) × mobility (MO) × host pathogenicity (HP) × clinical availability (CA). The highest risk ARGs were classified as Q1 (top 25 %); followed by Q2 (50–75 %) and Q3 (25–50 %), while the lowest risk ARGs were belonged to Q4 (bottom 25 %). Therefore, the percentage and abundance of ARGs belonging to different risk ranks for each food type stored in refrigerator were calculated.

In addition, 72 primer sets targeting 68 markers for 33 pathogens were used for a TapMan probe-based HT-qPCR. The primers, amplification reaction system and conditions were applied according to a previous study (An et al., 2020). The sample was discarded if the melting curve had multi-peaks. The absolute abundance of pathogen markers was calculated according to standard curves. All the sequence raw datasets and qPCR data can be found in the ScienceDB database at https://doi.org/10.57760/sciencedb.o00012.00001.

2.5. Statistical analysis

The antibiotic resistome and microbial community data were organized and calculated in Microsoft Excel 2013, and graphing was performed with Origin 8.0 and GraphPad Prism 8.0. Significant differences between food types and time intervals were compared by one-way ANOVA using SPSS 22.0 (IBM, USA) at a $P < 0.05$ level of significance. The adonis test, NMDs analysis, and Mantel test and Procrustes tests based on the Bray-Curtis distances were conducted in the R version 2.0 statistical environment with vegan 2.6–2 and ggplot2 packages. The network analysis was performed in R with "Hmisc 4.7–1" package and then visualized by the Gephi version 9.0 to show the co-occurrence between unique ARG/MGE and bacterial community. The variation partitioning analysis (VPA) of the contributions of bacterial communities and MGEs to the variations in ARGs were conducted on the R environment with vegan package (Zheng et al., 2018).

3. Results

3.1. Changes in microbial and physiochemical properties of refrigerated food

In order to extend the food shelf life, domestic refrigerator is a commonly used appliance in household to keep food fresh and inhibit its spoilage. As show in Fig. S1, the color and morphology of food did not obviously change before 20 days, while some white molds appeared in fish at 20 days and in pork and fish at 30 days, suggesting that microbiology spoilage slowly occurred although it is hard to be observed by naked eyes at early stage. In contrast, the total bacterial cells in fish rapidly reached to 10^8 CFU/g after only 3 days. Moreover, the number of total bacteria in pork firstly increased about 2 orders of magnitude (from 10^2 to 10^4 CFU/g) at 3 days, and peaked to 10^6 CFU/g after 5 days, and in vegetable increased to 10^8 CFU/g after 10 days (Fig. S2). However, the bacterial density after only 1-day storage at room temperature already exceeded 10^7 CFU/g in Rt control. These findings indicated that bacteria proliferated even at 4 °C despite the rate was slower than that in room temperature. As for the physicochemical properties of refrigerated foods, pH value significantly decreased from 6.62 to 5.95 in fish at 5 days, but did not change much in other samples; the moisture content was the highest in vegetable (81.62 %), followed by fish (63.83 %) and pork (54.91 %); the total nitrogen content was higher in animal samples of fish (10.25 %) and pork (11.19 %) than in vegetable (2.15 %), resulting in lower C/N ratios in fish (5.26 %) and pork (4.76 %) than in vegetable (24.05 %).

3.2. Temporal changes of ARG profiles and their health risk

During the cold storage in refrigerator, a large number of ARGs and MGEs were detected in vegetable (21–123), fish (36–134), and pork (27–108) samples (Fig. 1a). Moreover, the number of detected ARGs and MGEs significantly increased ($P < 0.05$) at 5 days in all food types. In order to examine the effect of cold storage on the development of food microorganisms, food samples kept at room temperature (maximum 28–29 °C) in summer for 1 day were also measured for comparison (Rt control). At the early 3–10 days, the diversities of ARGs and MGEs were remarkably lower than those in Rt control ($P < 0.05$), but they reached to the same level as that of Rt control in vegetable and fish afterwards ($P > 0.05$), and even exceeded Rt control in pork ($P > 0.05$). The detected ARGs conferred resistance to 11 major classes of antibiotics, including aminoglycosides, β-lactams, fluoroquinolones, glycopeptide, macrolide-lincosamide-streptogramin B (MLSB), multidrug, phenicol, rifamycin, sulfonamide, tetracycline, and trimethoprim. Among the detected ARGs, up to 105 were shared by the three food types, while the number of unique ARG in each food type was <10, indicating that most ARGs (76.09 %) were shared among different food types during cold storage (Fig. 1b and Table S1).

Moreover, the abundance of ARGs after 3 days of cold storage was highest in fish (1.64×10^9 copies/g), followed by pork (1.25×10^7 copies/g), while vegetable harboring the least (2.37×10^6 copies/g) (Fig. 2a). With the time increasing, the ARG abundances increased most rapidly (at 5 days) in fish samples to 1.84×10^{10} copies/g, but they significantly increased to 1.74×10^9 copies/g and 4.74×10^9 copies/g in vegetable and pork at 20 days (one-way ANOVA, $P < 0.05$), respectively. Also, the abundance of MGEs presented a similar change although there were some fluctuations at 10 days in vegetable and pork. Notably, in comparison with Rt control, although the ARG abundances were significantly lower at early stage, e.g., 3–10 days for vegetable and 3 days for fish and pork, they increased to a similar level of Rt control at late stage. In addition, the abundance of ARGs conferring to each class of antibiotic was different in each food type (Fig. S3). ARGs conferring resistance to multidrug were most dominant (43.4–94.7 %) among the three food types, while those carrying resistance to beta lactam and fluoroquinolone were more abundant in vegetable and others presenting resistance to MLSB predominated in fish and pork. Transposon was identified as the most abundant type of MGEs (89.9–99.2 %) among all food types. More specifically, the heatmap depicted the top 50 abundant ARGs and MGEs (Fig. S4), and it was found that pcoA and IS 6100 was most abundant in vegetable samples, while tcrB and tnpA_6 was most abundant in pork and fish samples. Moreover, NMDs analysis showed that ARG profiles were significantly different across three food types ($P < 0.05$, Fig. 2b) and between early (3 d and 5 d) and late (10, 20, 30 d) storage time ($P < 0.05$, Fig. S5). We also observed the abundance of total ARGs was positively correlated with MGEs (Fig. S6 and Table S2), suggesting MGEs played important role in the shifts of ARGs. In addition, the total ARG abundance increased with the abundance of 16S RNA genes the across three food types, indicating that ARGs proliferated with biomass in refrigerated food matrix (Fig. S7). As for the relative abundance of total ARGs, it increased significantly after 20 days in vegetable and after 5 days in fish ($P < 0.05$), but did not change significantly in

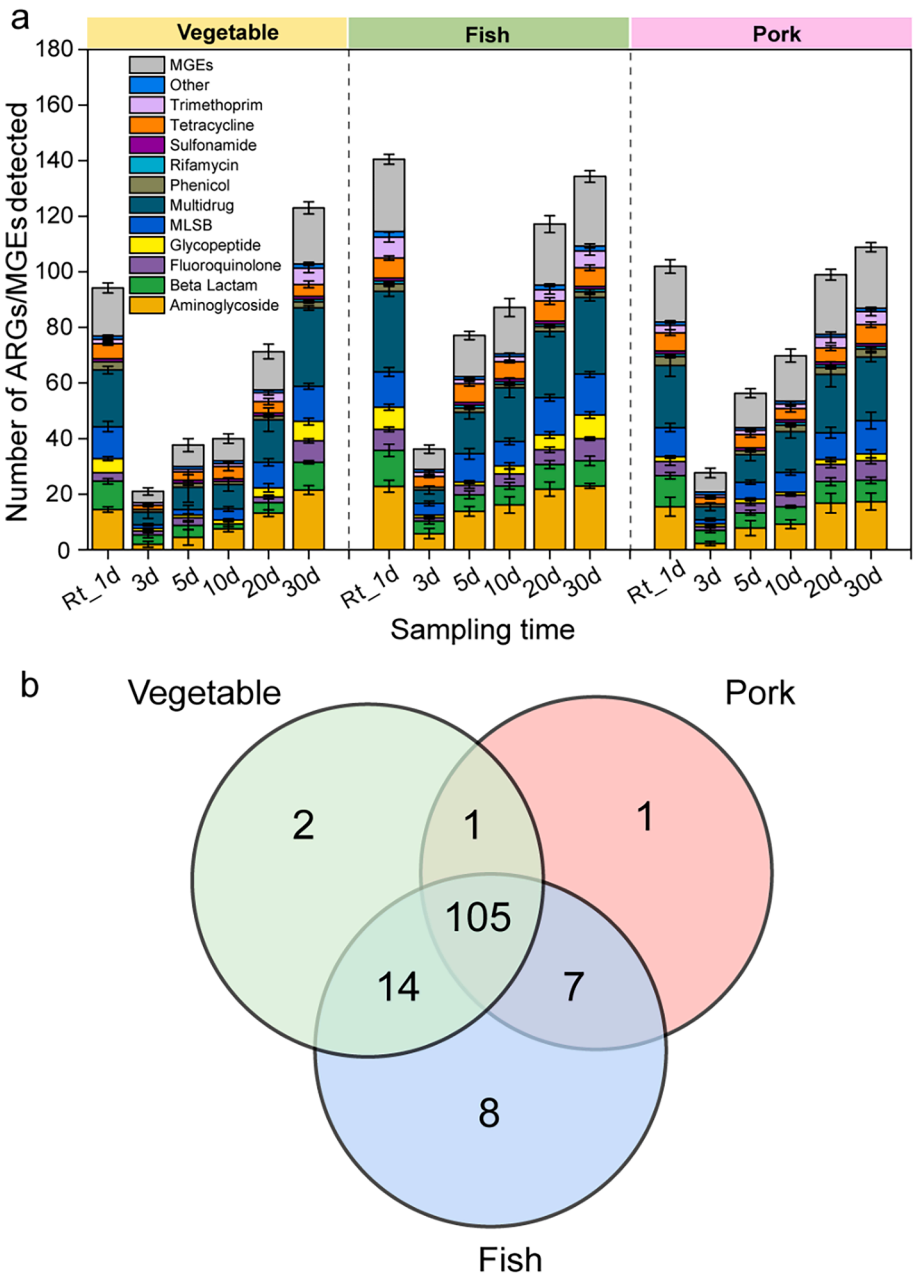

Fig. 1. The composition of antibiotic resistome in food during cold storage in refrigerator. (a) Detected number of ARGs and MGEs. (b) Shared ARGs across three food types including vegetable, fish, and pork. Rt_1d presents food stored at room temperature (maximum 28–29 °C) in summer for 1 day and is used as a control.

pork during the whole storage period (Fig. S8) ($P > 0.05$).

Furthermore, according to the recently developed ARG risk rank categorized methods based on their human accessibility, mobility, human pathogenicity, and clinical availability (Zhang et al., 2021; Zhang et al., 2022), all detected ARGs were classified as four risk ranks. Q1 presented the highest risk ARGs; followed by Q2 and Q3, while Q4 belonged to the lowest risk ARGs. It was interesting to find that a total of 27 detected ARGs (15.98 %) were identified as Q1 and Q2 risk index (Fig. S9 and Table S3), mainly including tetM, aadA5, and mexE in Q1 and floR, mdtA, and aadA6 in Q2 ARG families. Among Q1 and Q2 ARG families, ARGs conferring resistance to multidrug occupied the highest abundance. We also compared the abundance of ARGs based on risk ranks in each food type, and found fish and vegetable harbored more high-risk ARGs (Q1 and Q2) than pork.

3.3. Temporal changes of microbial communities and pathogens

A total of 2115, 034 and 3142, 085 high quality bacterial and fungal sequences were obtained from three food types, respectively. During the cold storage period, the alpha diversity (Shannon index) of bacterial community rapidly increased in vegetable after 10 days, followed by fish after 20 days and pork at 30 days, while that of fungal community significantly decreased at 10 days in fish and at 30 days in vegetable with the exception of pork samples with no significant change (Fig. S10, ANOVA, $P < 0.05$). Firmicutes (14.36–99.99 %) and Proteobacteria (0.16–42.57 %) were the most dominant phyla across the three food types, while Cyanobacteria (0.12–82.54 %) was only abundant in vegetable samples (Fig. 3a). In addition, the bacterial community composition displayed distinct temporal variations during this period. For example, the proportion of Proteobacteria rapidly increased from 3.01 % to 42.57 % in vegetable, from 0.15 % to 13.65 % in fish and from

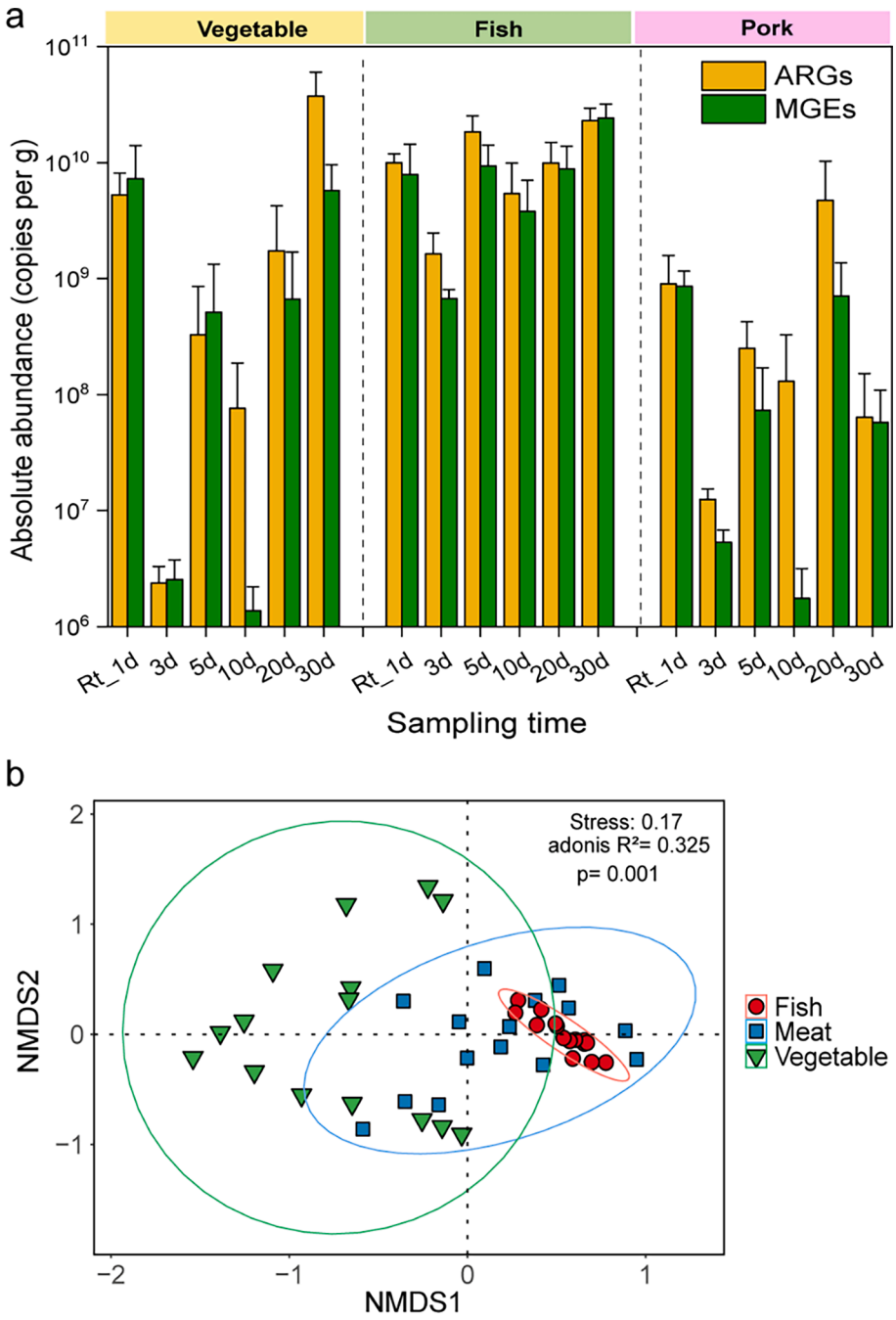

Fig. 2. The distribution of ARGs and MGEs in food during cold storage in refrigerator. (a) Absolute abundance of total ARGs and MGEs. (b) NMDs showing the patterns of ARGs across three food types including vegetable, fish, and pork. Definition of Rt_1d is as in Fig. 1.

0.01 % to 37.11 % in pork during 30-day storage. Regarding the fungal community, Ascomycota and Basidiomycota were the two dominating phyla during cold storage, and the proportion of Basidiomycota (0.58–77.39 %) was higher in pork than the other two food types (Fig. 3b). In addition, the NMDs further suggested that both the storage stage and food types significantly influenced bacterial and fungal communities ($P < 0.05$, Fig. 3c and d, Fig. S11).

Moreover, 59 core OTUs (22.1 % of the total OTUs) were detected among all food types (Fig. S12, Table S4), and they were mainly assigned to phyla Proteobacteria (22 OTUs), Firmicutes (33 OTUs), Cyanobacteria (2 OTUs), Actinobacteriota (1 OTU), and unclassified bacteria (1 OTU). The difference of relative bacterial abundances among the three food types and at each spoilage stage was analyzed at the genera level (Fig. S13). It was found that Chloroplast (0.01–82.47 %) and Bacillus (7.14–36.29 %) were the most abundant in vegetable samples, while Weissella (47.30–91.38 %) and Lactococcus (2.71–15.59 %) predominated in fish samples and Weissella (15.92–92.91 %) and Kurthia (0.84–69.74 %) dominated in pork samples. The abundance of Klebsiella, Staphylococcus, and Kurthia increased at late stage. The dominant fungal genera were also different across three food types. For example, Fusarium (6.84–44.68 %) and Penicillium (0–33.84 %) were more abundant in vegetable, while Candida (25.98–59.09 %) and Ogataea (2.88–34.45 %) were the predominant genera in fish, and the pork was dominated by Aspergillus (2.63–80.86 %). Notably, the abundance of Candida and Cladosporium distinctly increased over time, while Fusarium and Ogataea gradually decreased throughout this period. Fig. S14 showed the potential fungal pathogens in food, and it was found that the top 6 most abundant genera were Candida, Cladosporium, Fusarium,

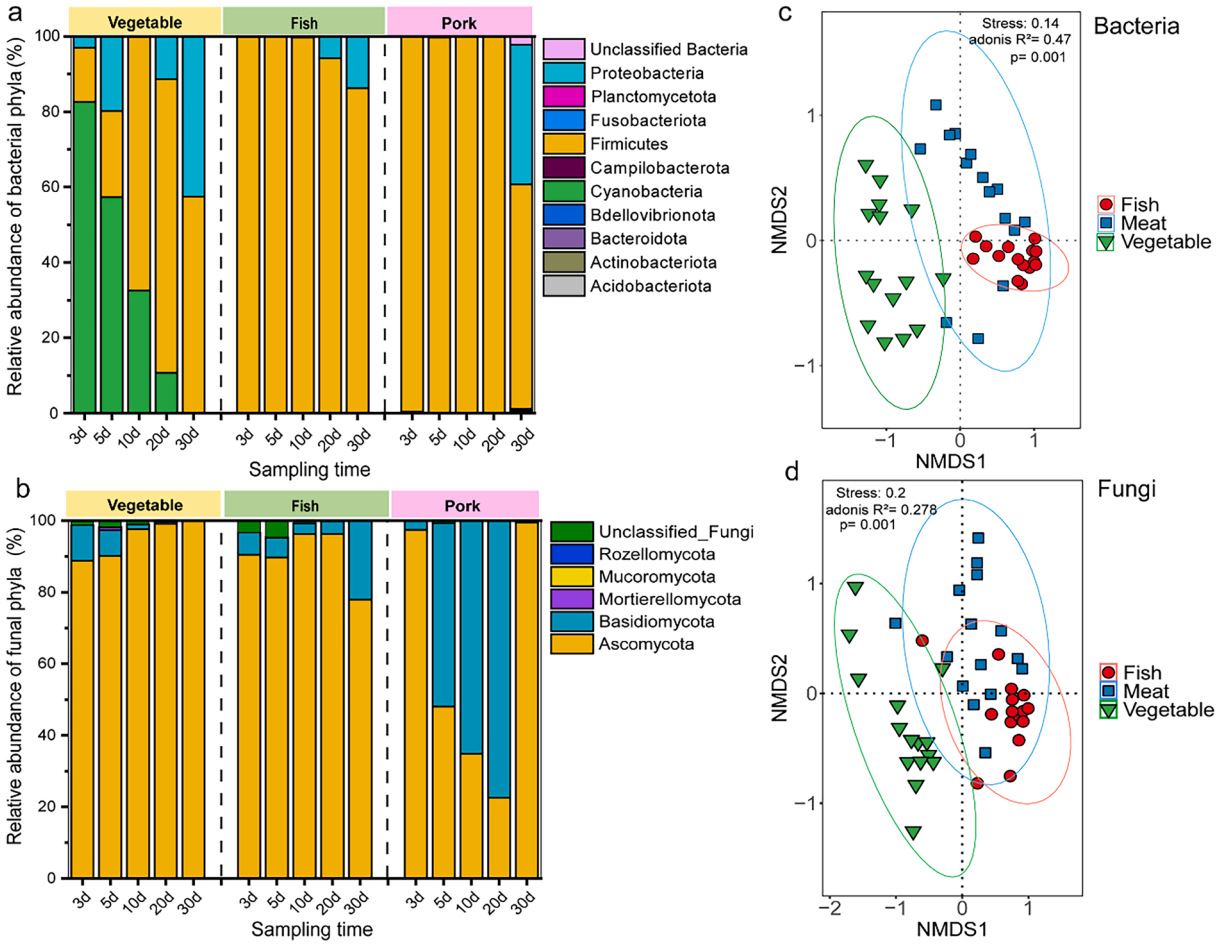

Fig. 3. The profile of microbial communities in food during cold storage in refrigerator. The relative abundance of bacterial phyla (a) and fungal phyla (b). NMDs showing the patterns of bacterial communities (c) and fungal communities (d) at the OTU level across three food types including vegetable, fish, and pork.

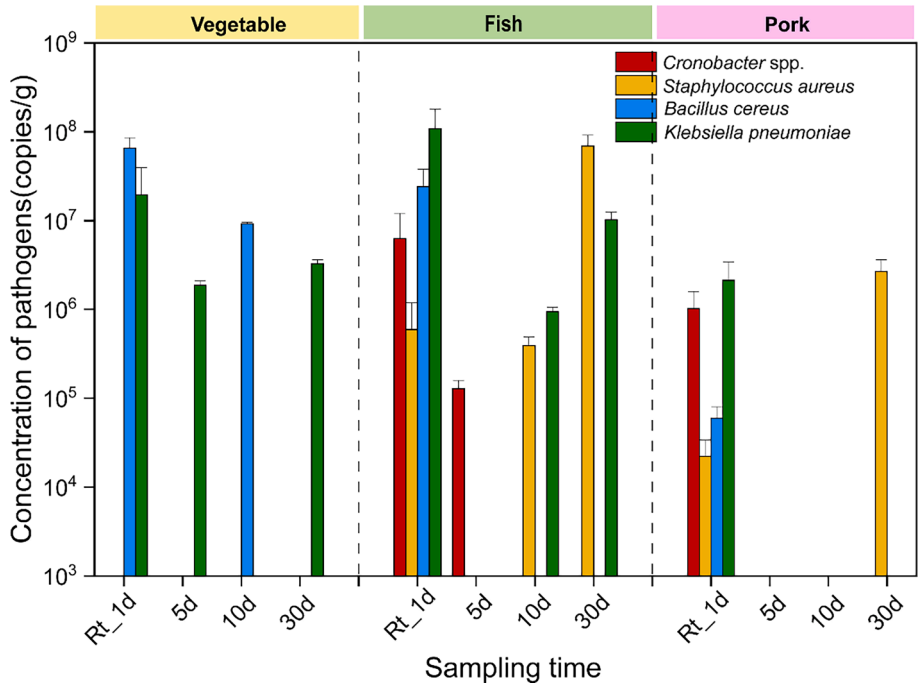

Fig. 4. Abundance of pathogens harboring virulence factor across three food types including vegetable, fish, and pork during cold storage in refrigerator. Data from three replicates and are presented the means ± SD. Definition of Rt_1d is as in Fig. 1.

Aspergillus, Penicillium, and Alternaria. In particular, the fungal pathogens were most frequently detected in vegetable, followed by pork, and fish harbored the least.

Furthermore, four pathogens harboring virulence factor, including Bacillus cereus (9.23×10^6 copies/g), Cronobacter spp. (1.28×10^5 copies/g), Klebsiella pneumoniae (9.45×10^5–1.02×10^7 copies/g), and Staphylococcus aureus (3.91×10^5–6.89×10^7 copies/g) were detected using the HT qPCR technology (Fig. 4). Across the three food types, fish samples contained the highest number of pathogens (3 types), followed by vegetable samples (2 types) and pork samples (1 type). S. aureus was only found in animal food types and Cronobacter spp. was only detected in fish samples at 5-day of storage. Over the cold storage period, the abundance of K. pneumoniae and S. aureus significantly increased by 10.79-fold and 176-fold at 30-day in fish samples, respectively. It was also worth noting that, both the type and abundance of pathogens detected in each food during cold storage were less than the Rt control. For example, four types of pathogens including B. cereus (5.99×10^4 copies/g), Cronobacter spp. (1.02×10^6 copies/g), K. pneumoniae (2.13×10^6 copies/g), and S. aureus (2.22×10^4 copies/g) were detected in Rt pork. In contrast, only one pathogen of S. aureus (2.67×10^6 copies/g) was found in pork during cold storage. Therefore, pathogens harboring virulence factor were indeed inhibited during cold storage, but they could still be frequently detected, suggesting that pathogens might survive or even breed under cold storage condition and pose health risk to human beings.

3.4. Correlation between the ARGs, MGEs, and bacterial communities

Both Procrustes analysis and mantel statistic test based on OTUs and ARGs showed that the bacterial communities were significantly correlated with the antibiotic resistomes in spoiled vegetable ($M^2 = 0.4151$, $r = 0.6523$, $P < 0.001$, 9999 permutations) and fish ($M^2 = 0.7189$, $r = 0.3406$, $P < 0.05$, 9999 permutations) during cold storage, although it presented slightly relevant in pork by mantel statistic test ($M^2 = 0.7472$, $r = 0.1017$, $P > 0.05$, 9999 permutations) (Fig. 5a). In addition, the bacterial communities at phylum level were found to correlate with certain class of ARGs as revealed by Spearman analysis (Fig. S15). For example, in fish, Proteobacteria were positively correlated with Beta Lactam and Glysopeptide. Moreover, the Variation partitioning analysis (VPA) was used to differentiate the effects of bacterial communities (BCs) and MGEs on ARG patterns. It was found that bacterial community contributed most to ARG variation in vegetable (18 %) and pork (35 %) samples, while the combination (16 %) of BCs and MGEs was the major contributor in fish (Fig. 5b).

Furthermore, the co-occurrence patterns between bacterial communities, ARGs, and MGEs were revealed by network analysis ($\rho > 0.8$, $P < 0.01$). A larger number of nodes were found in vegetable (172 nodes) and fish (185 nodes) than pork (48 nodes) (Fig. 6). In addition, the potential dominant hosts of ARGs and MGEs were distinctly different across three food types. For example, Kosakonia, Providencia, and Paenibacillus were the most dominant potential hosts in vegetable. However, some other genera such as Kurthia, Staphylococcus, and Klebsiella were potential hosts in fish. As for pork food matrix, Novosphingobium and Ochrobactrum were identified as dominant potential hosts. In particular, some potential pathogens such as Klebsiella and Cronobacter were shared hosts among different food matrix. Moreover, several ARGs and MGEs might be harbored in more than one potential host. For instance, ARGs including aac_6, ermD, and QnrB4, as well as the transposon tnpA5, were associated with up to 6 potential bacterial hosts in vegetable. In fish, ARGs such as qepA, acrR, and oqxA were correlated with>9 genera, while ARG lnuB was associated with 15 genera in pork. Notably, the keystone MGE (tnpA5) might act as hubs for horizontal gene transfer in both vegetable and pork during cold storage in refrigerator.

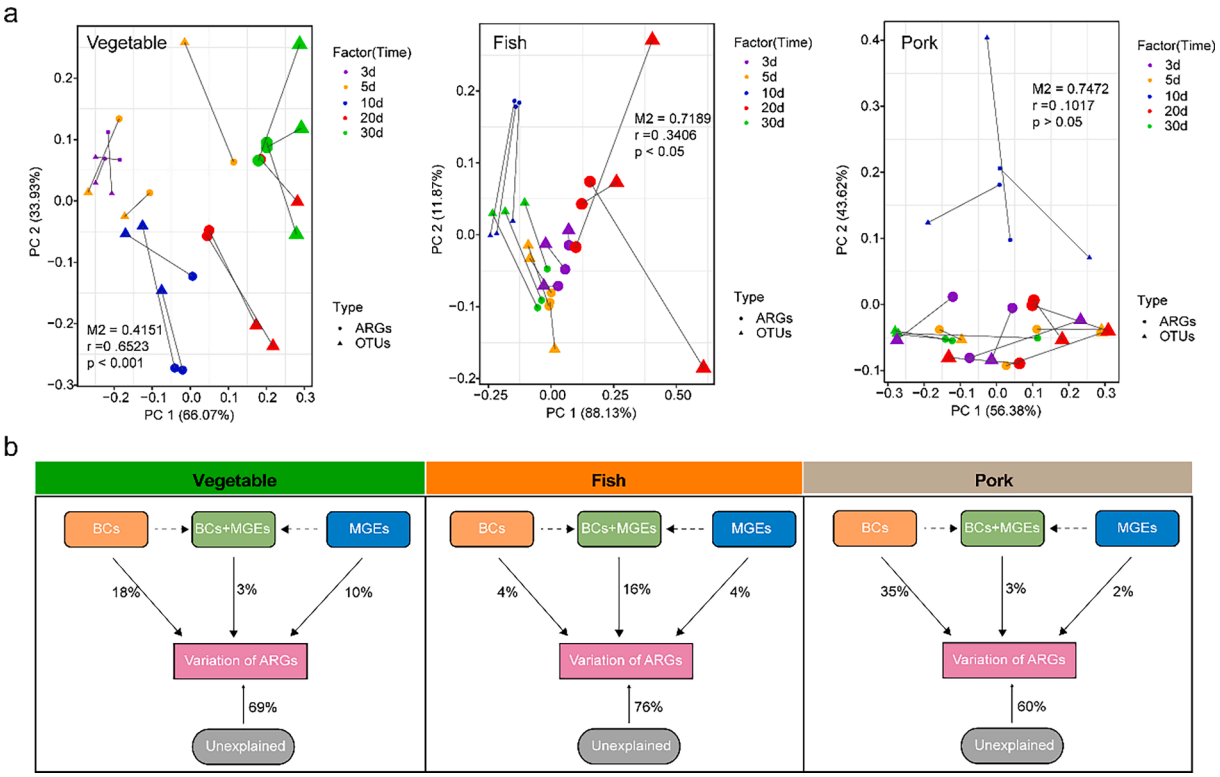

Fig. 5. Correlation between the ARGs and bacterial communities in food during cold storage in refrigerator. (a) Procrustes analysis showing the significant correlation between ARGs and the bacterial composition at OTU level. (b) Variation partitioning analysis (VPA) identifying the effect of bacterial communities (BCs) and MGEs on ARG patterns across three food types including vegetable, fish, and pork.

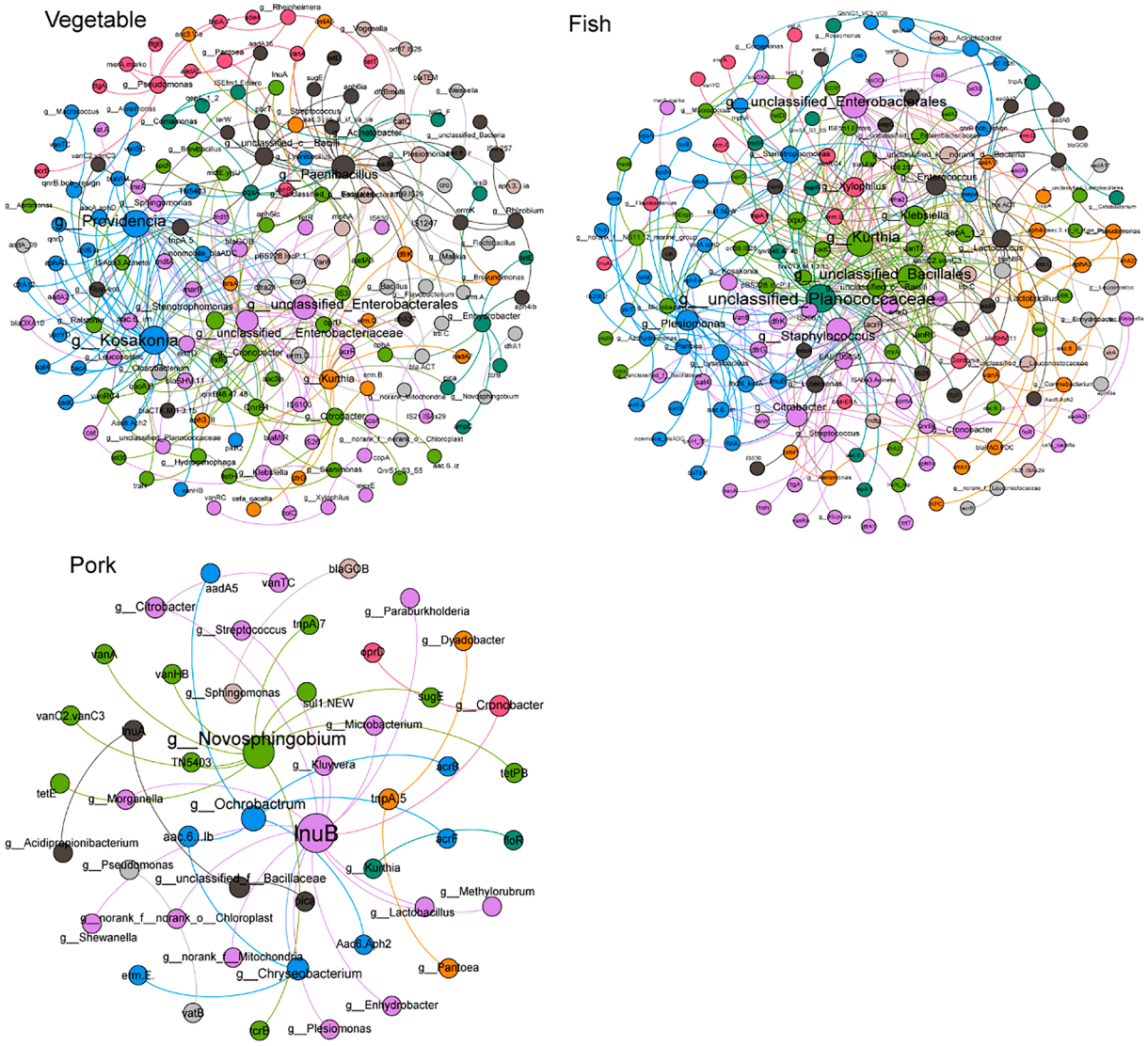

Fig. 6. Co-occurrence network of ARGs, MGEs, and bacterial communities at genus level across three food types including vegetable (a), fish (b), and pork (c) during cold storage in refrigerator.

4. Discussion

4.1. Antibiotic resistome and pathogens during cold storage

Food containing rich nutrient favors the growth of various microorganisms and easily get spoil, posing a big challenge to food waste and safety. Preserving food under cold condition in refrigerator is considered a desirable way to extend its shelf life. To date, few studies focused on the prevalence and transmission of microbial antibiotic resistance and pathogens under this circumstance. According to the national standard of the People's Republic of China for food safety (GB 4789.1–2016), the total bacterial number of the fresh food should range from 10^4–10^7 CFU/g. In the present study, the bacterial density of Rt control (1-day storage at room temperature) exceeded 10^7 CFU/g, indicating the early spoilage occurred. In contrast, the total abundance of 16S rRNA and ARGs at the early stage (3–5 days) in refrigerator was significantly lower than that in Rt control, demonstrating that cold storage suppressed the microbial growth and propagation of antibiotic resistance. However, the number and abundance of ARGs remarkably increased during cold storage. In particular, the ARG abundance boosted or even exceeded to the level of Rt control at the late stage (10–30 days) of food spoilage. These results indicated that refrigerators could be an overlooked breeding ground for proliferation and spread of antibiotic resistance. It was also worth noting that, the ARG relative abundances in vegetable and fish significantly increased during cold storage, suggesting the enrichment of ARGs under this circumstance. One possible reason might be due to the microbial growth occurred on the cooked leftover food under the cold storage as the abundance of ARGs was significantly correlated with that of 16S rRNA genes (Fig. S7). Another reason might attribute to cross-contaminate of the unwrapping raw food, leakage of cooked food, and unclean container in the refrigerator (Iquo and Itodo, 2017; Ye et al., 2019). Furthermore, the horizontal transmission rate of ARGs can also be promoted under the low-temperature stress condition (Hasegawa et al., 2018). In the present study, transposon was the most abundant MGE, which might play important role in transmitting ARGs under cold storage. Previous studies suggested that MGEs such as integrons, transposon, or plasmid were widely detected in food waste-related circumstances (Liao et al., 2019a; Yu et al., 2021).

Moreover, a large number of ARGs conferring resistance to almost all major classes of antibiotics was detected in all food types. Among these, ARGs conferring resistance to multidrug were most abundant, which was in line with a previous study showing that multidrug-resistance genes were the most predominant ARGs in ready to eat foods (Li et al., 2020). In addition, multidrug-resistant foodborne pathogens were

prevalent in food supply based on a report by the Centers for Disease Control (CDC), thus posing serious threats (Doyle, 2015). Notably, based on recent studies (Zhang et al., 2021; Zhang et al., 2022), ARGs can be identified as four health risk ranks. Q1 and Q2 were classified as the high-risk ARGs, which were harbored in pathogens currently or carried by mobile ones and could transfer to pathogens in the future, respectively. In the present study, 15.98 % of detected ARGs belonged to high-risk family including Q1 (10.06 %) and Q2 (5.92 %), especially with high abundance in fish and vegetable. In addition, ARGs conferring resistance to multidrug were most likely defined as high-risk ranks, which were consistent with the above investigations on various environmental habitats. Therefore, our results demonstrated a considerable health risk of food stored under cold conditions.

Furthermore, although the number and abundance of detected pathogens in cold storage were lower than that in Rt control, the breeding of pathogens is still a big problem under cold storage in the refrigerator. In the present study, the marker genes targeting on pathogen-specific genes or virulence factor genes of four pathogens including B. cereus, Cronobacter spp., K. pneumoniae, and S. aureus were found. Based on previous studies, pathogens such as K. pneumonia, S. aureus et al., was most frequently detected bacteria in food contamination (Baek et al., 2009; Iquo and Itodo, 2017; Makinde et al., 2021). In particular, Staphylococci isolates exhibiting multi-antibiotic resistance were found on the frozen food (Baek et al., 2009). In addition, the psychrotolerant B. cereus harboring enterotoxin genes was isolated from lettuce during cold chain and demonstrated to be resistant to three antibiotics. More importantly, some of them could even form biofilms, increasing health risk to human beings (Park et al., 2020). Although Cronobacter spp. was more frequently detected in formula and infant food (Carvalho et al., 2020), a recent study also suggested their high contamination rate in ready-to-eat food (Arslan and Erturk, 2021). It also should be noted that the abundance of K. pneumoniae and S. aureus significantly increased to 10.79-fold and 176-fold in fish samples during cold storage, respectively, suggesting that storage period under cold condition could still increase the level of pathogens. It is lucky to find that the notorious bacteria Listeria was not be observed in this study although it is the most prevalent foodborne pathogen, which was congruent with a previous result showing no Listeria monocytogenes and Yersinia enterocolitica were found in domestic refrigerators in Italy (Brown et al., 2018). Furthermore, we found some fungal pathogens (e.g., Fusarium, Candida, and Aspergillus). Fungal pathogens are also a serious problem in food spoilage, which may not only secrete mycotoxin but also form spores to survive stressed resistance such as cold condition (Dijksterhuis, 2017). For example, the dominant genera Candida and Fusarium isolated from food matrix, could cause human infections by virulence factors (Rajkowska and Kunicka-Styczynska, 2018) and contribute to fumonisin accumulation (Cendoya et al., 2017), respectively.

4.2. Food type-dependent antibiotic resistome and pathogens

By analyzing the shared ARGs across three food types, it was interesting to find that most ARGs (76.09 %) were shared among all food types. The high similarity of ARG subtypes might be explained by the shifts of microbial communities and the spread of ARGs via human's activities or natural environmental cycles (Singer et al., 2016; Li et al., 2020). However, the abundance of each ARG subtype was significantly different across three food types. For example, at the initial time (3 days of storage), the highest abundance of ARGs and MGEs was observed in fish, followed by pork, while vegetable harboring the least. According to previous studies, the nutrient and moisture content in food matrix affects microbial spoilage process (Bautista, 2014; Odeyemi et al., 2020), thus it was easily to understand that fish containing high protein and rich water content got spoiled most rapidly (Fig. S1). Some reports also demonstrated that a high level of ARGs and pathogens were detected in spoiled fish food matrix (Yu et al., 2021; Zhou et al., 2021).

Moreover, the dominant ARGs were also different in each food type. ARGs conferring resistance to beta lactam and fluoroquinolone were more abundant in vegetable, and those conferring resistance to MLSB predominated in fish and pork. This result might be due to their different living environments. For example, application of animal manures and sewage sludge in agriculture changed the ARG profiles in soil and vegetable (Chen et al., 2017), while animal feeding with antibiotics in livestock or aquaculture promoted the occurrence of ARB and ARGs in related food products (Doster et al., 2020; Zhou et al., 2021). Finally, food could also be contaminated by antibiotic resistance at each step of food chain "from farm to fork" (Founou et al., 2016). Among the three food types, fish samples contained the highest number and abundance of pathogens. In particular, S. aureus was only found in animal food types and Cronobacter spp. was only detected in fish samples, which was supported by a previous survey indicating positively test of methicillin resistant S. aureus in frozen shrimp (Elbashir et al., 2018). Notably, the abundance of K. pneumoniae and S. aureus significantly increased in fish samples over the cold storage period, respectively, indicating the potential health risk in spoiled fish. In addition, the potential fungal pathogen Candida was dominant in all food types, but Fusarium and Penicilium were more predominated in vegetable, and Aspergillus was frequently detected in pork. That was in consistent with previous studies showing that Candida, Aspergillus, and Penicilium were predominated in refrigerated foods (Yang et al., 2013; Ye et al., 2019). All these results suggested that more attention should be paid to the microbial health risk of seafood storage under cold condition.

4.3. Factors affecting the variation of antibiotic resistome during cold storage

Based on Procrustes analysis and mantel statistic test, bacterial communities were significantly correlated with the antibiotic resistome. Moreover, VPA analysis also identified the importance of bacterial community and MGEs on the ARG variation under cold storage in refrigerator, which was also demonstrated in other environments including river water (Zheng et al., 2018), microplastics (Li et al., 2021a), and food waste (Liao et al., 2019b). That was consistent with the result of spearman analysis showing that the bacterial communities at phylum level were significantly correlated with different classes of antibiotic resistome. For instance, in fish, the dominant phylum Proteobacteria were positively associated with Beta Lactam and Glysopeptide, possibly because they are suitable for copiotrophic and rapidly proliferate during food spoilage (Zhou et al., 2021). In addition, the most dominant transposon tnpA5 was identified as the keynotes in both vegetable and pork under cold storage, which was different from the room temperature condition as IS600 played important role in dissemination of ARGs in Rt control (Lin et al., 2022).

More specifically, the co-occurrence analysis revealed that the potential dominant host of ARGs and MGEs were affected by food types. As mentioned above, the waste fertilization on crop production and antibiotic treatment while animal feeding might influence the distribution of ARGs and bacterial microbial communities (Nguyen et al., 2016; Gao et al., 2020), thus further leading to the distinct potential ARGs and MGEs hosts. Except that, some genera, such as the potential pathogens Klebsiella and Cronobacter were shared hosts across different food types. Based on previous studies, Klebsiella spp. belonging to Enterobacteriaceae, were considered as common food-borne pathogens and had been detected in different spoiled food matrix. In particular, they might exhibit multiple resistance to antibiotics (Gram et al., 2002; Batdorj et al., 2007; Mladenovic et al., 2021). In addition, a high abundance of Cronobacter harboring virulence factors were frequently isolated from ready to eat foods including desserts, cereals, et al., and even resistant to >7 different antibiotics, thus posing health risk to human beings (Bao et al., 2017; Odeyemi and Abdullah Sani 2019; Arslan and Erturk 2021).

Finally, the components of different food types also influence the

spoilage processes. For instance, the high moisture and nitrogen facilitated the spoilage in fish (Fig. S2). In contrast, the reduced pH in fish implied that the acid hydrolysis products were produced by amino acid decomposition during spoilage process (Yu et al., 2016). Therefore, the physicochemical properties and the hydrolysis products during food spoilage might also contribute to the change of microbial communities and ARG abundance.

5. Conclusions

This study revealed the accumulation of ARGs and pathogens among three typical food types during cold storage in refrigerators, suggesting that preserving the leftover food in the refrigerator for >10 days remarkably increased microbial health risk. In particular, the seafoods (e.g., fish) contained high moisture and protein, and easily got spoiled after only 3 days. Considering the food types, fish harbored highest ARG and pathogen abundance, followed by vegetable and pork. In addition, the variation of ARGs was demonstrated to be mainly contributed by bacterial communities and MGEs. Our study would provide some useful suggestions to the residents in household management. For example, storing food in the refrigerator within three days, especially for fish; wrapping the raw and cooked food in refrigerator separately with crisper or plastic boxes; and cleaning the refrigerator regularly.

CRediT authorship contribution statement

Wenfang Lin: Methodology. **Fei Xu:** . **Hongqin Guo:** . **Li Cui:** Supervision, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107647.

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