RECENT DISCOVERIES IN HUMAN SERIOUS FOODBORNE PATHOGENIC BACTERIA: RESURGENCE, PATHOGENESIS, AND CONTROL STRATEGIES

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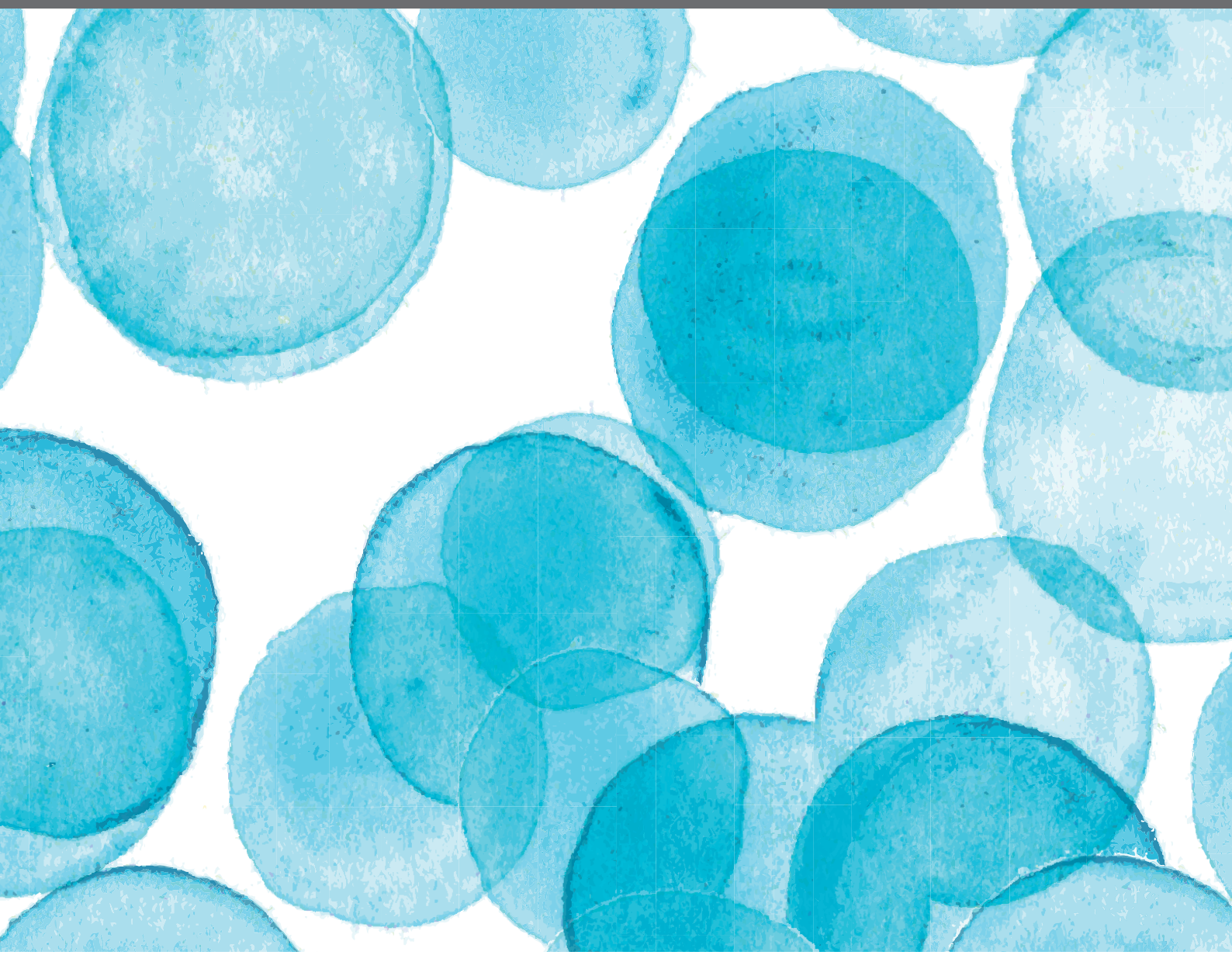

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RECENT DISCOVERIES IN HUMAN SERIOUS FOODBORNE PATHOGENIC BACTERIA: RESURGENCE, PATHOGENESIS, AND CONTROL STRATEGIES

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Food is the first necessity for humans to survive with huge amounts of food consumed daily worldwide. Globalization of food industry results in an increasingly complex food chain, making food safety a universal issue. Many millions of people in the world become sick while hundreds of thousand die annually due to consumption of contaminated food. Pathogenic bacteria contaminate food at any stages in the food chain, including production, processing, supplying, and storage. The most commonly known bacterial pathogens associated with human foodborne diseases worldwide are *Salmonella enterica*, *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, *Cronobacter sakazakii*, *Vibrio cholerae*, and *Vibrio parahaemolyticus*.

This eBook includes publications on recent discoveries in genetic diversity, prevalence, resistance and novel transmission vectors; molecular mechanisms underlying the pathogenesis; and new compounds and treatment strategies for better control of the human foodborne pathogenic bacteria. The information in the articles supports the urgent need for improving food safety and public health, particularly in globalization background.

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EDITORIAL
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Editorial: Recent Discoveries in Human Serious Foodborne Pathogenic Bacteria: Resurgence, Pathogenesis, and Control Strategies

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Editorial on the Research Topic

Recent Discoveries in Human Serious Foodborne Pathogenic Bacteria: Resurgence, Pathogenesis, and Control Strategies

Billions of people in the world are at risks of unsafe food. Many millions become sick while hundreds of thousand die annually due to consumption of contaminated food (Fung et al., 2018). Outbreaks and prevalence of foodborne diseases are not only a major burden on global healthcare systems, but also result in huge negative impact on economic growth and social stability. Along with the rapid development of web-based and mobile-ready electronic commerce, the fast-paced national and international trades in foodstuffs around the globe present new challenges to food safety systems, particularly in developing nations. Pathogenic bacteria contaminate food at any stages in the entire food chain from farm to dining-table. The most commonly known bacterial pathogens associated with foodborne diseases worldwide are *Salmonella enterica, Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, *Cronobacter sakazakii*, *Vibrio cholerae*, and *Vibrio parahaemolyticus*. This Research Topic reported recent discoveries in resurgence, pathogenesis, and control strategies of these foodborne pathogens.

Continuous monitoring of food contaminants and identification of risk factors are crucial for assuring food safety. Many original research articles included in this Research Topic addressed issues related to the genetic diversity, prevalence, resistance, and novel transmission vectors of pathogenic bacteria. For example, *C. jejuni*, a leading cause of gastroenteritis in humans, can reside in food animal reservoirs such as chickens, pigs, and cattle. The genetic diversity and frequency of antimicrobial resistance of *C. jejuni* recovered from 214 cattle at three Michigan herds in USA were examined and determined in a cross-sectional study (Cha et al.). *Cronobacter* spp. can cause necrotizing enterocolitis, bacteremia, and meningitis in neonates and infants, with a 40–80% mortality rate (Holy and Forsythe, 2014). Powdered infant formula (PIF) is the most significant source of *Cronobacter* spp. resulting in the infections. The antimicrobial and desiccation resistance of 70 *Cronobacter sakazakii* and *Cronobacter malonaticus* isolates from PIF and processing environments in China were determined (Fei et al.). Non-typhoidal *Salmonella* (NTS) can infect a wide range of hosts, including humans, poultry, cattle, and other domesticated and wild animals worldwide. It is estimated that 93.8 million people experience salmonellosis annually, with nearly 155,000 deaths (Majowicz et al., 2010). The potential virulence profile, genetic relatedness, and host adaptation of avian and mammalian NTS isolates based on the bacterial antigens FimA (adhesin) and IroN (receptor) were investigated in a large number of NTS

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isolates of different host origins (Alshalchi et al.). The authors revealed the possible influence of non-synonymous point mutations within the FimA adhesin of the NTS isolates in the host adaptation (Alshalchi et al.). Enteroaggregative *Escherichia coli* (EAEC) is a common cause of foodborne disease. EAEC strains express a heterogeneous array of putative virulence factors; therefore, the recognition of specific pathogenic factors remains challenging. Jønsson et al. monitored a collection of 162 clinical Danish EAEC strains and identified a novel pAA virulence plasmid encoding toxins and two distinct variants of the fimbriae of EAEC. *Enterococcus faecalis* is frequently detected in mineral and spring water and can cause human urinary tract infections, endocarditis, and neonatal sepsis. The prevalence, potential virulence genes, antimicrobial resistance, and genetic diversity of *E. faecalis* isolates from 314 mineral water and spring water samples surveyed in China were also investigated (Wei et al.). Collectively, these studies highlighted a high prevalence of antimicrobial or desiccation resistant pathogenic bacteria associated with diverse genotypes.

Decades of research has identified a number of virulence determinants produced and secreted by pathogenic bacteria (Martinović et al., 2016). To establish infections in humans, pathogens must sense and respond to newly encountered host environments to regulate the expression of critical virulence factors that allow for niche adaptation and successful colonization (Bäumler and Sperandio, 2016; Lustri et al., 2017). In this Research Topic, the recently described molecular mechanisms underlying the outcompeting resident microbiota within the gastrointestinal tract by non-typhoidal serovars of *S. enterica* was extensively reviewed by Anderson and Kendall. The bacterium, such as serovar *Typhimurium* (S. Tm) directly eliminates close competitors via bacterial cell-to-cell contact as well as by stimulating a host immune response to eliminate specific members of the microbiota. Additionally, S. Tm tightly regulates the expression of key virulence factors that enable S. Tm to withstand host immune defenses within macrophages. In this context, the authors also highlighted chemical and physical signals that S. Tm senses as cues to adapt to each of these host environments (Anderson and Kendall).

Some other original articles included in this Research Topic also reported new findings in bacterial pathogenesis. For instance, the detailed functions of a cellulose biosynthesis-related gene (*bcsR*) of *C. sakazakii* was investigated using a gene knockout technique, and the results demonstrated that the *bcsR* is a negative regulator of cellulose biosynthesis but positively regulates biofilm formation and the adhesion/invasion ability of *C. sakazakii* (Gao et al.). RpoS is a key stress-inducible sigma factor that regulates stress resistance genes in *E. coli*. A novel missense point mutation at RpoS residue 128 in a clinical Shiga toxin-producing *E. coli* (STEC) isolate was identified. The hydrophobicity of the amino acid at residue 128 is critical for RpoS activity and is consequently important for bacterial survival at cold temperature and oxidative stresses (Iwase et al.). STEC strains also differ in acid resistance. When grown in minimal medium at pH 3.3, STEC strain B201 exhibiting flocculation was more acid sensitive, while STEC strain B241 was planktonic and acid resistant. Transcriptomic and targeted gene expression data showed that the expression of curli and acid induced chaperone genes *csg* and *hde* positively correlated with the phenotypic differences (Kay et al.). *Bacillus cereus* is increasingly reported to be a causative agent of human gastrointestinal disease. Jeßberger et al. investigated enterotoxin production, secretion, and cytotoxicity in a set of 19 enteropathogenic and non-pathogenic *B. cereus* strains of diverse origins by using cell culture medium pre-incubated with human colon epithelial cell line CaCo-2. The authors suggested that the currently used methods in *B. cereus* diagnostics based on standard culture medium should be complemented by cultivation procedures simulating intestinal host conditions (Jeßberger et al.).

Biological and non-biological innovation in technologies has emerged for better control of foodborne pathogens. The contemporary advances in DNA sequencing technologies have not only enabled finer characterization of bacterial genomes but also provided deeper taxonomic identification of complex microbiomes inhabiting an environment, such as a particular body econiche (e.g., human intestinal contents) and a food manufacturing facility econiche (e.g., floor drain) (reviewed by Cao et al.). In the past years, the discovery of small non-coding RNAs (sRNAs) unraveled a new world of post-transcriptional regulatory networks, which cooperate with Ribonucleases (RNases) in the control of gene expression. With the development of new technologies, many sRNA molecules were identified and shown to be important players in bacterial virulence. RNA metabolism has recently been exploited for the development of new therapeutic applications (reviewed by Matos et al.). During food processing and preservation, many foodborne pathogens can be induced to enter a viable but non-culturable (VBNC) state by limiting environmental conditions such as extreme temperatures, drying, irradiation, pulsed electric field, and high pressure stresses, as well as the addition of preservatives and disinfectants. After entering the VBNC state, foodborne pathogens cannot be detected using conventional plate counting techniques and introduce big challenges to food safety. Various features of the VBNC state was extensively reviewed by Zhao et al. including biological characteristics, induction and resuscitation factors, formation and resuscitation mechanisms, detection methods, and relationship to food safety.

New compounds and treatment strategies have been explored for the control of bacterial pathogens. For instance, Shi et al. analyzed the effects of thymoquinone, a principal active ingredient in volatile oil of Nigella sativa seeds, on the suppression of virulence-related traits of *C. sakazakii* ATCC 29544, and *in vitro* tests showed that sub-inhibitory concentrations of thymoquinone significantly decreased motility, quorum sensing, and endotoxin production of the bacterium. Furthermore, thymoquinone substantially reduced the adhesion and invasion of *C. sakazakii* ATCC 29544 to human colonic cell line HT-29 cells and decreased the number of intracellular bacterial cells within the RAW264.7 macrophage cells. *V. cholerae* can cause cholera, a severe diarrheal disease that can be quickly fatal if untreated and is typically transmitted via contaminated water and person-to-person contact (Baker-Austin et al., 2018). Outbreaks of cholera are reported every year in developing nations (World Health Organization, http://www.who.int/). The

effects of dietary minerals zinc, selenium, and manganese on virulence attributes of *V. cholerae* was studied (Bhattaram et al.), and *in vitro* tests indicated that all the three minerals significantly reduced *V. cholerae* motility, adhesion to intestinal epithelial cells (Caco-2), and cholera toxin production (*ctxAB, fliA*, and *toxR*) *in vitro*, and decreased adhesion and toxin production in mouse intestine *ex vivo*. However, *in vivo* studies in an animal model are necessary to validate these results.

Bacterial pathogens persist in food processing facilities via growing predominantly as biofilms rather than in planktonic mode (Bae et al., 2012). Biofilms are complex microbial communities embedded in the protective extracellular polymeric substances (EPS). The efficiency of acidic electrolyzed water (AEW) to remove biofilms was evaluated for foodborne bacterial pathogens including *E. coli*, *V. parahaemolyticus*, and *L. monocytogenes*. *V. parahaemolyticus* infections (i.e., vibriosis) are normally acquired through exposure to sea water or through consumption of raw or undercooked contaminated seafood (Baker-Austin et al., 2018), while *L. monocytogenes* (a pathogen that can grow at refrigeration temperatures) infections (i.e., listeriosis) are frequently associated with unpasteurized dairy products and various ready-to-eat food (Fung et al., 2018). *In vitro* experiments showed that AEW triggered EPS disruption by the deformation of carbohydrate C-O-C bond and aromatic rings in amino acids tyrosine and phenylalanine. The authors suggested that AEW could be an eco-friendly alternative to sanitizers traditionally used in the food industry. Additionally, differential survival of hyper-aerotolerant *C. jejuni* under different gas conditions was also studied, and the resulting data suggested that modified atmosphere packaging using CO_2 may help to controlling poultry contamination with hyper-aerotolerant *C. jejuni* (Oh et al.).

In summary, this Frontiers Research Topic includes 21 articles, and 146 authors from Austria, Canada, China, Denmark, Germany, Ireland, Japan, Portugal, South Korea, United Kingdom, and United States. It provides an overview of recent discoveries in resurgence, pathogenesis, and control strategies of the human serious foodborne pathogenic bacteria, and supports the urgent need for improving food safety and public health, particularly in globalization background. The information presented in the articles not only underscores future research areas and needs for scientists, but also benefits governments, food producers, food suppliers, and food consumers to work together toward eliminating and controlling pathogen persistence in food and resistant infections in humans.

AUTHOR CONTRIBUTIONS

LC drafted the editorial. WA contributed to the editorial revision. All authors approved the final paper for publication.

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ORIGINAL RESEARCH
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Comparing the Genetic Diversity and Antimicrobial Resistance Profiles of *Campylobacter jejuni* Recovered from Cattle and Humans

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Campylobacter jejuni, a leading cause of gastroenteritis in humans, is a foodborne pathogen that can reside in chickens, pigs, and cattle. Because resistance to fluoroquinolones and macrolides, which are commonly used to treat human infections, has emerged in *C. jejuni*, it is imperative to continously monitor resistance patterns and examine the genetic variation in strains from human infections and animal reservoirs. Our previous study of *C. jejuni* from human campylobacteriosis cases showed a significantly higher rate of tetracycline resistance compared to national trends, and identified multilocus sequence type (ST)-982 and a history of cattle contact to be associated with tetracycline resistance. To further investigate these associations, we conducted a cross-sectional study to determine the frequency of antimicrobial resistance and examine the genetic diversity of *C. jejuni* recovered from 214 cattle at three Michigan herds. Overall, the prevalence of *C. jejuni* was 69.2% (range: 58.6–83.8%) for the three farms, and 83.7% ($n = 113$) of isolates were resistant to one or more antimicrobials. Resistance to only tetracycline predominated among the cattle isolates ($n = 89$; 65.9%) with most resistant strains belonging to ST-459 (96.5%) or ST-982 (86.4%). Among the 22 STs identified, STs 459 and 982 were more prevalent in one feedlot, which reported the use of chlortetracycline in feed upon arrival of a new herd. PCR-based fingerprinting demonstrated that the ST-982 isolates from cattle and humans had identical banding patterns, suggesting the possibility of interspecies transmission. Resistance to macrolides (1.5%) and ciprofloxacin (16.3%) was also observed; 14 of the 22 ciprofloxacin resistant isolates represented ST-1244. Together, these findings demonstrate a high prevalence of antimicrobial resistant *C. jejuni* in cattle and identify associations with specific genotypes. Continuous monitoring and identification of risk factors for resistance emergence are imperative to develop novel methods aimed at decreasing pathogen persistence in food animal reservoirs and the frequency of resistant infections in humans.

Keywords: *Campylobacter jejuni*, antimicrobial resistance, cattle, multilocus sequence typing, molecular epidemiology of infectious diseases, zoonotic pathogen

INTRODUCTION

Campylobacter jejuni is one of the most common causes of human gastroenteritis in the U.S., affecting roughly 1.3 million people annually (Scallan et al., 2011). Most *Campylobacter* isolates recovered from patients with gastroenteritis are classified as *C. jejuni* (89%) followed by *C. coli* (8%) (Centers for Disease Control and Prevention [CDC], 2014a). *Campylobacter* spp. can asymptomatically colonize the intestines of various food animals including chickens, cattle, and pigs (Altekruse et al., 1999; Harvey et al., 1999; Stanley and Jones, 2003). Previous studies have been more focused on chickens, as they are considered to be the major reservoir for human campylobacteriosis cases (Harris et al., 1986; Fitch et al., 2005). Recent molecular genotyping and statistical modeling studies (Pritchard et al., 2000; Wilson et al., 2008; Sheppard et al., 2009), however, have shown that cattle also represent an important source for human infections. In Finland and the U.K., for instance, cattle were found to contribute equally to human infections when compared to chickens (Wilson et al., 2008; de Haan et al., 2010). Another study conducted by the Centers for Disease Prevention and Control (CDC) reported that dairy products, mostly raw milk and cheese, contributed to 66% of the campylobacteriosis outbreaks in the U.S. (Interagency Food Safety Analytics Collaboration Project, 2015). *Campylobacter* shed by cattle can also contribute to contamination of the environment via run-off water from farming and meat processing operations, which represent additional sources for human infections. One prior study found identical genotypes of *C. jejuni* recovered from dairy cattle and ground water (Kwan et al., 2008), while our study of 7,182 human campylobacteriosis cases in Michigan identified epidemiological associations between disease and contact with ruminants, well water, and living in a rural area with higher cattle densities (Cha et al., 2016a).

A major concern with regard to treating campylobacteriosis in humans is antimicrobial resistance, particularly resistance to the fluoroquinolones and macrolides, which has increased significantly over the past two decades (Centers for Disease Control and Prevention [CDC], 2014b). Because of the association between fluoroquinolone use in poultry and increasing rates of resistance in human infections, fluroquinolones were banned for use in poultry in the U.S. in 2005 (Nelson et al., 2007). Nonetheless, increasing frequencies of fluoroquinolone resistant *Campylobacter* have been reported in both chickens and humans (Centers for Disease Control and Prevention [CDC], 2014b; United States Department of Agriculture [USDA], 2014), though few studies have been conducted in cattle. Fluoroquinolones such as enrofloxacin, were licensed for use in beef cattle in 1998 (Anderson et al., 2001) and macrolides including tulathromycin and tilmicosin, are commonly used for treating respiratory diseases in both beef and dairy cattle (Cahn and Line, 2010; United States Department of Agriculture [USDA], 2013).

The use of tetracyclines, like chlortetracycline or oxytetracycline, is also common in the cattle industry. According to the national study conducted by the U.S. Department of Agriculture (USDA) in 2007, over one-half (57.5%) of dairy operations in the U.S. were feeding medicated milk replacer, often containing tetracycline, to calves and pre-weaned heifers (Animal and Plant Health Inspection Service [APHIS], 2008). Tetracycline was also reported to be the primary antimicrobial used for treating lameness. It is noteworthy that our prior study of *C. jejuni* strains from Michigan patients demonstrated a significantly higher rate of tetracycline resistance (61.7%) compared to the national average (47.8%) (χ^2 $P < 0.01$) and resistance frequencies were higher in cases reporting contact with ruminants, specifically cattle, prior to the onset of symptoms (Cha et al., 2016b). We also demonstrated that strains belonging to multilocus sequence type (ST)-982 were more likely to be resistant to tetracycline and associated with infections among cases reporting ruminant contact. Together, these data suggest that a pathogenic, tetracycline-resistant lineage may be circulating in the Michigan cattle population. To investigate this possibility, we sought to examine the genetic diversity and antimicrobial resistance profiles of cattle-derived *C. jejuni*, which were collected during the same time period as the strains associated with human infections. We hypothesized that there was a high prevalence of *C. jejuni* in cattle and that the strains had a similar frequency of tetracycline resistance and resistance to other important antimicrobials when compared to the human strains. We also hypothesized that similar genotypes with identical antimicrobial resistance profiles could be detected in both cattle and humans.

MATERIALS AND METHODS

Sample Collection and *C. jejuni* Identification

Fecal samples were collected from 214 cows at one dairy (Farm A) and two beef herds (Farms B and C) in mid-Michigan in 2012 as part of an epidemiologic study to investigate fecal shedding of Shiga toxin-producing *Escherichia coli* in cattle (Venegas Vargas et al., 2016). A questionnaire, which was administered by interviewing each farm manager, was used to obtain data regarding farm demographics, management practices, and herd health management strategies (Supplementary Table S1). The distance from Farm A to Farm C was about 62 miles, while Farm B was located roughly in between the two farms. The average age of cows from Farm A was 3.9 years while all beef cows were ∼1 year old. This study was carried out in accordance with the recommendations and approval by the Institutional Animal Care and Use Committee of Michigan State University (AN12/10-223-00).

To isolate *Campylobacter* from cattle, 10 μl of each fecal sample was directly plated on blood agar containing cefoperazone (20 μg/ml), vancomycin (20 μg/ml), and amphotericin B (4 μg/ml) for 48 h at 37°C in microaerophilic conditions using the Oxoid™ CampyGen (Thermo Scientific, Waltham, MA, USA). Single colonies were selected from each sample based on morphology and appearance while focusing on small pinpoint gray colonies without hemolysis. After growth at 37°C for 48 h using the same conditions, DNA was extracted using the Wizard® genomic DNA purification kit (Promega, Madison,

WI, USA) and stored at −20°C. Confirmation and classification of *Campylobacter* were performed by multiplex PCR as described (Yamazaki-Matsune et al., 2007); all strains were stored in trypticase soy broth with 10% glycerol at −80°C until use. A previously characterized set of 94 *C. jejuni* clinical strains from humans with campylobacteriosis were used for comparison (Cha et al., 2016b). These human strains, which were recovered between 2011 and 2012 by the Michigan Department of Health and Human Services, were compared to the cattle-derived strains in this study. All of the human strains were cultured on the same agar media as the cattle samples and were confirmed with multiplex PCR.

Antimicrobial Susceptibility Profiling and Determination of *tet(O)* Gene

As described previously (Cha et al., 2016b), the minimum inhibitory concentrations (MICs) of nine antimicrobials were determined using broth microdilution with the CAMPY plate from Sensititre (Trek Diagnostic Systems, Cleveland, OH, USA) according to the manufacturer's instruction. Antimicrobials included ciprofloxacin, nalidixic acid, azithromycin, erythromycin, tetracycline, florfenicol, telithromycin, clindamycin, and gentamicin. *C. jejuni* ATCC 33560 was used as a control and the antimicrobial breakpoints were determined using the epidemiologic cut-off values (ECOFFs) following the guidelines of the European Committee on Antimicrobial Susceptibility Testing [EUCAST] (2015) and National Antimicrobial Resistance Monitoring System (Centers for Disease Control and Prevention [CDC], 2014b). Multiple drug resistance (MDR) was defined as resistance to three or more classes of antimicrobials. For all tetracycline resistant strains, the presence of *tet(O)* that confers resistance to tetracycline in *Campylobacter*, was detected using a published PCR protocol (Gibreel et al., 2004).

Multilocus Sequence Typing (MLST)

PCR amplification of seven multilocus sequence typing (MLST) genes was performed using the Kapa2G fast PCR kit (KapaBiosystmes, Boston, MA, USA) with primers listed on the *C. jejuni* and *C. coli* PubMLST website[1]. Amplified products were cleaned using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced at the Research Technology Support Facility at Michigan State University. Sequences were assembled and checked for overall quality using the SeqMan program in the Lasergene software suite (DNASTAR Inc., Madison, WI, USA), while alleles, STs, and clonal complex (CC) assignments were made using the PubMLST database (Jolley and Maiden, 2010). New alleles ($n = 2$) and STs ($n = 8$) identified in this study were deposited in the PubMLST database (ids 32712-32721, 33135-33155, 33157-33260). A Neighbor-joining tree (p-distance) with 1,000 bootstrap replications was constructed in MEGA 6 (Tamura et al., 2013) to examine the evolutionary relationships between *C. jejuni* strains. Clusters were classified for STs that grouped together with >75% bootstrap support, and were further evaluated for genetic recombination using Splitstree4 (Huson and Bryant, 2006).

Repetitive Sequence-Based PCR (Rep-PCR)

To compare the *C. jejuni* recovered from cattle within the same herd and compare a subset of cattle- and human-derived isolates, rep-PCR was utilized with ERIC1R-ERIC2 primers as described (Patchanee et al., 2012). Template DNA concentrations were standardized to 25 ng/μL prior to PCR and 0.8 μM of each primer was used with the KAPA2G Fast Readymix at the following cycling conditions: one initial cycle at 95°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 1 min, and extension at 65°C for 10 min, with a single final extension cycle at 65°C for 20 min. The amplified products were separated by electrophoresis at 80 V for 2 h using a 1.5% agarose gel. Fingerprint patterns were analyzed visually and the Dice coefficient with a 2.0% band position tolerance was used to calculate the similarity matrices in Bionumerics ver. 5.10 (Applied Maths, Inc., Austin, TX, USA). Dendrograms were created using the unweighted pair group method with arithmetic averages (UPGMA).

Data Analysis

Statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC, USA). The χ^2 test was used to examine differences in the prevalence of *C. jejuni* and antimicrobial resistance between farms and identify associations between antimicrobial resistance profiles and molecular traits (e.g., ST, CC, and cluster) as well as farm characteristics. The Fisher's exact test was used for variables with ≤5 in at least one cell. Odds ratios (OR) and their 95% confidence intervals (CI) were calculated in EpiInfo version 7.2.0.1[2] and SAS; $P < 0.05$ was considered significant.

RESULTS

Prevalence of *Campylobacter* in Cattle from Three Herds

The prevalence of *Campylobacter* was 71.0% among the 214 cows examined at the three farms. Most of the *Campylobacter* isolates were classified as *C. jejuni* (97.4%; $n = 148$), however, one *C. coli* isolate was recovered from Farm A. Four isolates could not be speciated and were therefore classified as neither *C. jejuni* or *C. coli*. Among all three farms, the highest prevalence of *C. jejuni* was observed at Farm C (83.8%), which was significantly higher than both Farm A (58.6%) and Farm B (62.2%) ($P < 0.01$) (**Table 1**). Virtually all of the animals in Farms B ($n = 82/83$) and C ($n = 74/75$) were sampled, while only 10.9% ($n = 58/530$) of the total animals were sampled at Farm A, which had a considerably larger herd size. After culture and speciation, only 135 of the 148 (91.2%) *C. jejun*i strains were viable and could be tested for susceptibility to nine antimicrobial agents.

[1] http://pubmlst.org/campylobacter

[2] www.cdc.gov

Antimicrobial Resistance Profiles of *C. jejuni* Isolates across Farms

Among the 25, 50, and 60 *C. jejuni* strains tested from Farms A, B, and C, respectively, 22 (16.3%) were susceptible to all nine antimicrobials (pan-susceptible), while 113 (83.7%) were resistant to one or more agent. Tetracycline resistance predominated ($n = 113$; 83.7%) followed by nalidixic acid (17.0%) and ciprofloxacin (16.3%). Among the tetracycline resistant isolates, 89 (78.8%) were exclusively resistant to tetracycline, while the remainder ($n = 24$; 21.2%) were resistant to one or more additional antimicrobial (**Table 2**). Twenty one of these tetracycline resistant strains were also resistant to ciprofloxacin and nalidixic acid (CipNalTet), while one strain was only resistant to gentamicin in addition to the tetracycline. Resistance to the macrolides, azithromycin, and erythromycin, was observed in two strains (1.5%), which were also resistant to tetracycline and another antimicrobial class; these strains were classified as MDR. All 113 tetracycline resistant isolates harbored *tet(O)*.

Differences in resistance frequencies were observed between the three farms with dairy Farm A having a significantly lower frequency of resistant *C. jejuni* than the two beef farms ($P < 0.00001$). The frequency of resistance to specific antimicrobials and antimicrobial classes also varied by farm. Farm C, for instance, had a significantly higher proportion of strains (95.0%) resistant solely to tetracycline (Tet) when compared to both Farms A (16.0%) and B (56.0%) (Fisher's exact $P \leq 0.00001$). The frequency of any Tet resistance, which included an additional 24 strains with varying profiles, did not differ between Farms C (97.7%) and B (90.0%) (Fisher's exact $P = 0.24$). Farms C and B combined, however, were more likely to have any Tet resistance than the dairy farm (OR: 22.1; 95% CI: 7.29, 66.80) as only 40% of the 25 Farm A isolates were resistant. By contrast, Farm B had the highest rate (32%) of resistance to CipNalTet when compared to Farms A (24%) and C (0%). Although the frequency of CipNalTet was not significantly higher in Farm B relative to Farm A (OR: 1.4; 95% CI: 0.45, 4.07), the beef Farm B had significantly more CipNalTet resistance than the beef Farm C (Fisher's exact $P \leq 0.00001$) and compared to Farms A and C combined (OR: 6.2; 95% CI: 2.23, 17.20). Associations could not be examined for macrolide resistance as only two resistant strains were identified.

Associations between Farm Characteristics and *C. jejuni* Resistance

In the univariate analysis, significant associations were observed between farm characteristics and the presence and type of antimicrobial resistant *C. jejuni* (Supplementary Table S2). Farms classified as beef operations (OR: 22.1, 95% CI: 7.29, 66.80) or that reported use of preventive antimicrobials like chlortetracycline (OR: 10.5, 95% CI: 2.35, 47.24) were significantly more likely to have resistance to any of the antimicrobials evaluated. On the contrary, use of a mixture of antibiotics for treatment (OR: 0.1, 95% CI: 0.02, 0.42), fly control on the farm (OR: 0.1, 95% CI: 0.02, 0.14), and power washing (OR: 0.1, 95% CI: 0.02, 0.42) significantly lowered the likelihood of resistant *C. jejuni*. For CipNalTet resistance, associations were identified with Holstein breed (OR: 5.6, 95% CI: 2.02, 15.76), mixed antibiotic use for treatments (Fisher's $P < 0.0001$) and power washing (Fisher's $P < 0.0001$) in the univariate analysis. Because only three farms were sampled and management practices vary depending on the type of cattle operation studied, our ability to conduct a

TABLE 1 | Prevalence of *Campylobacter jejuni* in cattle and antimicrobial resistance frequencies by farm.

Farm	Farm type	Total No. of cattle	No. (%) of cattle sampled		No. (%) of cattle with *C. jejuni*		OR	95% CI	*P*	No. (%) of animals with resistant *C. jejuni**		OR	95% CI	*P***
A	Dairy	530	58	(10.9%)	35	(58.6%)	ref	–	–	10	(40.0%)	ref	–	–
B	Beef	83	82	(98.8%)	50	(61.0%)	1.0	0.52, 2.04	0.94	45	(90.0%)	13.5	3.98, 45.83	<0.00001
C	Beef	75	74	(98.7%)	62	(83.8%)	3.4	1.51, 7.65	0.002	58	(96.8%)	43.5	8.60, 220.01	<0.00001

*OR, odds ratio; ref, reference group; 95% CI, 95% confidence interval; P, P-value. *Ten of the 35 isolates from Farm A and 2 of the 62 isolates from Farm C were not viable and were excluded from the analyses. **Fisher's exact test P-value.*

TABLE 2 | Resistance profiles of *C. jejuni* recovered from cattle by farm.

Antimicrobial class	Resistance profile	Total		Farm A		Farm B		Farm C	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
0	Susceptible	22	(16.3)	15	(60.0)	5	(10.0)	2	(3.3)
1	Tet	89	(65.9)	4	(16.0)	28	(56.0)	57	(95.0)
2	TetGen	1	(0.7)	0	(0)	1	(2.0)	0	(0)
2	CipNalTet	21	(15.6)	6	(24.0)	15	(30.0)	0	(0)
3	AziEryNalTet	1	(0.7)	0	(0)	0	(0)	1	(1.7)
5	AziEryCipNalTetTelClin	1	(0.7)	0	(0)	1	(2.0)	0	(0)
	Total	135		25		50		60	

No., Number; Tet, tetracycline; Gen, gentamicin; Cip; ciprofloxacin; Nal, nalidixic acid; Azi, azithromycin; Ery, erythromycin; Tel, telithromycin; Clin, clindamycin.

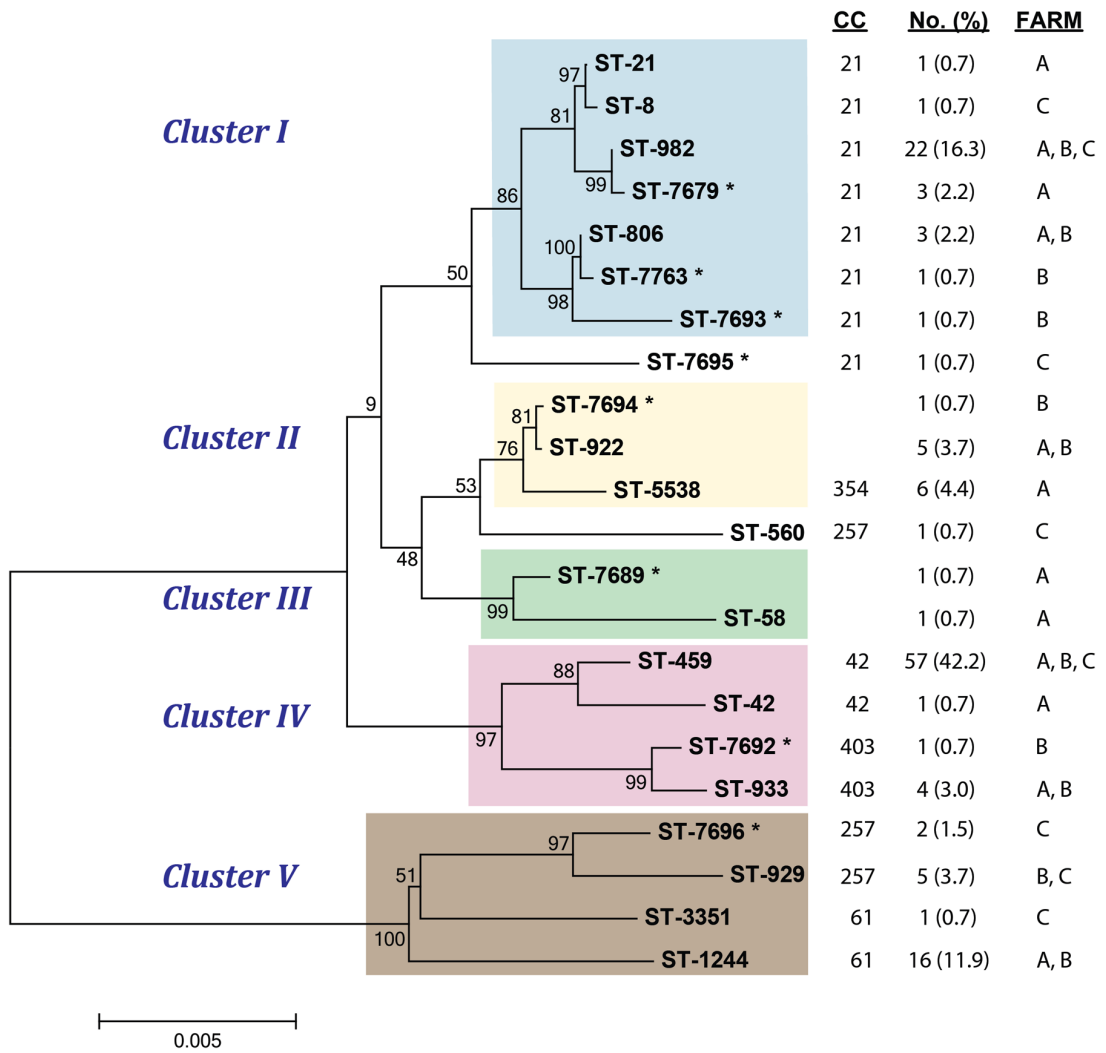


FIGURE 1 | Neighbor-joining phylogeny of sequence types (STs) recovered from cattle at three farms. The cluster is noted as well as the previously defined clonal complex (CC) and distribution of strains representing each lineage. *denotes novel STs recovered in this study.

more comprehensive, multivariate analysis was limited in this study.

Genetic Diversity and Frequency of *C. jejuni* Genotypes in Cattle

Multilocus sequence typing was used to investigate the genetic diversity of *C. jejuni* strains recovered from cattle in all three farms. A total of 22 different STs, including eight novel types, were represented among the 135 *C. jejuni* strains recovered. Eighteen of the 22 STs were assigned to six previously defined CCs, while the remaining four STs were classified as singletons. The Neighbor-joining algorithm grouped all 22 STs in to five clusters (I–V) with bootstrap values > 75% (**Figure 1**). The four STs, which were classified as singletons via PubMLST, grouped together into two distinct clusters, Clusters II and III. Cluster III contained one genotype (ST-5538) that was previously classified as CC-354. It is notable that strains representing multiple CCs defined in PubMLST were found to group together within a given cluster. For example, Cluster IV contains strains representing CCs 42 and 403, which grouped together with 97% bootstrap support, while CCs 257 and 61 of Cluster V grouped together with 100% support. All of the STs comprising Cluster I were assigned to CC-21, though one CC-21 isolate was classified as a singleton in this analysis. Although the Neighbor-net analyses on all sites and 162 parsimonious informative sites indicated significant evidence of recombination among the 22 STs [pairwise homoplasy index (PHI) = 0.0], the five clusters identified in the Neighbor-joining phylogeny were similar in the Neighbor-net phylogeny (Supplementary Figure S1).

Among the 135 *C. jejuni* isolates, the most prevalent STs were ST-459 (n = 57) and ST-982 (n = 22). Both STs were found in all three farms (**Figure 1**) though the frequency varied. For ST-459, Farm C had the highest number of strains (n = 35)

followed by Farm B ($n = 20$). Farm C also had the highest number of ST-982 strains ($n = 17$), while Farms A and B had 4 and 1 strains, respectively. ST-1244 ($n = 16$) was recovered from Farms A and B, however, a significantly greater number of strains were found at Farm B ($n = 13$). STs 922 ($n = 5$), 933 ($n = 4$), and 806 ($n = 3$) were also observed at Farms A and B, while ST-929 ($n = 5$) was found at Farms B and C. The remaining 15 STs identified were exclusive to specific farms and most were represented by only one strain. Notably, ST-5538 ($n = 6$) was found only at dairy Farm A and two of the novel STs, ST-7679 ($n = 3$) and ST-7696 ($n = 2$), were recovered only from Farms A and C, respectively. Among the previously defined CCs, CC-42 predominated ($n = 58$; 42.9%) in this study followed by CC-21 ($n = 33$; 24.4%) and CC-61($n = 17$; 12.6%). In all, Farm C had the lowest level of genetic diversity with eight STs, while 12 STs were recovered from Farm A and 11 were recovered from Farm B.

Association between Phylogenetic Lineage and Antimicrobial Resistance Profiles

Several significant associations were observed between specific STs and antimicrobial resistance profiles (**Figure 2**). The most prevalent ST detected, ST-459 ($n = 57$), was significantly associated with tetracycline resistance (OR: 22.00; 95% CI: 3.28, 923.78) as the majority ($n = 55$; 96.5%) of strains were resistant to tetracycline only. Most of the 22 isolates assigned to ST-982 ($n = 21$; 95.5%) were also resistant to tetracycline (**Figure 2**), although the association was not significant in the overall analysis because of the high proportion of tetracycline resistance among ST-459 strains. For fluoroquinolone resistance, isolates belonging to ST-1244 were more commonly resistant to ciprofloxacin and nalidixic acid (Fisher's $P < 0.0001$); 14 of the 16 ST-1244 strains were resistant to both antimicrobials. Thirteen of these strains were also resistant to tetracycline, yielding a significant association between ST-1244 and CipNalTet resistance (Fisher's $P < 0.0001$). Similarly, strains assigned to ST-929 had a higher likelihood of resistance to CipNalTet (Fisher's $P < 0.05$) as did isolates assigned to ST-7679 (Fisher's $P < 0.01$). Significant associations were also observed between specific STs and pan-susceptibility, as all of the strains belonging to ST-5538 ($n = 6$) and ST-922 ($n = 5$) were susceptible to all antimicrobials evaluted (Fisher's $P < 0.0001$) (**Figure 2**). The associations between ST-459 and Tet resistance as well as ST-1244 and CipNalTet resistance remained significant even when the farm was considered.

Similar associations were observed across the clusters identified in the Neighbor-joining phylogeny. Cluster IV, for instance, mostly consisted of ST-459 ($n = 57$) strains and was significantly associated with tetracycline resistance (OR: 13.1; 95% CI: 2.91, 118.53). Likewise, Cluster V was associated with CipNal (Fisher's $P < 0.0001$) and CipNalTet resistance (Fisher's $P < 0.0001$) as ST-1244 ($n = 16$) comprised the majority (66.7%) of strains within this cluster. On the other hand, Cluster II, which is mostly comprised of ST-5538 and ST-922, was significantly associated with pan-susceptibility (Fisher's $P < 0.0001$) as was Cluster III (Fisher's $P < 0.01$), though the number of strains in the latter group was small ($n = 2$).

DNA Fingerprinting Analysis of *C. jejuni* Isolates to Investigate Transmission

Rep-PCR was performed on all 135 *C. jejuni* isolates to assess the genetic diversity of isolates that were assigned to the same STs, and examine transmission of *C. jejuni* within and between farms. Overall, the fingerprinting patterns correlated well with the MLST results and most strains assigned to the same STs had identical patterns or clustered together (**Figure 3**). Strains belonging to ST-922 ($n = 5$), for instance, were indistinguishable by rep-PCR despite being found at two different farms; the same was true for strains representing ST-806 ($n = 3$), ST-933 ($n = 2$), and ST-1244 ($n = 16$). Most of the STs within each farm were identical by rep-PCR, though there were exceptions as six pairs of strains with different STs had identical fingerprint patterns (e.g., ST-806 and ST-21).

The most common genotypes, ST-459 and ST-982, grouped into two major clusters. When each ST was examined by UPGMA analysis separately, most ST-459 strains shared a fingerprinting pattern (459-C) consisting of nine bands on average regardless of the resistance profile and farm of origin (**Figure 4A**). Even though several unique patterns differing by 1–8 bands were observed for these ST-459 strains, only one strain clustered outside of the main ST-459 cluster (**Figure 3**). Importantly, the 459-C pattern representing strains with varying resistance profiles was found at all three of the farms, although the 459-C strains with resistance to only tetracycline varied in prevalence across farms with most coming from Farms B ($n = 17$) and C ($n = 31$).

A similar result was observed for the 22 ST-982 strains with most possessing the same rep-PCR pattern (982-A). Four other patterns represented by one strain each (**Figure 4B**) were clustered together in a different part of the phylogeny. Although strains with the 982-A rep-PCR pattern were found at all three farms, they varied by resistance profile. Farms B and C had 982-A strains with only tetracycline resistance, while strains from Farm A were either pan-suceptible ($n = 1$) or resitant to CipNalTet ($n = 2$). Interestingly, all three CipNalTet resistant isolates belonging to ST-7679 were recovered from Farm A and had the same fingerprint pattern as 982-A (**Figure 3**). This genotype differed from ST-982 by one single nucleotide polymorphism (SNP) in one of the seven MLST housekeeping genes.

Comparison of *C. jejuni* Isolates from Humans and Cattle and Associations with Antimicrobial Resistance Profiles

A Neighbor-joining phylogeny was also constructed to compare the evolutionary relationships of 94 *C. jejuni* strains recovered from human patients to the 135 cattle strains. The genetic diversity was lower in the cattle strains as 22 cattle-derived STs were identified relative to to the 49 human-derived STs (Supplementary Figure S2), though eight STs (ST-8, ST-21, ST-982, ST-806, ST-922, ST-459, ST-42, and ST-929) were found in both the humans and cattle. Although the bootstrap support was not high enough to identify specific clusters in the combined analysis, the more closely related STs were assigned to the same CCs by PubMLST. Notably, four of the eight overlapping STs (ST-21, ST-8, ST-982, and ST-806), were assigned to CC-21.

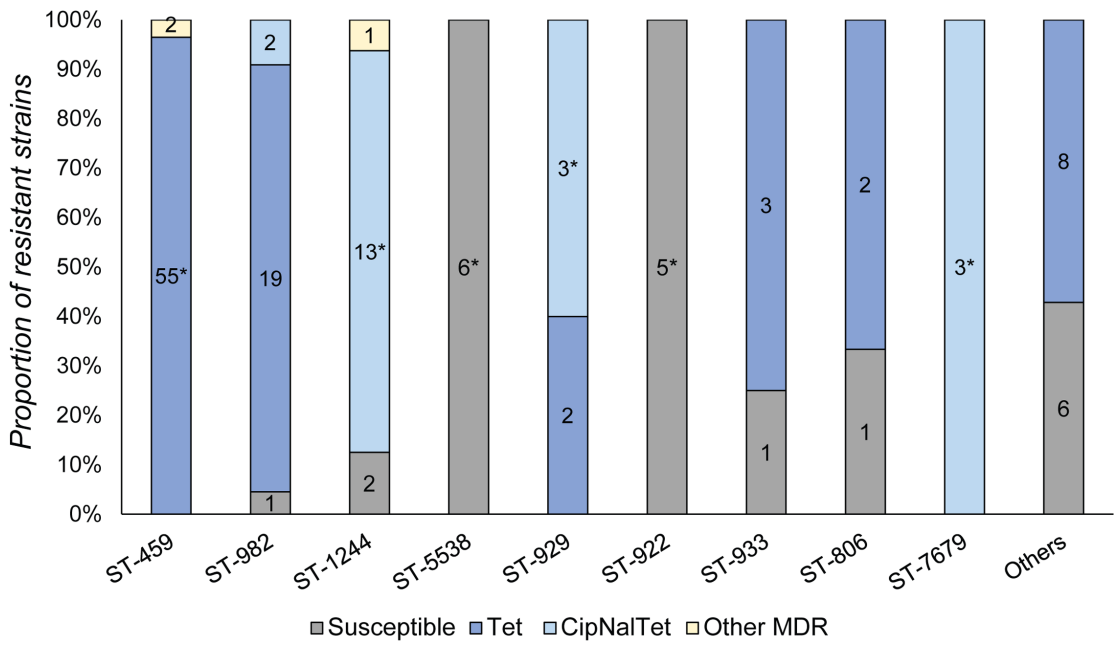


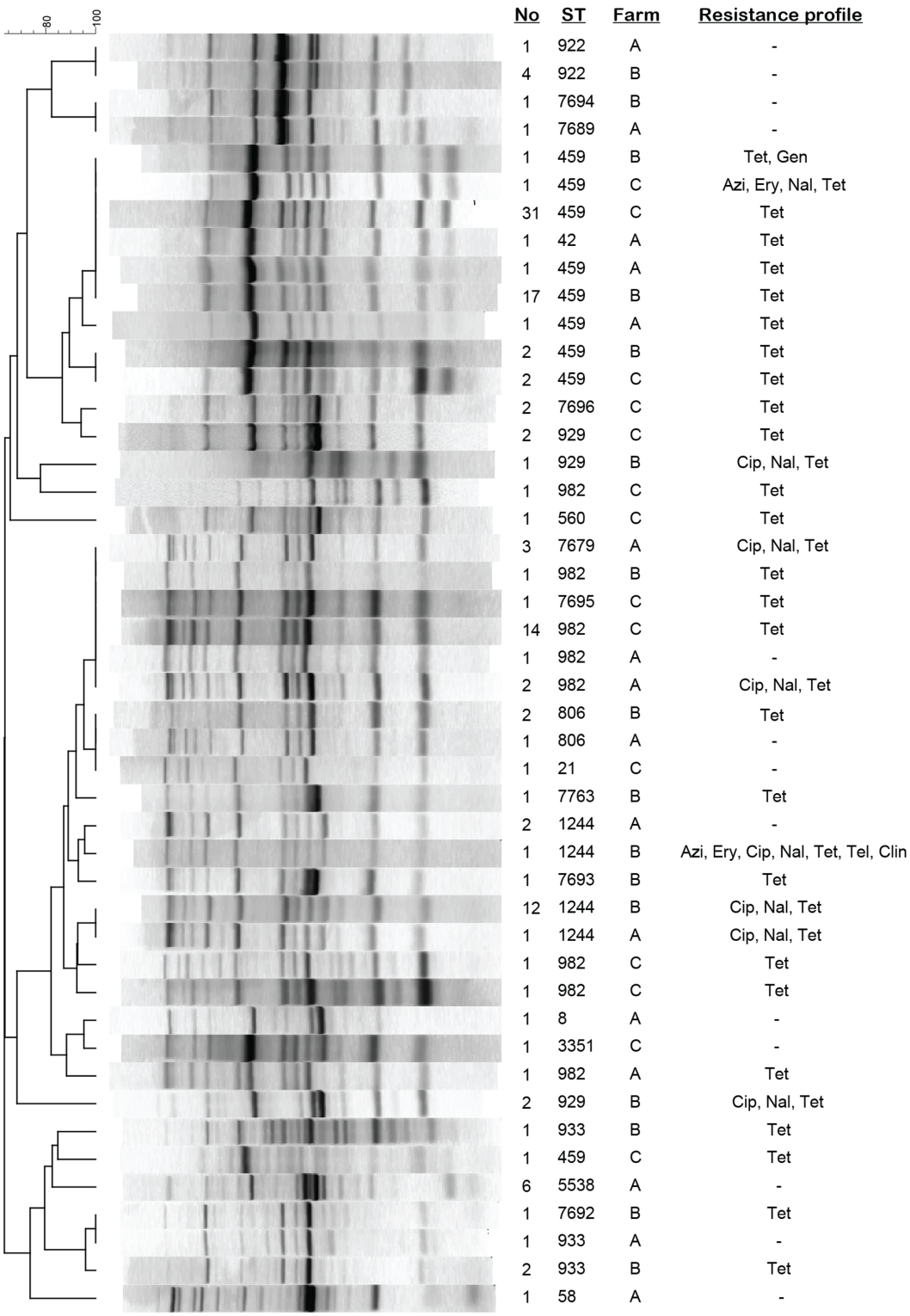
FIGURE 2 | Distribution of resistance profiles in *Campylobacter jejuni* strains by ST. Number of isolates belonging to each resistance profile are noted within each bar and *denotes significant associations between a ST and specific antimicrobial resistance profile. Tet, tetracycline resistance; CipNalTet, ciprofloxacin, nalidixic acid and tetracycline resistant; MDR, multiple drug resistance.

Another phylogenetic tree was constructed to compare the 22 cattle-derived STs to the 36 STs representing strains recovered only from patients residing in Michigan; STs from patients reporting travel outside of Michigan were excluded. The Neighbor-joining phylogeny demonstrated adequate bootstrap support (>78%) for 12 clusters (**Figure 5**). The eight STs that were shared between both sources were included in the analysis, and three of these STs (ST-8, ST-21, and ST-982) were assigned to CC-21 by PubMLST. STs 806, 922, and 929 grouped together in to cattle-specific clusters, while STs 459 and 42 did not cluster together with bootstrap support exceeding 78%. Rep-PCR of human ST-982 ($n = 10$) and ST-459 ($n = 1$) strains were similar to the cattle-derived strains representing the same ST (**Figure 5**, inset). The human ST-982 and ST-459 strains had fingerprint patterns 982-A and 459-C, respectively, which predominated among the cattle-derived isolates recovered from each of the three farms.

The antimicrobial resistance profiles of the eight STs shared between humans and cattle were also examined (Supplementary Figure S3). Notably, similar resistance profiles were observed among strains with the same STs. All strains belonging to ST-21, for example, were pan-susceptible, while ST-42 and ST-459 strains were mostly resistant to tetracycline. ST-982, which consisted of 10 strains from humans and 22 from cattle, also shared a similar resistance pattern. In detail, the majority of strains (81.3%) representing ST-982 were resistant to tetracycline only, however, two ST-982 isolates from both species were CipNalTet resistant. Combining all of the susceptibility and genotyping data from cattle ($n = 135$) and humans ($n = 94$) confirmed the associations identified separately for each species. Specifically, ST-982 was significantly associated with tetracycline resistance (OR:6.41; 95% CI: 1.53, 56.83), while STs 21, 922, and 5538 were more likely to be pan-susceptible (Fisher's $P < 0.01$). ST-464 and ST-1244, which were found exclusively in humans and cattle, respectively, were significantly associated with CipNal and CipNalTet resistance (Fisher's $P < 0.01$). The same was true for ST-459 and resistance to tetracycline (OR:14.75; 95% CI: 3.64, 128.14) and ST-7679 with CipNalTet resistance (Fisher's $P < 0.01$).

DISCUSSION

This study demonstrates a high prevalence (69.2%) of *C. jejuni* in cattle, indicating that cattle are an important reservoir in Michigan. Because seasonality has been shown to affect *C. jejuni* prevalence in cattle with higher peaks in warmer months (Grove-White et al., 2010), it is possible that higher temperatures contributed to the high frequencies observed given that sampling took place during the summer. Among the three farms sampled, Farm C, a beef operation, had a significantly higher proportion of cattle with *C. jejuni* compared to Farm A (dairy) and Farm B (beef). Previous studies have reported a higher prevalence of *C. jejuni* in beef compared to dairy cattle (Englen et al., 2007) as well as an increased frequency in feedlot cattle over time during their feeding period (Minihan et al., 2004; Besser et al., 2005). It is therefore possible that the significant difference observed between Farm C and other farms in this study is due to the different operation type, proportion of the herd sampled (Farm A) or sampling times (Farm B). Longitudinal



No	ST	Farm	Resistance profile
1	922	A	-
4	922	B	-
1	7694	B	-
1	7689	A	-
1	459	B	Tet, Gen
1	459	C	Azi, Ery, Nal, Tet
31	459	C	Tet
1	42	A	Tet
1	459	A	Tet
17	459	B	Tet
1	459	A	Tet
2	459	B	Tet
2	459	C	Tet
2	7696	C	Tet
2	929	C	Tet
1	929	B	Cip, Nal, Tet
1	982	C	Tet
1	560	C	Tet
3	7679	A	Cip, Nal, Tet
1	982	B	Tet
1	7695	C	Tet
14	982	C	Tet
1	982	A	-
2	982	A	Cip, Nal, Tet
2	806	B	Tet
1	806	A	-
1	21	C	-
1	7763	B	Tet
2	1244	A	-
1	1244	B	Azi, Ery, Cip, Nal, Tet, Tel, Clin
1	7693	B	Tet
12	1244	B	Cip, Nal, Tet
1	1244	A	Cip, Nal, Tet
1	982	C	Tet
1	982	C	Tet
1	8	A	-
1	3351	C	-
1	982	A	Tet
2	929	B	Cip, Nal, Tet
1	933	B	Tet
1	459	C	Tet
6	5538	A	-
1	7692	B	Tet
1	933	A	-
2	933	B	Tet
1	58	A	-

FIGURE 3 | Repetitive sequence-based PCR profiles of *C. jejuni* strains recovered from cattle. Patterns represent strains of a given ST that vary by resistance profile or farm. If the same ST was recovered from more than one farm, then both strains were included for comparison. The number (No) of strains representing each pattern and ST is indicated. Azi, azithromycin; Cip, ciprofloxacin; Clin, clindamycin; Ery, erythromycin; Gen, gentamicin; Nal, nalidixic acid; Tel, tetracycline; Tel, telithromycin.

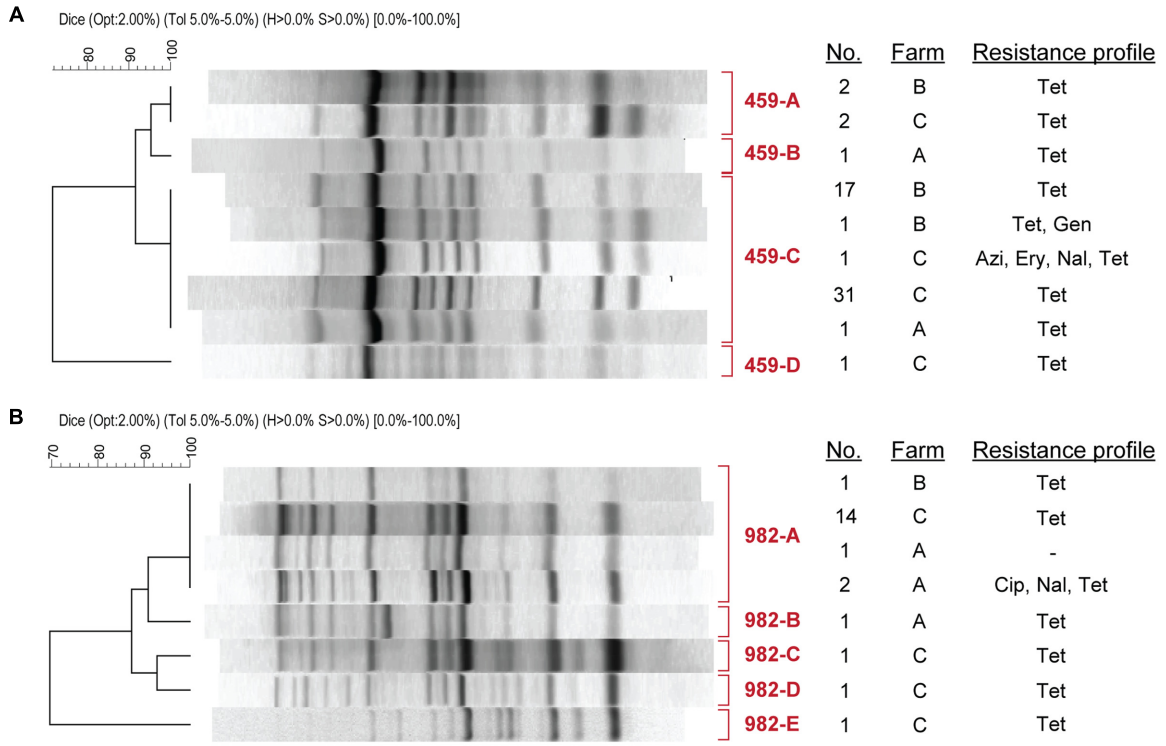


FIGURE 4 | Repetitive sequence-based PCR pattern of (A) ST-459 strains and **(B)** ST-982 strains. Patterns represent strains of the same ST that vary by resistance profile or farm. The number (No) of strains representing each pattern (labeled in red) is indicated. Azi, azithromycin; Cip, ciprofloxacin; Ery, erythromycin; Gen, gentamicin; Nal, nalidixic acid; Tel, tetracycline.

studies in additional cattle farms, which utilize a standardized sampling scheme, are warranted to better quantify the prevalence of *C. jejuni* by operation over time.

A high frequency of genotypes ST-459, ST-982, and ST-1244 was observed among the cattle-derived *C. jejuni* isolates. These STs are classified as CC-42, CC-21, and CC-61, respectively, which are the most commonly reported lineages from cattle around the world (Minihan et al., 2004; Kwan et al., 2008; de Haan et al., 2010). According to the PubMLST database, more than 60% of the reported *C. jejuni* isolates from cattle can be classified into these three CCs, suggesting that the genotypes may be better adapted to cattle and the farm environment. To enhance our ability to assess the genetic diversity of *C. jejuni* and investigate transmission of strains with the same ST within and across farms, we utilized rep-PCR, a highly discriminatory tool for characterizing *Campylobacter* (Wilson et al., 2009; Patchanee et al., 2012). Although another single primer targeting $(GTG)_5$ was used to increase the discriminatory power, ERIC1R-ERIC2 primers yielded higher discriminatory power for all STs examined in this study. The rep-PCR patterns by ERIC primers correlated well with the STs, confirming that this genotyping tool is useful for epidemiological studies, particularly those conducted in resource limited settings. It is important to note, however, that some discrepancies were found as isolates with different STs were indistinguishable by rep-PCR. Nevertheless, these STs were closely related in the phylogenetic analysis and belonged to the same CCs. Rep-PCR was particularly useful in differentiating the isolates assigned to the predominant STs, ST-459 and ST-982, and showed that some fingerprint patterns were shared across all three farms. One similarity among the farms was the reported contact with other animals including dogs, cats, and wildlife like starlings, pigeons, raccoons, deer (Supplementary Table S1), which could be important for pathogen transmission. Because a recent study in Ohio detected the same genotypes circulating in cattle and starlings in the same area (Sanad et al., 2013), it is possible that some strains are more readily transmitted between farms via birds or other wildlife. Besides ST-459 and ST-982, however, the other STs were detected in Farms A and B or B and C. Since Farms A and C were 62 miles away from north to south and Farm B was located roughly in between (24 miles south of A and 37 miles north of C), more frequent transmission may have occurred between farms in close proximity.

The significantly higher frequency (95%) of tetracycline resistant isolates recovered from Farm C is notable and may be partly due to the distribution of circulating genotypes. Tet resistance was associated with ST-459, or ST-459-C by rep-PCR, and was found at all three farms, though the Farm C beef operation had the highest prevalence. Importantly, Farm C was the only farm to report the use of chlortetracycline, which was added to the feed of new animals upon arrival and was continuously administered at 2 grams/head for 5 days per month. It is therefore possible that the selection pressure associated with

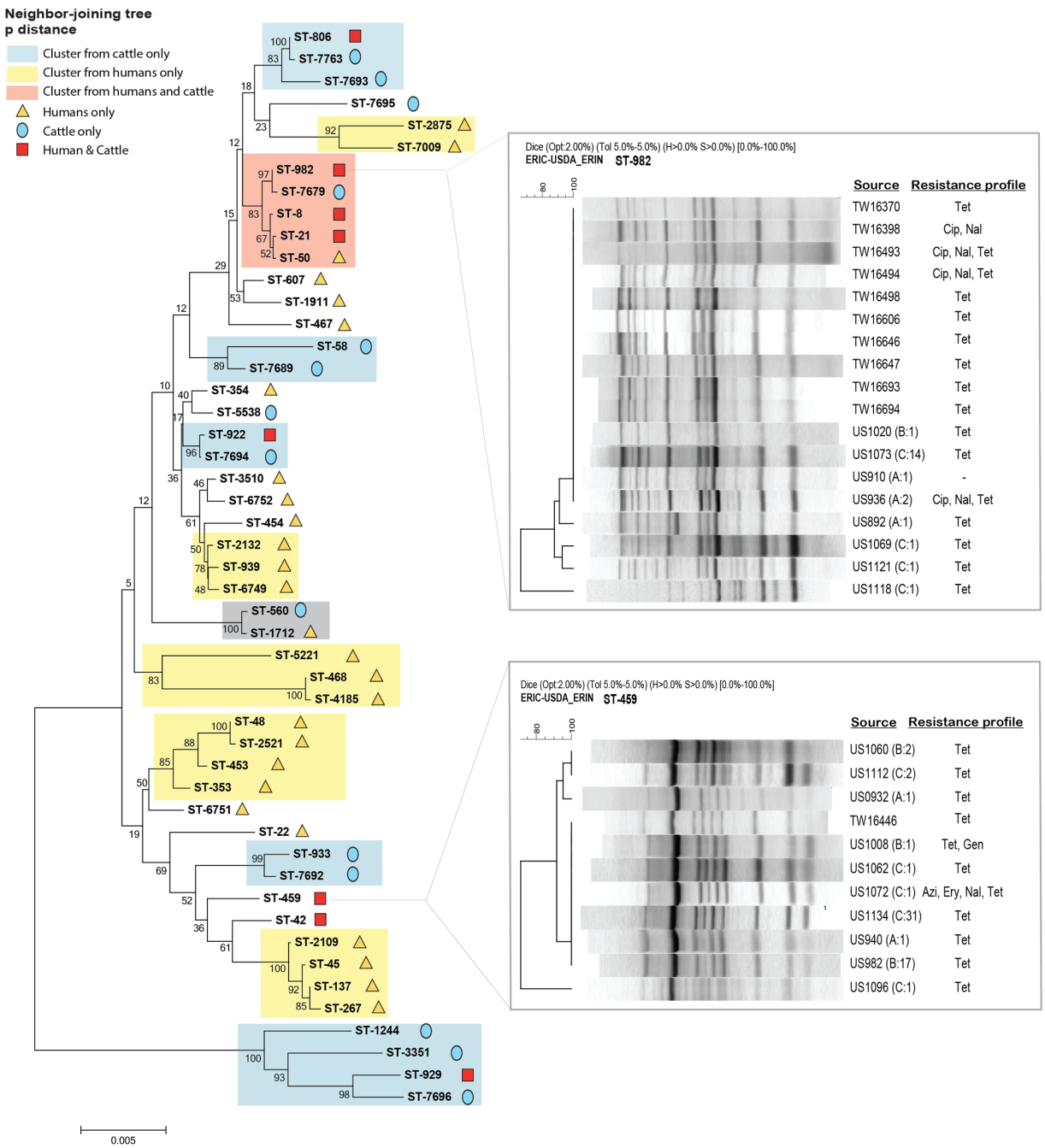


FIGURE 5 | Neighbor-joining phylogeny of STs recovered among cattle and human patients from Michigan. Clusters are colored based on bootstrap support for STs within each cluster and source as some clusters are specific only to humans (yellow) or cattle (blue) and others overlap (orange). The gray box highlights a new cluster containing strains from both sources. Rep-PCR banding patterns are shown for the predominant overlapping STs, ST-982 (n = 10) and ST-459 (n = 1), representing strains from both humans and cattle. Identification numbers starting with TW were recovered from humans, while numbers starting with U.S. are from cattle. The farm and number of cattle strains (No) representing each pattern are indicated in parentheses.

chlortetracycline use at Farm C resulted in the dissemination of one Tet resistant genotype that is well adapted to cattle and the environment. This genotype may also be responsible for the high prevalence of *C. jejuni* at Farm C and is in line with previous studies that have reported high levels of tetracycline resistance in *C. jejuni* from cattle given therapeutic and subtherapeutic doses of the antimicrobial (Inglis et al., 2005; Asai et al., 2007). Farm C also reported use of disinfectant spraying as the sole cleaning method every 6 months, which differed from the other farms that used power washing more frequently (Supplementary Table S1).

Indeed, a previous study in cattle farms has reported more frequent cleaning of water troughs in barns reduced the risk of *Campylobacter* (Ellis-Iversen et al., 2009). Additional molecular epidemiological studies are greatly needed to further investigate the association between farm management practices in cattle operations, especially antibiotic use and cleaning protocols, and the frequency of *C. jejuni* and antimicrobial resistant *C. jejuni*.

The frequency of macrolide resistance (2%) observed in this study was similar to prior studies in the U.S. with ranges between 0 and 2.9% (Sato et al., 2004; Bae et al., 2005). A higher frequency (16%) of fluoroquinolone resistance, however, was observed relative to other cattle studies (range: 0.6 and 5.0%). Because none of the farms reported use of fluoroquinolones, these data suggest that resistance is maintained in the population in the absence of antimicrobial use. This hypothesis is supported by studies involving the mechanism of fluroquinolone resistance, which typically involves a single point mutation in the chromosomal *gyrA*. Indeed, fluoroquinolone resistant *C. jejuni* strains were found to have increased fitness in chickens even when the selective pressure was removed (Luo et al., 2005). In support of this finding, Farm B had the highest frequency of fluoroquinolone resistance (69.6%), which was associated with one genotype (ST-1244). The clonal spread of fluoroquinolone resistant ST-1244 in Farm B warrants further investigation to explore the *in vivo* fitness of fluoroquinolone resistant *C. jejuni* in cattle and the continuous monitoring of resistance frequencies in the farm environment.

Our previous study with human *C. jejuni* isolates in Michigan showed a significantly higher rate of tetracycline resistance (61.7%) compared to national reports (47.8%) (χ^2 $P < 0.01$) and further, that resistance was associated with ST-982 and history of cattle contact (Cha et al., 2016b). To our knowledge, this is the first report of a ST-982 *C. jejuni* infection in humans in the U.S., as the ST-982 isolates highlighted in PubMLST were recovered from cattle, a lamb, and the farm environment. Compared to the human strains, which were collected during an overlapping time frame and in the same vicinity as the cattle strains, a significantly higher frequency of tetracycline resistance was observed in the cows (χ^2 $P < 0.001$). Twenty two cattle strains (16.3%) belonged to ST-982, confirming a high prevalence of this genotype in Michigan cattle. Nineteen (86.4%) of the ST-982 isolates from cattle were resistant to tetracycline, and the association between ST-982 and Tet resistance was significant after combining the human ($n = 94$) and cattle ($n = 135$) isolates (OR: 6.41; 95% CI: 1.53, 56.83). Notably, all 10 ST-982 isolates from humans had the same rep-PCR pattern as the predominant 982-A pattern found in cattle at all three farms. It is important to note, however, that genotypic variation may exist by year and the lack of cattle sampling in 2011 may have limited our ability to detect additional overlapping STs that were present in both species. Nonetheless, these findings suggest that a tetracycline-resistant ST-982 lineage, which has contributed to a high frequency of human infections, is also circulating in Michigan cattle.

Among the CipNalTet resistant isolates from Farm A, we identified a novel genotype, ST-7679, which contains one SNP in one of the seven MLST loci relative to ST-982. Because the rep-PCR patterns for these ST-7679 strains were identical to the ST-982 strain patterns, it is likely that diversification of this resistant lineage has occurred in this farm environment. Further characterization by whole genome sequencing will be useful to elucidate the evolutionary relationship between these two genotypes as well as more comprehensively monitoring transmission patterns within and across herds and humans. Although these isolates were confined to Farm A, ST-982 was widespread in both humans and cattle in Michigan and hence, additional monitoring of these resistant lineages is warranted.

Since this study is cross-sectional in design with samples collected from only three farms, it is possible that our results are not generalizable to all Michigan cattle or even to cattle outside of Michigan. Nonetheless, common associations were identified between resistance profiles and specific lineages in strains from all three farms as well as in humans with campylobacteriosis. Taken together, these data suggest that a subset of resistant genotypes are circulating in both the cattle and human populations in a given geographic location and that cattle serve as an important reservoir for both *C. jejuni* and resistant *C. jejuni*. Given the impact of antimicrobial resistance on human and animal health, it is clear that longitudinal studies are needed to better define risk factors for emergence, persistence, and transmission of resistant *C. jejuni* in cattle and the environment while focusing on those lineages that are more readily linked to human infections.

AUTHOR CONTRIBUTIONS

WC, RM, DG, and SM conceived the study and contributed materials; RM, CV-V, SR, PB, DG, and SM planned the study and sampled the animals; WC, RM, and SW performed the experiments; WC and SM analyzed the data and drafted the paper; all authors approved the final version.

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SUPPLEMENTARY MATERIAL

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Antibiotic and Desiccation Resistance of *Cronobacter sakazakii* and *C. malonaticus* Isolates from Powdered Infant Formula and Processing Environments

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This study evaluated the antimicrobial and desiccation resistance of *Cronobacter sakazakii* and *Cronobacter malonaticus* isolates from powdered infant formula and processing environments. The antimicrobial susceptibility tests showed that the 70 *Cronobacter* strains, representing 19 sequence types, were susceptible to the most of the antibiotics except for amoxicillin-clavulanate, ampicillin, and cefazolin. Furthermore, the growth of six *C. sakazakii* and two *C. malonaticus* strains from different sequence types (STs) in hyperosmotic media was measured. The growth of the two *C. sakazakii* strains (CE1 and CE13) from the neonatal pathovars ST4 and ST8, were significantly higher ($p < 0.05$) than that of other strains. *C. malonaticus* strain CM35 (ST201) was the slowest grower in all strains, and most could not grow in more than 8% NaCl solution. Also the survival of these strains under desiccation conditions was followed for 1 year. The viable count of *Cronobacter* spp. under desiccation conditions was reduced on average by 3.02 log cycles during 1 year, with CE13 (ST8) being the most desiccation resistant strain. These results will improve our understanding of the persistence of the two closely related species *C. sakazakii* and *C. malonaticus* which are of concern for neonatal and adult health.

Keywords: *C. sakazakii*, *C. malonaticus*, desiccation, antibiotic susceptibility, powdered infant formula

INTRODUCTION

Cronobacter spp. (formerly *Enterobacter sakazakii*) is an emerging opportunistic bacterial pathogen comprised of seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter condimenti*, *Cronobacter universalis,* and *Cronobacter dublinensis* (Joseph et al., 2012b; Lu et al., 2014). This organism can cause necrotizing enterocolitis, bacteremia, and meningitis in neonates and infants, with a 40–80% mortality rate (Forsythe et al., 2014; Holy and Forsythe, 2014; Li et al., 2015). To date, only the three species *C. sakazakii*, *C. malonaticus*, and *C. turicensis* have been isolated from neonatal infections (Sonbol et al., 2013). *Cronobacter* spp. has been recovered from a wide variety of foods, with powdered infant formula (PIF) being of particular concern as it is the most significant source of

Cronobacter strains resulting in neonatal infections (Iversen and Forsythe, 2004; Craven et al., 2010). *Cronobacter* strains cannot survive after the standard pasteurization procedures, therefore, the addition of non-heat treated materials and environmental contamination during filling and packaging are the plausible causes of *Cronobacter* contamination of PIF (Nazarowec-White and Farber, 1997). Besides, the strong resistant ability of the organism in dry PIF factory environments can increase the likelihood of post-pasteurization contamination (Riedel and Lehner, 2007).

Currently, antibiotic therapy is considered to be the common and preferred way to prevent the *Cronobacter* infection in humans (Depardieu et al., 2007). Many studies have confirmed that *Cronobacter* strains can be effectively eliminated by antibiotics, however, prolonged use of antibiotics is undesirable as it may result in the development of *Cronobacter* antibiotic resistance (Yoneyama and Katsumata, 2006; McMahon et al., 2007). Hochel et al. (2012) reported that all of 53 *Cronobacter* strains isolated from 399 retail food samples were resistant to erythromycin, and two of them were resistant to both erythromycin and tetracycline. Chon et al. (2012) found that 77.8% *Cronobacter* strains isolated from desiccated foods in Korea were not susceptible to cephalothin. The *Cronobacter* strains resistance to cefotaxime and streptomycin have also been isolated from various foods in Korea and commercial PIF in China (Lee et al., 2012). Caubilla-Barron and co-workers reported that the *C. sakazakii* strains isolated from two fatal neonatal infections expressed β–lactamase activity (Caubilla-Barron et al., 2007). In addition, the effective dose of antibiotics for treatment may change after long-term antibiotic use, therefore screening environmental and PIF isolates of *Cronobacter* for antibiotic resistance would be a useful comparison with clinical isolates.

The greater resistance of *Cronobacter* spp. to desiccation compared with other *Enterobacteriaceae* will enable their long term survival under a low water activity (a_w) condition such as in PIF (a_w: 0.2–0.5) which has a long shelf life of up to 1 year (Gurtler and Beuchat, 2007). In general, the viability of *Cronobacter* spp. in PIF decreases by 0.5–0.6 log cycles per month (Edelson-Mammel et al., 2005; Gurtler and Beuchat, 2007). Caubilla-Barron et al. (2007) found there was considerable reduction in the viable count of *Cronobacter* strains (about 3.34 $\log_{10}$ cycles) in the first 6 months, whereas after the following 24 months, the average reduction in viability decreased by 1.88 $\log_{10}$ cycles. It is important to note that some capsulated *C. sakazakii* strains were still recovered after 2.5 years, by contrast, both *Salmonella enteritidis*, and *Escherichia coli* were undetectable after 15 months (Barron and Forsythe, 2007). Some difference in desiccation resistance can be found among the different *Cronobacter* strains, for example, *C. sakazakii* NCTC11467^T has atypical growth characteristics compared with most of *Cronobacter* strains, and cannot be recovered from desiccated condition after 1 year (Iversen et al., 2004). *Cronobacter* spp. produce highly mucoid colonies on milk agar plates and the capsular material could be linked to virulence traits such as macrophage survival as well as desiccation resistance (Ogrodzki and Forsythe, 2015). Particular capsule profiles correlate with the meningitic *C. sakazakii* pathovar clonal complex (CC) 4 (Ogrodzki and Forsythe, 2015).

Cronobacter is a diverse genus and has been extensively studied using multilocus sequence typing for over 1,000 strains (Forsythe et al., 2014). This has revealed the high clonality within the genus as well as identified particular pathovars. *C. sakazakii* sequence type (ST) four identified using multi-locus sequence typing (MLST) was the dominant ST, and was associated with neonatal meningitis (Joseph and Forsythe, 2011). In addition, *Cronobacter* pathovars, associated with clonal complex are now recognized. Of particular relevance is the *C. sakazakii* CC4 pathovar which is associated with neonatal meningitis (Hariri et al., 2013; Forsythe et al., 2014). *C. sakazakii* ST8 and *C. sakazakii* ST1 were mainly isolated from clinical sources and PIF, respectively (Forsythe et al. 2014). *C. sakazakii* CC4 was also the genotype of ∼25% of isolates recovered from the processing environment of PIF manufacturing plants (Sonbol et al., 2013). Why this CC predominates in PIF and neonatal infections is uncertain since no particular virulence genes have been detected (Joseph et al., 2012a). Therefore, a better understanding of the environmental fitness and antibiotic resistance of *Cronobacter* isolates with significant sequence type from PIF production areas is warranted.

In previous studies, our group isolated 66 *Cronobacter sakazakii* and four *C. malonaticus* strains from PIF and processing environments from 2009 to 2012, and identified their sequence type by MLST (Fei et al., 2015). In this study, we further characterized the strains according to their antibiograms and desiccation tolerance, and considered whether the differences corresponded with their sequence type.

MATERIALS AND METHODS

Bacterial Strains

A total of 70 *Cronobacter* strains (66 *C. sakazakii* and four *C. malonaticus* strains) were studied. All strains had been isolated from PIF and processing environments, as previously reported (Fei et al., 2015). All 70 *Cronobacter* strains were used for the antimicrobial assays, and six *C. sakazakii* (CE21, CE1, CE13, CE38, CE52, and CE25) and two *C. malonaticus* (CM3 and CM35) strains with different sequence types were selected for studying the survivals under desiccation condition.

Strains were recovered from storage at −80°C in 40% (v/v) glycerol, by inoculating 0.1 mL portions of thawed cultures into 10 mL Luria-Bertani (LB) broth, followed by cultivation at 37°C for 12 h. The cultures were streaked onto Tryptic Soy Agar (TSA) plates and incubated at 37°C for 24 h for single colony isolation. A single colony of each strain was inoculated into the LB and incubated at 37°C for 18 h for following study.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility determination was performed using the BD PhoenixTM^{100} Automated Microbiology System (BD Diagnostic Systems, Sparks, MD) as according to the manufacturer's instructions. Twenty-one antibiotics were selected for the susceptibility test, including amikacin,

amoxicillin-clavulanate, ampicillin, ampicillin-sulbactam, aztreonam, cefotaxime, ceftazidime, cefazolin, cefepime, chloramphenicol, ciprofloxacin, colistin, gentamicin, imipenem, levofloxacin, meropenem, moxifloxacin, piperacillin, piperacillin-tazobactam, tetracycline, and trimethoprim-sulfamethoxazole. The results were expressed as sensitive (S), intermediate (I), and resistant (R), and resistant according to the PhoenixTM^{100} guidelines. *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were used as the quality control organisms, and were included in each run according to the manufacturer's recommendations.

Resistance to Osmotic Stress

For determining bacterial growth in a hyperosmotic environment, cultures (0.1 mL) were inoculated into brain–heart infusion (BHI) broth containing molar equivalents of either sodium chloride (NaCl) (4, 6, 8, 10% w/v) or sorbitol (12.5, 19, 25, 31% w/v). The above cultures were incubated at 37°C for 24 h, and subsequent growth was measured the growth according to the absorption values at 600 nm (OD 600) using an ultraviolet spectrophotometer (Biochrom Ltd., Cambridge, England). The "time to detection" (TTD), defined as the time (in h) to reach an OD 600 of 0.2, and "growth rate" (in h^{-1}) were used to determine the growth of each strain under the different treatments, as previously reported (Alvarez-Ordonez et al., 2014).

Resistance to Dry Stress

C. sakazakii and *C. malonaticus* were grown in LB for 18 h at 37°C before analysis. For dry stresses, the cultures were harvested by centrifugation at 8,000 g for 10 min, then the cell pellets were diluted in 0.85% sterile normal saline (NS) to obtain a cell density of 8.00 $\log_{10}$ cfu/mL.

The *C. sakazakii* and *C. malonaticus* cell suspensions were desiccated as previously described with minor modifications (Breeuwer et al., 2003). Unstressed cultures (50 μL) were transferred to sterile petri dishes without their lids, which were placed in a constant temperature humidity chamber, and incubated at 25°C with 20.7% air relative humidity (RH) for air-drying. After drying for 1.5 h, the petri dishes were covered and kept at 21°C with 20.7% RH for 1 year. Periodically, the bacterial survival was determined by conventional colony counting.

Statistical Analysis

Mean values and standard deviations were obtained from three replicate experiments with duplicated plating ($n = 6$). Statistical analysis was performed by Analysis of Variance (ANOVA) with the SPSS 20.0 software. Tukey's multiple range test was used to determine the significant differences ($p < 0.05$) between treatments.

RESULTS

Antimicrobial Susceptibility Tests

The antimicrobial susceptibility pattern and Minimal Inhibitory Concentration (MIC) of the 70 *Cronobacter* strains are shown in **Tables 1, 2**. All *Cronobacter* strains were susceptible to most antibiotics, including amikacin, ampicillin-sulbactam, aztreonam, cefepime, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, imipenem, levofloxacin, meropenem, moxifloxacin, piperacillin, piperacillin- tazobactam, tetracycline, and trimethoprim-sulfamethoxazole. However, they were resistant to amoxicillin-clavulanate, ampicillin, and cefazolin. The MIC of antibiotics, except cefazolin, did not vary across the 70 *Cronobacter* strains. Ciprofloxacin and trimethoprim-sulfamethoxazole were considered to be the most effective antibiotics against the 70 *Cronobacter* strains at MIC of ≤ 0.5 and $\leq 0.5/0.95$ μg/mL. In contrast, there were some differences in their resistance to cefazolin; **Table 2**. The 70 *Cronobacter* strains were divided into four groups according to their cefazolin MIC-values ($\leq$4, >4, $\leq$8, >8, $\leq$16, >16 μg/mL). A majority of *Cronobacter* isolates with the same ST had a same MIC-value.

Resistance to Osmotic Stress

Six *C. sakazakii* and two *C. malonaticus* strains were incubated in BHI with various molar equivalents of either NaCl or sorbitol at 37°C for 24 h. The TTD values and growth rates of eight *Cronobacter* strains are shown in **Table 3**. Compared with growth in BHI without high concentration solutes, the growth of all eight strains in BHI with different concentrations of NaCl and sorbitol was significantly delayed and slower. The TTD-values of all eight strains growing at >8% NaCl were >24 h, and the growth rates of those ranged from 0.0004/h to 0.0035/h. Meanwhile, in BHI with sorbitol, all strains reached an OD 600 of 0.2 within 12 h. This result showed that the growth of strains was not completely inhibited in <31% sorbitol, whereas there was no growth in the molar equivalent NaCl. In addition, there was variation between strains in their resistance to osmotic stress. Among the eight strains in this study, the growth of CE25 (ST64) was the most significantly delayed ($p < 0.05$), while CE13 (ST8) had more resistance to the delayed growth compared with other strains. The growth rates of CE13 (ST8) and CE1 (ST4) were significantly higher ($p < 0.05$) than that of other strains, and CM35 (ST201) was the slowest grower ($p < 0.05$) in all strains.

Resistance to Dry Stress

The survival of six *C. sakazakii* and two *C. malonaticus* strains stored under desiccation condition (21°C, RH = 20.7%) was monitored for up to 1 year (**Table 4**). In the first 60 days, the average recovery declined by about 1.55 log cycles, then, a smaller decrease was observed during the next 10 months, ranging from 1.07 log cycles (CE13, ST8) to 1.85 log cycles (CE25, ST64). Within 1 year, the reduction of all strains ranged from 2.43 log cycles (CE13, ST8) to 3.46 log cycles (CE25, ST64). The survival of *C. malonaticus* CM3 (ST258) and *C. sakazakii* CE25 (ST64) were significantly less ($p < 0.05$) than the other six *Cronobacter* strains, and *C. sakazakii* CE13(ST8) was the highest ($p < 0.05$) survival in all eight *Cronobacter* strains. In addition, during the different time-phase in 1 year, the CM3 (ST258) had significant lower ($p < 0.05$) survival value compared with other strains.

TABLE 1 | Antimicrobial susceptibility and MIC of 70 *Cronobacter* strains isolated from PIF and processing environments.

Antibiotics	MIC[a] (μg/mL)	SIR[b]	Antibiotics	MIC[a] (μg/mL)	SIR[b]
Amikacin	≤8	S (100%)	Colistin	≤0.5	S (100%)
Amoxicillin-Clavulanate	≤4/2	R (100%)	Gentamicin	≤2	S (100%)
Ampicillin	≤4	R (100%)	Imipenem	≤1	S (100%)
Ampicillin-Sulbactam	≤4/2	S (100%)	Levofloxacin	≤1	S (100%)
Aztreonam	≤2	S (100%)	Meropenem	≤1	S (100%)
Cefotaxime	≤1	S (100%)	Moxifloxacin	≤1	S (100%)
Ceftazidime	≤1	S (100%)	Piperacillin	≤4	S (100%)
Cefepime	≤2	S (100%)	Piperacillin-tazobactam	≤4/4	S (100%)
Chloramphenicol	≤4	S (100%)	Tetracycline	≤2	S (100%)
Ciprofloxacin	≤0.5	S (100%)	Trimethoprim-sulfamethoxazole	≤0.5/0.95	S (100%)

[a]*MIC: (Minimal Inhibitory Concentration) the lowest concentration of the antibiotics that can inhibit effectively growth of the tested microorganism.*
[b]*S, Susceptible; I, Intermediate; R, Resistant.*

DISCUSSION

C. sakazakii and *C. malonaticus* are the dominant species of *Cronobacter* spp. isolated from PIF and processing environment, and can infect infants and adults, respectively (Fei et al., 2015). Given the long term shelf-life of PIF (up to 1 year) and repeated isolation from PIF production plants (Craven et al., 2010; Sonbol et al., 2013), environmental fitness of *Cronobacter* is very important trait to understand as it may lead to increased persistence and neonatal exposure.

The phylogenetic relationship of concatenated sequences (3,036 bp) based on conventional MLST (7 loci), ribosomal-MLST (53 loci), and core genome MLST (1865 loci) reflects the whole genome phylogeny of the *Cronobacter* genus (Joseph et al., 2012b; Forsythe et al., 2014). The application of MLST has also led to the recognition of stable clonal complexes and sequence types associated with certain clinical presentations. Subsequently the diversity and clonal stability within the *Cronobacter* genus needs to be taken into consideration when interpreting laboratory studies. Therefore, *Cronobacter* strains with different sequence types were chosen for detailed study here.

In this study, 70 *Cronobacter* strains, previously isolated from PIF and processing environments, were used. These represented 19 sequence types of *C. sakazakii* and *C. malonaticus*, detailed information can be obtained from *Cronobacter* MLST databases (http://pubmlst.org/cronobacter/) (Fei et al., 2015). The main STs from PIF and processing environments, were the neonatal pathovars *C. sakazakii* ST4, ST1, and ST8, as well as ST64, ST12, ST21, ST258, and ST201.

The isolates were susceptible to most antibiotics, except for amoxicillin-clavulanate, and ampicillin. Previous reports revealed that the occurrence of susceptibility to cefotaxime, chloramphenicol, ciprofloxacin, gentamicin, and tetracycline in *Cronobacter* strains isolated from different sources (Chon et al., 2012; Xu et al., 2014). Chon et al. (2012) reported that only 5.6% strains from desiccated foods in Korea were resistance to ampicillin whereas in our study, all isolates were resistant to this antibiotic. Furthermore, we found that the MIC values of cefazolin varied across different STs, but were similar within each ST. This may reflect a connection between the ST and antimicrobial resistance.

The ability of eight *Cronobacter* isolates with different STs to resist the osmotic stress was evaluated in this study. Compared with *C. malonaticus* isolates, *C. sakazakii* isolates had a higher capacity to grow in the hyperosmotic media, in agreement with previous studies (Caubilla-Barron et al., 2007; Avelino Alvarez-Ordóñez et al., 2014). *C. sakazakii* CE13 (ST8) was the most resistant to osmotic stress in the eight strains, interestingly, *C. sakazakii* ATCC 29544^T reported to have atypical growth characteristics is also ST8 (Osaili and Forsythe, 2009). *C. sakazakii* CE1 (ST4) also showed a more osmotic stress resistance, which might be one of the major reasons why *C. sakazakii* CC4 is frequently isolated from infant food, ready-to-eat foods, potable water. This could result in higher infant exposure and therefore risk of infection (Forsythe et al., 2014).

Cronobacter spp. tend to persist more in PIF with a water activity (a_w) 0.25–0.30, compared with a_w 0.43–0.50, and the survival rate was not associated with whether the PIF was milk-based or soybean-based (Osaili and Forsythe, 2009). In general, this organism can persist in PIF for more than 1 year, therefore, monitoring of survival under desiccated conditions is warranted for the two main *Cronobacter* species, *C. sakazakii* and *C. malonaticus*. Breeuwer et al. (2003) reported that the reduction of *Cronobacter* strains (species undetermined) which had been dried in air and incubated for 46 days at 25°C ranged from 1.0 to 1.5 log cycles. Using similar desiccation conditions, the viable counts of *Cronobacter* strains maintained at 25°C for 45 days decreased by 0.86–1.76 log cycles in our study. Barron and Forsythe (2007) reported that the number of survivors of *C. sakazakii*, *C. muytjensis,* and *C. turicensis* under desiccated conditions in the first 30 days reduced by 0.58 log cycles on average, and decreased 3.34 log cycles

TABLE 2 | The differences of resistance to cefazolin in 70 *Cronobacter* strains isolated from PIF and processing environments.

MLST	Stain	MIC-1	MIC-2	MIC-3	MIC-4	MLST	Stain	MIC-1	MIC-2	MIC-3	MIC-4
ST1	CE21					ST12	CE41				
	CE24						CE44				
	CE43					ST17	CE28				
	CE47						CE58				
	CE59					ST21	CE51				
	CE60						CE52				
	CE63						CE53				
	CE69					ST22	CE15				
	CE70						CE50				
	CE71					ST31	CE56				
	CE72					ST40	CE29				
	CE73					ST50	CE32				
	CE74					ST64	CE25				
	CE79						CE30				
ST4	CE1						CE31				
	CE7						CE33				
	CE9						CE34				
	CE10						CE36				
	CE11						CE54				
	CE12						CE62				
	CE17						CE68				
	CE18						CE77				
	CE19						CE78				
	CE20					ST83	CE8				
	CE22					ST201	CM35				
	CE23					ST258	CM2				
	CE27						CM3				
	CE48						CM5				
	CE49					ST259	CE16				
	CE61					ST268	CE26				
	CE64					ST260	CE55				
	CE67					ST261	CE75				
ST8	CE13						CE76				
	CE14					ST269	CE65				
ST12	CE38						CE66				

MIC-1 (≤4 μg/mL)
MIC-2 (> 4, ≤8 μg/mL)
MIC-3 (> 8, ≤16 μg/mL)
MIC-4 (> 16 μg/mL).

during the first 6 months. While, under the similar desiccation conditions, the results in current study indicated that the reduction of *Cronobacter* strains declined by an average of 0.86 log cycles during the first month, and 2.25 log cycles during the first 6 months. The differences in reduction of *Cronobacter* strains between different reports indicated that a continuous assessment of resistance to dry stress was very necessary.

Interestingly, after kept in the desiccation conditions for 1 year, the total decrease of CE13 (ST8) was the least, followed by CE38 (ST12) and CE1 (ST4). This trend was similar to osmotic stress resistance. Mechanisms of resistance to these environmental stressor have previously been reviewed by Osaili and Forsythe (2009). The higher resistance to both osmotic and desiccation stresses by *C. sakazakii* compared with *C. malonaticus* may in part account for the predominance of *C. sakazakii* in PIF and processing environments and subsequent greater infant exposure.

In summary, this study contributes to an improved understanding of the environmental persistence of *C. sakazakii* and *C. malonaticus* and subsequent risk of infant exposure through contaminated PIF. In addition, the relatively low antibiotic resistance is

TABLE 3 | Growth ability of the six *C. sakazakii* (CE21, CE1, CE13, CE38, CE52, and CE25) and two *C. malonaticus* (CM35 and CM3) strains under concentrations of NaCl (4, 6, 8, 10% w/v) and sorbitol (SB) (12.5, 19, 25, 31%w/v) conditions.

	Solute (w/v)	*Cronobacter* strains							
		C. sakazakii						*C. malonaticus*	
		CE21 (ST1)	**CE1 (ST4)**	**CE13 (ST8)**	**CE38 (ST12)**	**CE52 (ST21)**	**CE25 (ST64)**	**CM35 (ST201)**	**CM3 (ST258)**
TTD (h)	Control (0%)	3	3	3	3	3	3	3	3
	NaCl (4%)	7	5	5	7	5	9	9	6
	NaCl (6%)	10	6	6	12	8	17	14	11
	NaCl (8%)	>24	>24	>24	>24	>24	>24	>24	>24
	NaCl (10%)	>24	>24	>24	>24	>24	>24	>24	>24
	SB (12.5%)	5	3	4	5	4	5	5	5
	SB (19%)	7	5	5	6	6	7	7	7
	SB (25%)	8	7	7	7	7	8	9	9
	SB (31%)	11	9	7	9	9	12	12	12
Growth Rate	Control (0%)	0.064 ± 0.001^{a}	0.065 ± 0.003^{a}	0.064 ± 0.002^{a}	0.065 ± 0.002^{a}	0.064 ± 0.002^{a}	0.065 ± 0.002^{a}	0.065 ± 0.002^{a}	0.065 ± 0.001^{a}
	NaCl (4%)	0.029 ± 0.003^{adef}	0.036 ± 0.002^{bce}	0.038 ± 0.003^{bc}	0.032 ± 0.004^{ade}	0.036 ± 0.002^{bce}	0.034 ± 0.002^{abde}	0.029 ± 0.003^{adef}	0.026 ± 0.002^{af}
	NaCl (6%)	0.017 ± 0.002^{a}	0.030 ± 0.002^{b}	0.026 ± 0.001^{c}	0.020 ± 0.002^{d}	0.024 ± 0.002^{c}	0.018 ± 0.001^{a}	0.016 ± 0.002^{ae}	0.015 ± 0.002^{e}
	NaCl (8%)	0.002 ± 0.000^{a}	0.004 ± 0.000^{b}	0.003 ± 0.000^{c}	0.001 ± 0.000^{d}	0.001 ± 0.000^{d}	0.001 ± 0.000^{d}	0.001 ± 0.000^{d}	0.001 ± 0.000^{d}
	NaCl (10%)	0.001 ± 0.000^{a}	0.001 ± 0.000^{a}	0.001 ± 0.000^{a}	0.001 ± 0.000^{a}	0.001 ± 0.000^{a}	0.000 ± 0.000^{b}	0.0004 ± 0.000^{b}	0.000 ± 0.000^{b}
	SB (12.5%)	0.053 ± 0.002^{a}	0.059 ± 0.001^{b}	0.058 ± 0.002^{b}	0.060 ± 0.001^{b}	0.060 ± 0.001^{b}	0.059 ± 0.001^{b}	0.048 ± 0.002^{c}	0.046 ± 0.002^{c}
	SB (19%)	0.047 ± 0.001^{a}	0.051 ± 0.002^{bcd}	0.053 ± 0.002^{bc}	0.051 ± 0.001^{bcd}	0.052 ± 0.001^{bcd}	0.050 ± 0.002^{bd}	0.039 ± 0.001^{e}	0.037 ± 0.001^{e}
	SB (25%)	0.040 ± 0.001^{a}	0.043 ± 0.001^{b}	0.045 ± 0.001^{c}	0.038 ± 0.001^{a}	0.039 ± 0.001^{a}	0.039 ± 0.001^{a}	0.030 ± 0.001^{d}	0.030 ± 0.001^{d}
	SB (31%)	0.024 ± 0.001^{aeh}	0.035 ± 0.001^{b}	0.038 ± 0.001^{c}	0.026 ± 0.001^{de}	0.025 ± 0.001^{aeh}	0.028 ± 0.000^{f}	0.022 ± 0.000^{g}	0.025 ± 0.001^{aeh}

All strains have different STs. The averages of three replications ± standard deviations were given. Rows values with the different letters were significant different ($p < 0.05$); The p value for each comparison is showed on each row.

TABLE 4 | Survivors (log cfu/mL) of the six *C. sakazakii* (CE21, CE1, CE13, CE38, CE52, and CE25) and two *C. malonaticus* (CM35 and CM3) after dehydration for up to 1 year.

Time (days)	Survivors (log cfu/mL)							
	C. sakazakii						*C. malonaticus*	
	CE21 (ST1)	CE1 (ST4)	CE13(ST8)	CE38 (ST12)	CE52 (ST21)	CE25 (ST64)	CM35 (ST201)	CM3 (ST258)
0	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
6	7.81 ± 0.15^{a}	7.86 ± 0.11^{a}	7.88 ± 0.02^{a}	7.89 ± 0.03^{a}	7.81 ± 0.06^{a}	7.65 ± 0.03^{b}	7.77 ± 0.01^{a}	7.48 ± 0.05^{c}
18	7.62 ± 0.04^{ad}	7.51 ± 0.04^{b}	7.70 ± 0.04^{cd}	7.81 ± 0.03^{d}	7.67 ± 0.04^{acd}	7.28 ± 0.04^{e}	7.53 ± 0.03^{b}	7.05 ± 0.05^{g}
45	6.64 ± 0.16^{a}	7.10 ± 0.05^{b}	7.03 ± 0.05^{b}	7.14 ± 0.05^{b}	7.00 ± 0.04^{b}	6.65 ± 0.10^{a}	6.71 ± 0.06^{a}	6.24 ± 0.05^{c}
60	6.45 ± 0.10^{abde}	6.58 ± 0.10^{abcd}	6.60 ± 0.10^{abcd}	6.62 ± 0.08^{bcd}	6.51 ± 0.07^{acde}	6.39 ± 0.09^{ade}	6.48 ± 0.13^{abcde}	6.07 ± 0.11^{f}
90	6.29 ± 0.10^{abef}	6.47 ± 0.09^{abcdf}	6.51 ± 0.12^{bcdf}	6.50 ± 0.07^{bcdf}	6.38 ± 0.11^{abdf}	6.18 ± 0.10^{aef}	6.33 ± 0.08^{abcdef}	5.92 ± 0.16^{g}
120	6.11 ± 0.10^{ad}	6.28 ± 0.06^{bcd}	6.40 ± 0.07^{bc}	6.30 ± 0.09^{bcd}	6.11 ± 0.12^{ad}	6.07 ± 0.09^{ad}	6.21 ± 0.09^{abd}	5.81 ± 0.09^{e}
165	5.86 ± 0.10^{abe}	5.95 ± 0.11^{abd}	6.29 ± 0.13^{c}	6.11 ± 0.09^{bd}	5.85 ± 0.10^{abe}	5.77 ± 0.13^{ae}	5.89 ± 0.06^{abe}	5.31 ± 0.05^{f}
225	5.48 ± 0.12^{a}	5.38 ± 0.10^{a}	6.02 ± 0.10^{b}	5.85 ± 0.08^{c}	5.35 ± 0.11^{a}	5.38 ± 0.08^{a}	5.49 ± 0.06^{a}	5.02 ± 0.05^{d}
255	5.27 ± 0.13^{acd}	5.29 ± 0.11^{acd}	5.86 ± 0.10^{b}	5.72 ± 0.07^{b}	5.16 ± 0.08^{ac}	5.16 ± 0.06^{ac}	5.38 ± 0.11^{ad}	4.95 ± 0.09^{e}
365	4.96 ± 0.12^{a}	5.00 ± 0.08^{a}	5.57 ± 0.10^{b}	5.39 ± 0.10^{c}	4.86 ± 0.09^{a}	4.54 ± 0.09^{d}	4.97 ± 010^{a}	4.56 ± 0.11^{d}
Total decrease	3.04 ± 12^{a}	3.00 ± 0.08^{a}	2.43 ± 0.10^{b}	2.61 ± 0.10^{c}	3.14 ± 0.09^{a}	3.46 ± 0.09^{d}	3.03 ± 010^{a}	3.44 ± 0.11^{d}

The averages of three replications ± standard deviations are shown. All strains have different STs. Rows values with the different letters were significant different ($p < 0.05$). The p value for each comparison is showed on each row.

reassuring for the current treatment of *Cronobacter* infections.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CM and PF. Performed the experiments: PF, YJ, CM, JF, and YZ. Generated and analyzed the data: PF and RL. Wrote the paper: PF, CM, and SF.

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The Possible Influence of Non-synonymous Point Mutations within the FimA Adhesin of Non-typhoidal *Salmonella* (NTS) Isolates in the Process of Host Adaptation

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Non-typhoidal *Salmonella* (NTS) remains a global pathogen that affects a wide range of animal species. We analyzed a large number of NTS isolates of different host origins, including *Salmonella* Heidelberg ($n = 80$, avian), *S*. Dublin (50, bovine), *S*. Typhimurium var 5- ($n = 40$, porcine), *S*. 4,5,12,:i:- ($n = 40$, porcine), *S*. Cerro ($n = 16$, bovine), and *S*. Montevideo ($n = 14$, bovine), using virulence profiling of the *bcfC*, *mgtC*, *ssaC*, *invE*, *pefA*, *stn*, *sopB*, and *siiE* virulence-associated genes, a biofilm production assay, pulsed field gel electrophoresis, and the full-length sequencing of the *fimA* (adhesin) and *iroN* (receptor) genes. We determined a key amino acid substitution, A169 (i.e., threonine changed to alanine at position 169), in the FimA protein that changed ligand affinity of FimA toward N-acetyl-D-glucosamine. This finding clearly indicates the important role of non-synonymous single nucleotide polymorphism (nsSNPs) in adhesin functionality that may impact the host tropism of NTS. This nsSNP was found in *S*. Heidelberg and *S*. Cerro isolates. Although this was not the case for the IroN receptor, the phylogeny of this receptor and different host origins of NTS isolates were positively correlated, suggesting existence of specific host immune selective pressures on this unique receptor in *S. enterica*. We found that *pefA,* a gene encoding major fimbrial subunit, was the most-segregative virulence factor. It was associated with *S*. Heidelberg, *S*. Typhimurium var 5- and *S*. 4,5,12,:i:- but not with the rest of NTS strains. Further, we observed a significantly higher frequency of non-biofilm producers among NTS strains that do not carry *pefA* (42.5%) compared to *S*. Heidelberg (2.5%) and *S*. Typhimurium var 5- (7.5%)

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and *S*. 4,5,12,:i:- (0%). This study provides new insights into the host adaptation of avian and mammalian NTS isolates that are based on the bacterial antigens FimA and IroN as well as the interrelationships between host adaptation, overall genetic relatedness, and virulence potential in these NTS isolates.

Keywords: non-typhoidal *Salmonella*, host adaptation, adhesin FimA, receptor IroN, plasmid-encoded major fimbrial subunit PefA, salmonellosis

INTRODUCTION

Non-typhoidal *Salmonella* (NTS) remains a serious zoonotic pathogen worldwide (Bangtrakulnonth et al., 2004). The public health importance of NTS is underscored by the fact that from 2000 to 2008 infections caused by this pathogen accounted for ∼1.2 million illnesses, with 23,000 hospitalizations and 450 deaths each year in the US alone (Scallan et al., 2011). The global situation is even worse: in excess of 1.3 billion people experience salmonellosis annually, with nearly 3 million deaths (O'Reilly et al., 2012; Keestra-Gounder et al., 2015). This incredibly diverse species can infect a wide range of hosts, including humans, poultry, cattle, and other domesticated and wild animals. Interestingly, within the population of NTS, there are *Salmonella* lineages that exhibit the narrow-host range (i.e., host-specialists), whereas certain NTS lineages have the wide-host range (i.e., host-generalists). For instance, *Salmonella enterica* subs. *enterica* serovar Enteritidis is capable of invading numerous host species, ranging from mammals, birds to reptiles (Altekruse et al., 2006; Bosch et al., 2016; Feasey et al., 2016). In a sharp contrast to *S*. Enteritidis, *S*. Gallinarum, a genetically closely related lineage, is strictly restricted to galiforme birds. In contrast to host-range diversity of NTS lineages, typhoidal serovars (TS) of *S. enterica* have a predilection for specific hosts. For instance, *S. enterica* serovar Typhi, and Paratyphi (e.g., causative agents of the systematic typhoid fever) are strict human pathogens (Gal-Mor et al., 2014). Despite the fact that these two groups of *S. enterica* spp. I, NTS and TS, share >96% DNA sequence identity (McClelland et al., 2001), the molecular bases for their host specificity differ profoundly. The mechanisms by which NTS serovars cross host barriers remain elusive. Recently, Yue et al. (2015), examining patho-adaptation of *S. enterica* serovar Typhimurium in diverse hosts, reported that non-synonymous single-nucleotide polymorphisms (nsSNPs) in certain antigen genes play an important role in host adaptation of NTS. They found distinct host-specific nsSNP signatures within the *fimH* gene (i.e., gene encoding the type 1 fimbrial adhesion) that may determine NTS host tropism. Moreover, nsSNPs may play a role in the host adaptation process of a narrower host range pathogen, such as *Neisseria gonorrhoeae*. Vidovic et al. (unpublished data) found that host immunity, acting on antigenic gene such the *tbpB* gene—which encodes an outer-membrane lipoprotein responsible for gonococcal transferrin-iron acquisition—can generate a series of nsSNPs during a single outbreak of *N. gonorrhoeae*, resulting in genetic diversification and host adaptation of the outbreak isolate. Gene gain or loss, also play a role in host adaptation of zoonotic and human pathogens. It has been found that invasive strains of *S*. Typhimurium and Enteritidis undergo a genome degradation to adapt to the new extraintestinal lifestyle. The genome degradation occurs via the formation of pseudogenes and the shedding of genes involved in the gut colonization and anaerobic catabolism of inflammation-derived nutrients (Feasey et al., 2016).

The aim of the present study was to investigate the role of nsSNPs in two antigen genes, *fimA,* and *iroN*, as well as the role of eight virulence genes, *bcfC*, *mgtC*, *ssaC*, *invE*, *pefA*, *stn*, *sopB,* and *siiE,* in the process of the host adaptation of NTS isolates obtained from avian (*S*. Heidelberg), bovine (*S*. Dublin, *S*. Cerro, *S*. Montevideo), and porcine (*S*. Typhimurium var 5-, *S*. 4,5, 4,5,12,:i:-) hosts. The entire collection of NTS isolates was analyzed for the presence of three virulence genes, *bcfC*, *pefA,* and *siiE*, involved in NTS colonization/biofilm formation and five virulence genes, *ssaC*, *invE*, *stn*, *sopB,* and *mgtC*, involved in invasion of the host. In addition to the screening for the three genes implicated in colonization/biofilm formation, we tested the all NTS isolates for their ability to form a biofilm, a crucial virulence phenotype that leads to chronic carriage and shedding of NTS (Hurley et al., 2014). Furthermore, we analyzed the role of nsSNPs in the host adaptation of NTS serovars using the full-length gene sequencing of *fimA* (i.e., gene encoding fimbriae that enable bacteria to colonize the epithelium of specific host organs) and *iroN* (i.e., gene that encodes outer membrane receptor of iron salmochelin). Both of these antigenic genes, *fimA* and *iroN*, have a high potential to influence the host adaptation of NTS. The adhesion FimA is important for attachment to enterocytes and promotes intestinal colonization of the host (Althouse et al., 2003). The IroN, a unique receptor of *S. enterica*, promotes a growth advantage to NTS over other gut micro biota, as this receptor uptakes metabolites excreted from other bacteria (Baumler et al., 1998). Our findings reveal interrelationships between host adaptation, overall NTS genetic relatedness, and NTS virulence.

MATERIALS AND METHODS

Collection of Non-typhoidal *Salmonella* (NTS) Isolates

The University of Minnesota Veterinary Diagnostic Laboratory (VDL), which is fully accredited by the American Association of Veterinary Laboratory Diagnosticians, serves as the veterinary microbiological reference center for the state of Minnesota. The Minnesota Poultry Testing Laboratory (MPTL), located in Willmar, MN serves as the authorized laboratory for the National Poultry Improvement Plan in Minnesota. During 2015, the VDL identified NTS isolates in 2049 clinical samples of avian (n =

1,406), porcine (n = 516), and bovine (n= 127) origin. For the present study, we selected NTS serovars most commonly associated with each of these three animal hosts according to VDL records (**Table 1**). In total, the collection had 240 NTS isolates, including 80 clinical isolates of bovine origin [i.e., *S. enterica* serovar Dublin (n = 50); *S. enterica* serovar Cerro (n = 16) and *S. enterica* serovar Montevideo (n = 14)], 80 clinical isolates of porcine origin [i.e., *S. enterica* serovar Typhimurium var 5- (n = 40) and *S. enterica* serovar 4,5,12:i:– (n = 40)] and 80 isolates associated with the poultry barns [i.e., *S. enterica* serovar Heidelberg (n = 80)]. Isolates of the bovine and porcine origins were received from the VDL, and isolates of the avian origin were received from the MPTL. Primary identification was undertaken at the National Reference laboratory, Ames, Iowa, using standard microbiological and serological methods. Isolates were stored at −80°C in Luria-Bertani (LB) broth (Difco) containing 10% glycerol. For each experiment in this study, fresh cultures derived from the frozen stocks were used.

Extraction of DNA

Non-typhoidal *Salmonella* (NTS) isolates were plated from frozen stocks on LB agar plates (Difco), followed by an overnight incubation at 37°C. Growth from an overnight culture was collected by a sterile loop and resuspended in 1 mL of 0.9% saline. After centrifugation at 10,000 X g for 1 min. the supernatant was removed and genomic DNA was extracted using the Qiagen DNeasy tissue kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions.

TABLE 1 | The most common serovars of NTS, isolated from the clinical samples of avian, bovine and porcine origins during 2015 at the Veterinary Diagnostic Laboratory, University of Minnesota.

Name of serovars	Host origin of NTS isolates			Total (n)
	Avian (n)	Bovine (n)	Porcine (n)	
Salmonella Heidelberg	389	6	22	417
Salmonella Typhimurium var 5-		2	77	79
Salmonella 4,5,12:i:-	78	5	75	158
Salmonella Dublin		51		51
Salmonella Cerro		17	3	20
Salmonella Montevideo	1	16	2	19
Salmonella Kentucky	197			197
Salmonella Uganda	181		4	185
Salmonella Reading	133			133
Salmonella Hadar	133			133
Salmonella Senftenberg	42		13	55
Salmonella Enteritidis	55			55
Salmonella Agona	1	3	47	51
Salmonella Mueenchen	46		4	50
Salmonella 4, 12:i:-		1	49	50
Salmonella St. Paul	45		1	46
Salmonella Derby			42	42
Salmonella Anatum	31		9	40
Salmonella Typhimurium	11	1	20	32
Salmonella Schwarzengrund	18	6	5	29

Virulence Gene Profiling

The NTS isolates were screened by PCR for the presence of eight virulence-associated genes, including three genes involved in NTS colonization/biofilm formation: fimbrial usher (*bcfC*), plasmid-encoded major fimbrial subunit (*pefA*), and non-fimbrial adhesion (*siiE*) as well as five genes involved in invasion of the host, invasion protein InvE (*invE*), secretion system apparatus outer membrane protein SsaC (*ssaC*), magnesium transport protein MgtC (*mgtC*), enterotoxin (*stn*), and inositol phosphate phosphatase SopB (*sopB*). Three multiplex PCR reactions were used to detect: (1) *mgtC*/*bcfC*; (2) *pefA*/*siiE*; and (3) *stn*/*sopB*. Identification of remaining two virulence-associated genes, *ssaC* and *invE*, was performed individually by employing conventional PCR methods. All primer sequences used for the virulence-profiling assay were designed in this study. *S. enterica* serovar Enteritidis ATCC 4931 was used as a positive control and *Escherichia coli* O157 strain B-1 (Vidovic and Korber, 2006) was used as a negative control. PCR amplification was carried out in 50 μL using a T100™ thermal cycler (Bio-Rad, Hercules, CA). Primers for the PCR assays used in this study are presented in **Table 2**.

Pulsed-Field Gel Electrophoresis (PFGE)

All NTS isolates were characterized by the PFGE typing method, as previously described by the Centers for Disease Control and Prevention (CDC) PulseNet program (Ribot et al., 2006; Centers for Disease Control and Prevention, 2013; An et al., 2017). Briefly, genomic DNA was digested with 50 U of restriction enzyme XbaI (Roche Diagnostics, Indianapolis, IN, USA) for 2 h at 37°C. Electrophoresis was carried out using a 1% agarose gel in 0.5 X Tris-borate-EDTA buffer at 14°C in a CHEF Mapper XA System (BioRad Laboratories, Inc., Hercules, CA, USA) with the following conditions: 6 V/cm for 19 h with an initial switch time of 2.16 s and final switch time of 63.8 s. The analysis of the PFGE patterns was performed using BioNumerics software version 5 (Applied Maths, St.-Martens-Latern, Belgium). Similarities between PFGE patterns were determined based on the Dice similarity coefficient. The resulting similarities in the matrix were further processed by employing the unweighted-pair group method using average linkages to create a dendrogram that depicted the genetic relatedness between NTS isolates.

Biofilm Formation Assay

Abilities of the NTS isolates to produce biofilms were assessed as previously described (O'Toole and Kolter, 1998). Briefly, overnight cultures of tested isolates were diluted 1:100 into LB medium, then dispensed into wells of 96-well polyvinyl chloride microtiter plates (Costar 2797, Corning, NY), followed by incubation at 37°C for 24 h. The cultures in 96-well plates were stained with 0.1% crystal violet for 10 min, followed by solubilization with 125 μl of 30% glacial acetic acid for 10 min. After this, the cultures were transferred to flat-bottom polystyrene microtiter plates (Greiner bio-one, Germany) and quantified by measuring absorbance at 550 nm (A550) in an Epoch Microplate Spectrometer (Biotek, Winooski, VT). A negative control well (e.g., containing growth medium only) was included in each PVC microtiter plate, and the absorbance

TABLE 2 | Primers used for detection of eight virulence-associated genes in the population of NTS isolates.

Gene name	Protein function	Primer pair	Sequence of primers	Amplicon size (bp)
bcfC	Fimbrial usher	*bcfC*-F	CCAGTACGCTGGCGGATAAT	177
		bcfC-R	TGTCATCGTCATAGCCGCTC	
mgtC	Magnesium transport protein MgtC	*mgtC*-F	ATTGGCGCGGAAAGACAATG	458
		mgtC-R	ATCGCGGCCTCTTTTACGAT	
ssaC	Secretion system apparatus outer membrane protein SsaC	*ssaC*-F	ACCTGGTTTGATGGCAGCAT	680
		ssaC-R	CCACTAGCACCACCGTCATT	
invE	Invasion protein InvE	*invE*-F	TCCAGTCGACGGACGAAATG	948
		invE-R	TAGTACGACGCTGTTCTGCC	
pefA	Plasmid-encoded major fimbrial subunit	*pefA*-F	CAGGGTTGTGCAAATCTGGC	165
		pefA-R	GCTGGCGTTAGCGTTTACAG	
stn	Enterotoxin	*stn*-F	CCGCGCCTTTACCCTCAATA	361
		stn-R	CAGGATGCCCAAAGCAGAGA	
sopB	Inositol phosphate phosphatase SopB	*sopB*-F	TTGTGGATGTCCACGGTGAG	644
		sopB-R	TTATAGGGTTCGCCGCCATC	
siiE	Non-fimbrial adhesion	*siiE*-F	AGAATCGCCTCGCTTACTCG	910
		siiE-R	ACGCACATCTTCCCAACGAT	

value of this well was subtracted from the values of all test wells (Kadurugamuwa et al., 2003). Also, a positive control, using *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 4931, was included in each 96-well plate.

fimA and *iroN* Full-Length Genes Sequencing and Non-synonymous Single Nucleotide Polymorphism (nsSNP) Analysis

Primers for the *fimA* and *iroN* genes, *fimA* (forward 5′–CAG GAT GCA GAG ATA ACT TTT CTG and reverse 5′–CTA GCG CCG CGC CTT TCC TTA TCA) and *iroN* (forward 5′–TGC CTT TTC CTT AAT TGA ATG ATA and reverse 5′–GCA GTG CAT TGC TGG ATA TCA GTC), were designed to flank ∼ 120 bp up- and down-stream of the targeted genes, respectively. The amplicons were generated by Platinum *Taq* DNA polymerase (Thermo Fisher Scientific) and were prepared for DNA sequencing by the Prism BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences on both strands were determined using an ABI 3730x1 DNA analyzer (Genomics Center, University of Minnesota, Minneapolis, MN). Each strand was checked, then aligned with its complementary strand. A consensus DNA sequence was obtained using Clustal Omega (Larkin et al., 2007). The regions of homologous recombinations within the *fimA* and *iroN* genes were identified using a non-parametric recombination detection method, SiScan (Gibbs et al., 2000), as described earlier (Vidovic et al., 2011a). The annotated DNA sequences were exported into Molecular Evolutionary Genetics Analysis (MEGA) version 7 (Tamura et al., 2007) for the identification of nsSNPs. Phylogeny, based on nsSNPs, was inferred using the minimum evolution method (Rzhetsky and Nei, 1992). The neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial phylogenetic tree.

Nucleotide sequence translation was carried out using EMBOSS Transeq (Kearse et al., 2012) (the European Molecular Biology Laboratory—European Bioinformatics Institute; Hinxton, Cambridge, United Kingdom). Protein structure was predicted using Protter 2D prediction software (Omasits et al., 2014). Raptor X software was used to predict potential binding sites from an amino acid sequence input based on prevalent predetermined binding motifs that correlate to a bank of small molecules (Källberg et al., 2012).

Statistical Analysis

To determine statistically significant differences in the proportion of genes and important nsSNPs between avian, bovine, and porcine NTS isolates, we used the Agresti-Coull method together with Fisher's exact test (Agresti, 2002). To test for an association between host origin and the ability of NTS isolates to form biofilm, we used a chi-squared test, both overall and pairwise between hosts groups.

Nucleotide Sequence Accession Numbers

Nucleotide sequences were deposited in GenBank. Accession numbers for the DNA sequences of the *fimA* and *iroN* genes ranged from KY367267 to KY367280.

RESULTS

Virulence Gene Profiles of the NTS Isolates

The occurrence of the eight virulence-associated genes, *pefA*, *siiE*, *stn*, *sopB*, *bcfC*, *mgtC*, *ssaC,* and *invE*, among 240 NTS isolates is shown in **Table 3**, **Figure 1**. The *stn* gene, which encodes enterotoxin, had the highest frequency of occurrence in all three groups of NTS isolates. All S. Dublin, S. Montevideo, S. Cerro, and S. 4,5,12,:i:- isolates, almost all S. Typhimurium var 5- isolates and a majority of *S*. Heidelberg isolates were positive for the *siiE* gene that encodes giant non-fimbrial adhesion protein. Another

TABLE 3 | Distribution of virulence-associated genes within the collection of NTS isolates of avian, bovine and porcine origins.

Virulence genes	No. (%) of isolates positive for virulence-associated genes				
	Total ($n = 240$)	Avian ($n = 80$)	Bovine ($n = 80$)	Porcine ($n = 80$)	*P*-values
pefA	76 (31.6)	31 (38.7)	0	45 (56.2)	0.00
siiE	230 (95.8)	71 (88.7)	80 (100)	79 (98.7)	0.00
stn	234 (97.5)	74 (92.5)	80 (100)	80 (100)	0.01
sopB	214 (89.1)	64 (80)	71 (88.7)	79 (98.7)	0.00
bcfC	194 (80.8)	56 (70%)	72 (90)	73 (91.2)	0.00
mgtC	200 (83.3)	54 (67.5)	69 (86.2)	70 (87.5)	0.00
ssaC	206 (85.8)	71 (88.7)	65 (81.2)	70 (87.5)	0.19
invE	208 (86.7)	64 (80)	74 (92.5)	70 (87.5)	0.07

highly prevalent (>80%) virulence factor was the effector gene *sopB*, required for host invasion. High frequencies of occurrence (>80%) were also observed for the *ssaC* gene (i.e., secretion system apparatus outer membrane protein C) and the *invE* gene (i.e., invasion protein). The *mgtC* gene (i.e., magnesium transporter) was found in most bovine (95%), porcine (87.7%), and avian isolates (67.5%); this difference was significant ($P = 0.00$). The virulence-associated gene, fimbrial usher *bcfC*, had a high frequency of occurrence in *S.* Montevideo (100%), *S.* Dublin (92%), *S.* Typhimurium var 5- (90%), *S.* 4,5,12,:i:- (87.5%), but relatively lower prevalence in *S.* Cerro (75%) and *S.* Heidelberg (70%). Also, this difference was statistically significant ($P = 0.00$). Occurrence of the *pefA* gene (e.g., plasmid-encoded major fimbrial subunit) showed the most significant difference ($P < 0.00$) comparing *S.* Typhimurium var 5- (64.1%), *S.* 4,5,12,:i:- (48.8%), and *S.* Heidelberg (38.7%) to the bovine isolates *S.* Dublin. *S.* Montevideo and *S.* Cerro in which the gene was absent.

Ability of the NTS Isolates to Form Biofilms

The results of this assay are shown in **Figure 2** collectively, for each host-associated group (e.g., avian, bovine, and porcine) and each individual NTS strain in **Figure 1**. Populations of NTS isolates from the three hosts contained a similar percentage (7.5–8.75%) of the high biofilm-producing isolates. Among the group of moderate biofilm-producing isolates, *S.* Heidelberg were highest (41.2%), followed by S. Cerro (31.2%), *S.* Typhimurium var 5- (30%), S. 4,5,12,:i:- (30.0%), *S.* Montevideo (28.5%), and *S.* Dublin (0%) ($P < 0.05$). The low biofilm producers were similarly distributed among NTS of porcine (58.7%), avian (47.5%), and bovine (38.7%) origin. NTS isolates that did not produce biofilms represented a smallest fraction among *S.* Typhimurium var 5- (7.5%), *S.* Montevideo (7.1%), *S.* Heidelberg (2.5%), *S.* Cerro (0%), and *S.* 4,5,12,:i:- (0%), but were most frequent among *S.* Dublin (66%) ($P < 0.005$; **Figure 1**).

Overall Genetic Relatedness of the NTS Isolates

The 240 NTS isolates were resolved by PFGE into 137 pulsotypes, which were further grouped into 10 clusters and 1 outlier (**Figure 1**). The NTS isolates of bovine origin were resolved into 41 pulsotypes, grouped into three clusters and placed at the apical part of the dendrogram. Each of three clusters was exclusively comprised of isolates that belonged to a single serovar (i.e., except the top cluster which possesses 14 isolates of *S.* Montevideo and one isolate of *S.* Dublin). The top cluster was comprised of Montevideo isolates with 75% genetic similarity, the second top cluster contained Dublin isolates with 76% genetic similarity and the third, most homogeneous cluster was comprised of Cerro isolates with 83% genetic similarity. The NTS isolates of porcine origin were split into three monophyletic clusters, located at the central part of the dendrogram, and another cluster positioned at the basal part of the dendrogram. The three monophyletic clusters were comprised of Typhimurium var 5- isolates with 68% genetic similarity. Besides these three monophyletic clusters, one genetically distant isolate of S. Thyphimurium var 5- formed an outlier and shared 58% of genetic similarity with the rest of Salmonella isolates. The cluster located at the basal part of the dendrogram contained isolates of 4,5,12:i:- serovar with 78% genetic similarity. *Salmonella* Heidelberg isolates were resolved into two large, genetically related clusters and third, much smaller cluster. Two genetically related clusters were placed at the central part of the dendrogram, while the smaller cluster was positioned at the bottom of the dendrogram. Heidelberg shared 62% genetic similarity among themselves, which made this group of *Salmonella* the most heterogeneous among the entire collection of NTS isolates although comprised of one serotype. Interestingly, *S.* Heidelberg contained multiple pulsotypes that grouped different isolates within the same pulsotype, indicating that *S.* Heidelberg may have several clonal groups (**Figure 1**). Some of these clonal groups are genetically related (e.g., pulsotypes within two large clusters), whereas others are genetically distant (e.g., pulsotypes between the small and two large clusters) (**Figure 1**).

Identification of nsSNPs and Determination of Their Impact on the Fima Adhesin and the Iron Receptor

To avoid any effect of homoplasy on the SNP analysis, all DNA sequences were tested for regions of homologous recombination. A total of six alleles, among 240 NTS isolates, were identified for the *fimA* gene. The overall average pairwise distance for the *fimA* gene, an estimate of evolutionary divergence between sequences was 0.0082 among *S.* Dublin, *S.* Montevideo, and *S.* Cerro. Evolutionary divergence in the same gene among *S.* Heidelberg, *S.* Typhimurium var 5- and *S.* 4,5,12,:I:- did not exist. No SNP was found among the populations of *S.* Heidelberg, *S.* Typhimurium var 5- and *S.* 4,5,12,:I: isolates. Fourteen SNPs were identified among *S.* Dublin, *S.* Montevideo and *S.* Cerro isolates, resulting in a SNP density of 40 (one SNP per 40 bp). Out of these 14 SNPs, six were nsSNPs, further generating four haplotypes of protein FimA among the bovine isolates. These four haplotypes were compared with *S.* Heidelberg, *S.* Typhimurium var 5- and *S.* 4,5,12,:i:- haplotypes, and seven amino acid substitutions were identified at residues 24, 71, 72, 93, 148, 167, and 169 of the FimA protein (**Figure 3A**). An amino acid substitution at the 93 position of

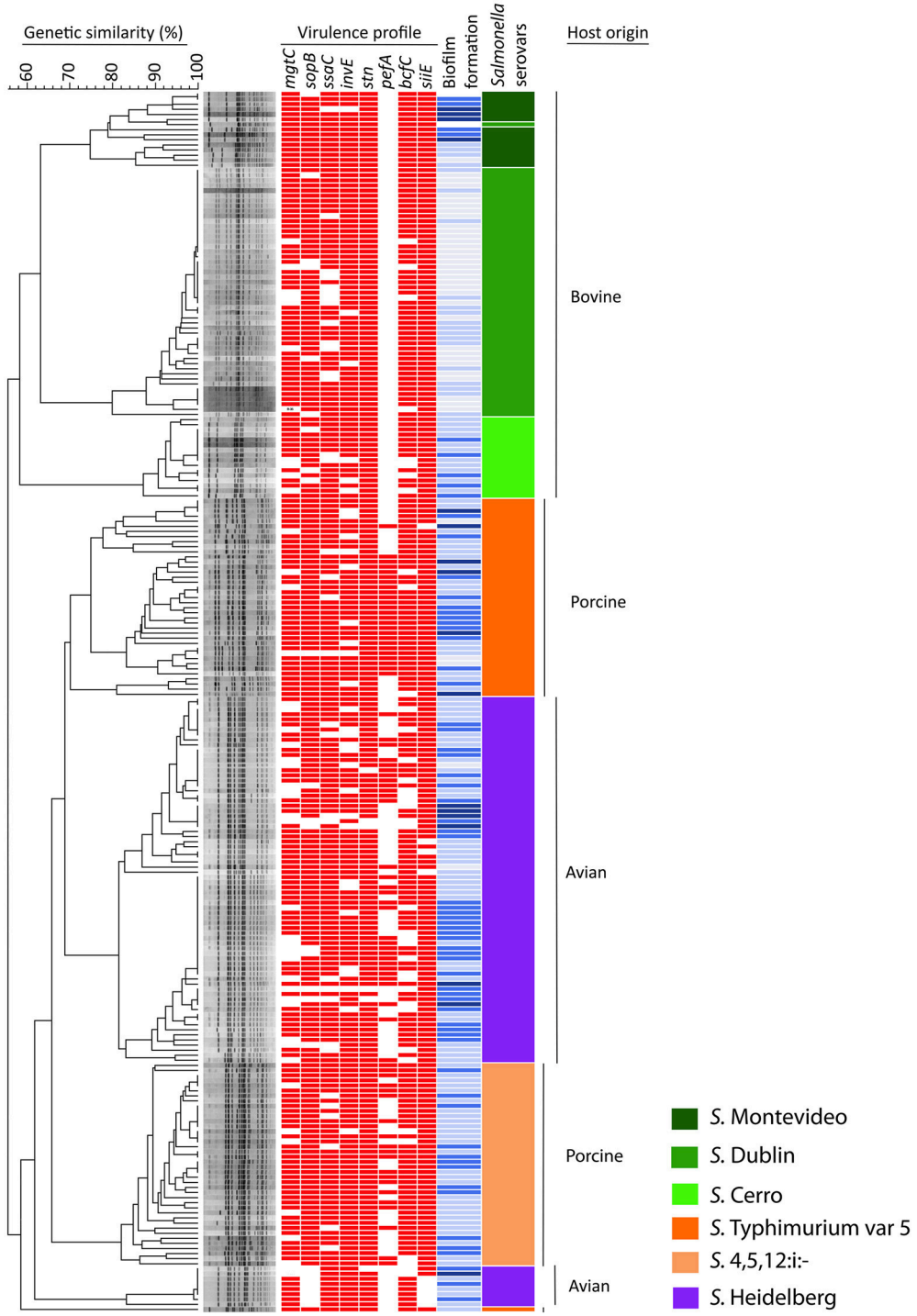

FIGURE 1 | Dendrogram, virulence profiles and biofilm production in 240 NTS isolates of avian, bovine and porcine origin. The dendrogram is based on PFGE analysis of 80 clinical isolates of bovine origin (serovars Montevideo, Dublin, and Cerro), 80 clinical isolates of porcine origin (serovars Typhimurium var 5–and 4,5,12:i:–) and 80 avian-associated isolates (serovar Heidelberg). The virulence profile of each NTS strain is portrayed by a color-coded pattern (red indicates presence and no color indicates absence of the virulence factor). The ability of each NTS strain to form biofilm is presented by different shades of blue color (dark blue = high biofilm producers, blue = moderate biofilm producers, topaz sky blue = low biofilm producers, and light blue = no biofilm produced). The virulence profile and ability to produce biofilm in each NTS strain was aligned with its position in the dendrogram. The serovar identity of each isolate is presented by one of six different colors. Vertical bars on the far right indicate the different host origins represented in this NTS collection.

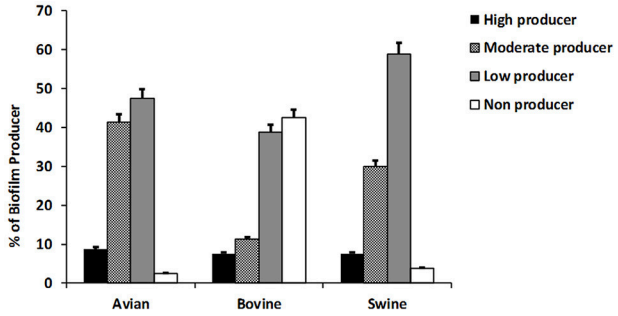

FIGURE 2 | The ability of NTS isolates of different host origins to form biofilms. The biofilm formation of all NTS isolates tested in this study was compared to that of *Salmonella enterica* subsp. Enterica serovar Enteritidis ATCC® 4931 (OD = 0.090333). The values of NTS isolates that correlated to the absorbance of ≤0.068 were considered as weak producers, 0.069–0.136 as moderate producers and ≥0.137 as strong producers. The results are representative of three independent experiments carried out in triplicate.

the FimA protein was unique to NTS isolates of porcine origin. Another six amino acid substitutions were shared between NTS isolates from all six *Salmonella* serovars (**Figure 3A**).

Protein-based phylogeny of the FimA adhesin showed that each *S.* Heidelberg and *S.* Typhimurium var 5- as well as *S.* 4,5,12,:i:- together was grouped into a single distinct cluster, whereas the bovine isolates were grouped into three major clusters and a single outlier (**Figure 3B**). The FimA adhesin of the great majority ($n = 67$, 84%) of bovine isolates was clustered into a clade, genetically most diverged from other NTS isolates. Most of the *S.* Cerro isolates were clustered together with *S.* Heidelberg isolates into a clade (**Figure 3B**). In contrast to the bovine isolates, NTS isolates of avian and porcine origins showed profound homogeneity of FimA adhesin (**Figure 3B**).

The full-length sequencing of the *iroN* gene (e.g., 2,175 nt) revealed the existence of eight alleles among the population of 240 NTS isolates. Each population of bovine and porcine isolates was composed of three unique alleles, while the population of avian isolates contained two alleles of the *iroN* gene. The overall average pairwise distance of the *iroN* gene was 0.00817, 0.00046, and 0.00001 for the bovine, porcine and avian isolates, respectively, indicating that any two bovine isolates would diverge, on average, ∼0.82%, porcine 0.04% and avian 0.001%. Among the isolates of bovine origin, 47 SNPs were identified within the *iroN* gene, while avian and porcine *iroN* sequences contained one and three SNPs, respectively. *Salmonella* Dublin, *S.* Montevideo, and *S.* Cerro isolates had the highest SNP density of 46, followed by *S.* Typhimurium var 5- and *S.* 4,5,12,:I:- isolates of 725; *S.* Heidelberg isolates had the lowest SNP density of 2,175. Out of 47 SNPs identified in *iroN* among *S.* Dublin, *S.* Montevideo and *S.* Cerro isolates, six were nsSNPs. All three *iroN* SNPs among *S.* Typhimurium var 5- and *S.* 4,5,12,:i:- isolates were nsSNPs, and no nsSNPs were detected among *S.* Heidelberg isolates. In total, a single IroN haplotype was found among *S.* Heidelberg isolates and three IroN haplotypes each were found among *S.* Dublin, *S.* Montevideo, *S.* Cerro, and *S.* Typhimurium var 5- as well as *S.* 4,5,12,:i:- isolates. When these seven haplotypes were compared, 13 amino acid substitutions were identified at residues 2, 4, 7, 85, 152, 289, 357, 385, 407, 507, 555, 585, and 604 of the IroN protein (**Figure 3A**). Amino acid substitutions at positions 4 and 7 were unique in *S.* Dublin, *S.* Montevideo, *S.* Cerro isolates and substitutions at positions 407 and 585 were unique in *S.* Heidelberg isolates (**Figure 3A**).

Phylogeny of the IroN receptor showed the existence of three clades, each representing a group of NTS isolates of distinct host origin (**Figure 3C**), indicating a greater interdependence between the phylogeny and the host origin of NTS isolates compared to that of FimA adhesin.

A summary of characteristics for each haplotype of FimA and IroN, including: solvent accessibility, type of ligand, predicted ligand-protein binding sites and *P* value of predicted model, is presented in **Table 4**. There is a major division among the haplotypes of FimA adhesin based on their predicted binding sites. FimA of *S.* Heidelberg isolates ($P = 6.27e\text{-}07$) and *S.* Cerro isolates ($P = 1.10e\text{-}07$) preferably bind to N-acetyl-D-glucosamine, whereas FimA adhesin of *S.* Typhimurium var 5 − *and S.* 4,5,12,:i:- isolates ($P = 4.24e\text{-}07$) and *S.* Dublin ($P = 5.19e\text{-}07$) and *S.* Montevideo ($P = 1.22e\text{-}07$) bind preferentially to a different ligand, chloramphenicol. Among the first group, the solvent accessibility analysis showed that the FimA protein of *S.* Heidelberg isolates was buried by 23% compared to 31% in the haplotype of *S.* Cerro, indicating better solvent accessibility for the FimA of *S.* Heidelberg isolates. Among the second group, the FimA of *S.* Montevideo haplotype was most buried (34%), compared to other two FimA haplotypes, Typhimurium var 5-, 4,5,12,:i:- and Dublin (22 and 23%, respectively). All haplotypes of the IroN receptor had profound affinity to Fe^{3+} ion (**Table 4**). The solvent accessibility analysis showed that only the IroN of *S.* Dublin haplotype had lower solvent accessibility, 35% of protein being buried, compared to 24 and 23% for other IroN haplotypes (**Table 4**).

DISCUSSION

In this host-NTS population based study, we examined the role of nsSNPs in the process of adaptation of NTS to their hosts and revealed genetic as well as virulence signatures of NTS groups based on their serovar identity. Using a well-defined population of 240 NTS isolates in combination with full gene sequencing of two important antigens, adhesin *fimA* and salmochelin receptor *iroN*, we discovered a key nsSNP in the *fimA* that profoundly shifts in ligand preference of the adhesin FimA. Multiple alignments of all FimA haplotypes clearly showed that amino acid substitution at position 169, threonine (T) to alanine (A), produced a significant shift in ligand affinity of adhesin FimA from chloramphenicol to N-acetyl-D-glucosamine, a glucose derivative involved in cell wall biogenesis of many prokaryotic and eukaryotic organisms. This nsSNP occurred exclusively in the FimA haplotype of *S.* Heidelberg and *S.* Cerro, indicating a further phylogenetic segregation of these two FimA haplotypes from other adhesin haplotypes, forming a single clade. It is noteworthy to mention that although Heidelberg and Cerro FimA haplotypes showed phylogenetic

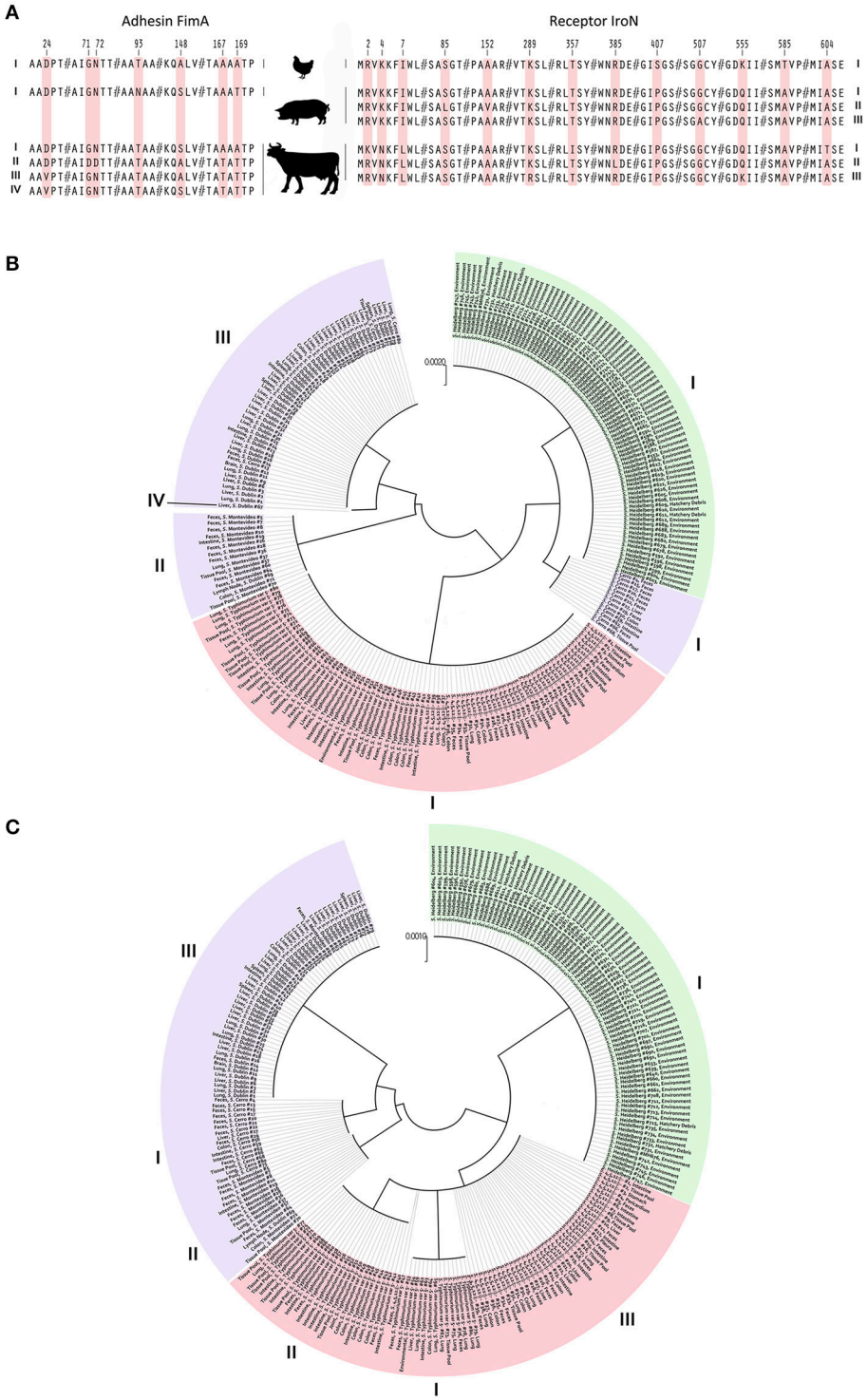

FIGURE 3 | Identification of nsSNPs and their influence on adhesin FimA and receptor IroN of NTS isolates from avian, bovine and porcine hosts. **(A)** Image portrays amino acid substitutions in different FimA and IroN haplotypes of NTS isolates obtained from avian, porcine and bovine hosts. Each amino acid substitution is coded by light red color and numbers above indicate the positions of point mutations within the FimA and IroN proteins. On the far left and far right are Roman numerals indicating FimA and IroN haplotype numbers, respectively. **(B)** Phylogeny of the FimA adhesin. Each of 240 NTS isolates is marked by its identification, name of serovar, and site of isolation. NTS of avian origin are colored green, porcine light red, and bovine light purple. Roman numerals positioned around the phylogenetic tree indicate the identity of different haplotypes. **(C)** Phylogeny of the IroN receptor. An identical approach was used to mark different NTS groups and their IroN haplotypes as was done with FimA.

TABLE 4 | Characteristics of avian, bovine and porcine FimA and IroN haplotypes.

Haplotype name	Solvent accessibility (%)			Ligand	Predicted binding sites	*P*-values
	Exposed	Medium	Buried			
FimA_Avian	29	47	23	N-acetyl-D-glucosamine	A145, Q147, A148, L149, V150, T153, N154, T155, L156	6.27e-07
FimA_Porcine	29	48	22	Chloram-phenicol	A75, Q76, V77, P78, R157, T159, A160, R161	4.24e-07
FimA_Bovine-1	27	41	31	N-acetyl-D-glucosamine	A145, Q147, A148, L149, V150, T153, N154, T155, L156	1.10e-07
FimA_Bovine-2	29	47	23	Chloram-phenicol	A75, Q76, V77, P78, T159, A160, R161	5.19e-07
FimA_Bovine-3	27	38	34	Chloram-phenicol	A75, Q76, V77, P78, R157, T159, A160, R161	1.22e-07
IroN_Avian	40	34	24	Fe^{3+}	N82, T87, R88, V119, R120, Y121, S122, W123, R124, G125, E126, R127, D128, R332, E335	1.29e-32
IroN_Porcine-1	39	35	24	Fe^{3+}	N82, T87, R88, V119, R120, Y121, S122, W123, R124, G125, E126, R127, D128, R332, E335	1.08e-32
IroN_Porcine-2	40	35	24	Fe^{3+}	N82, T87, R88, V119, R120, Y121, S122, W123, R124, E126, R127, D128, Q280, R332, E335	4.28e-33
IroN_Porcine-3	40	35	24	Fe^{3+}	N82, T87, R88, E102, V119, R120, Y121, S122, W123, R124, E126, R127, D128, Q280, E335	1.51e-32
IroN_Bovine-1	39	35	24	Fe^{3+}	N82, T87, R88, E102, V119, R120, Y121, S122, W123, R124, E126, R127, D128, R332, E335	1.05e-32
IroN_Bovine-2	40	35	23	Fe^{3+}	N82, T87, R88, E102, V119, R120, Y121, S122, W123, R124, E126, R127, D128, T129, E335	1.05e-32
IroN_Bovine-3	40	23	35	Fe^{3+}	N82 T87 R88 V119 R120 Y121 S122 W123 R124 E126 R127 D128 T129 Q280 E335	3.36e-33

similarity and identical ligand preference, these two haplotypes differ in solvent accessibility. The Heidelberg FimA haplotype is less buried and more exposed to solvents than Cerro FimA haplotype, which may indicate the host specificity of these two FimA haplotypes. To gain an insight into the influence of the three different hosts (i.e., immune selective pressure) on these two antigen genes, we performed the PFGE analysis and compared it to the phylogeny of the FimA and IroN proteins. The PFGE analysis provides an overall genetic relatedness of bacterial isolates based on their entire genomes, which is mostly not affected by the host but rather by a general genome make up of a bacterial isolate. In contrast to the PFGE analysis, the phylogeny of the FimA adhesion and the IroN receptor, provides a direct insight into the influence of the host's immune selective pressure and microbiome on these two outer membrane proteins. By comparing the phylogeny of the FimA adhesin with the PFGE dendrogram, we observed a profound homogeneity of the FimA adhesin of *S.* Heidelberg, *S.* Typhimurium var 5- and *S.* 4,5,12,:i:-. The PFGE analysis clustered *S.* Typhimurium var 5- and *S.* 4,5,12,:i:- isolates into two large clusters, separated by a large cluster of *S.* Heidelberg, whereas the FimA adhesin of the same NTS isolates was clustered as a single FimA allele, indicating a strong influence of the host on this adhesin. Similarly, the population of *S.* Heidelberg isolates was resolved into two clusters by the PFGE analysis, with genetic similarity of 62%, indicating an existence of the genetically very heterogeneous population. In contrast to the PFGE analysis, the phylogeny of the FimA protein of *S.* Heidelberg resulted in a distinct homogenous allele, again indicating a substantial influence of the particular host's immune pressure on this outer membrane protein. Interestingly, a single FimA allele of *S.* Cerro showed a closer relation to the FimA allele of *S.* Heidelberg, which may suggest that this small group of bovine isolates may equally affect the avian host. Another possible explanation is that this group of bovine isolates has been exposed to the bovine host for a short period.

It has been shown that nsSNPs in epitopes of certain antigens can lead to profound changes in biological fitness of different microbial pathogens, resulting in the emergence of highly virulent isolates (Kim et al., 2003; Choi et al., 2006; Fujimoto et al., 2008; Vidovic et al., 2011b) or determination of the pathogen's host tropism (Yue et al., 2015). For instance, Choi et al. (2006), examining genetic heterogeneity in the hexon gene of human adenovirus type 3 (Ad3) over a 9-year period in Korea, discovered novel, emerging Ad3a16 and Ad3a18 genotypes during an Ad3 outbreak of childhood pneumonia in Korea. Newly emerged Ad3a16 and Ad3a18 genotypes had three amino acid substitutions in loop 2 of the hexon gene. These three nsSNPs were associated with greater hydrophobicity of this protein, resulting in the replacement of the previously predominant Ad3 genotypes by newly emerged Ad3a16 and Ad3a18, apparently due greater fitness. Most recently, Yue and colleagues (Yue et al., 2015) found that a single amino acid substitution in the binding pocket of FimH, the type 1 fimbrial adhesin, can dramatically change ligand affinity of this adhesin and result in determination of the NTS host tropism toward mammalian or avian host receptors. It has been shown that the presence of isoleucine instead of threonine at position 78 of the FimH in Gallinarum and Pullorum serovars affects mannose-inhibitable binding (Kisiela et al., 2005), and may determine the avian host specificity of these two NTS serovars (Guo et al., 2009; Kisiela et al., 2012; Kuzminska-Bajor et al., 2012).

We identified numerous nsSNPs in the *iroN* gene that had no influence on Fe^{3+} affinity or solvent accessibility among the IroN haplotypes of NTS groups from avian, bovine and porcine hosts. All IroN haplotypes showed an extremely high affinity for Fe^{3+} and great solvent accessibility, which can be explained by the functional constraint of this receptor. In other words, the primary role of IroN is Fe^{3+} acquisition, regardless of NTS host origin. It is important to mention that despite a relatively high heterogeneity of the IroN receptor, all IroN haplotypes showed a strong correlation between their phylogeny and the host origin of NTS isolates. Each IroN haplotype was clustered together with other haplotypes from the same host-origin group (**Figure 3C**). Comparing the IroN phylogeny to that of the FimA, it is evident that the host influence is even greater on the IroN receptor of NTS isolates, as numerous IroN haplotypes were clustered exactly according to their host origin. This strong connection between phylogeny of IroN and the host origin of NTS isolates can be explained by the location and function of this protein. IroN, an outer membrane siderophore receptor unique for *S. enterica* subspecies I, II, IIIa, IIIb, IV, and VI (Baumler et al., 1998), represent an ideal target for the host immune system (Fernandez-Beros et al., 1989; Heithoff et al., 1997). Besides its primary role in Fe^{3+} acquisition, IroN is responsible for acquiring metabolites secreted by other bacteria, such as myxochelin A (Corbin and Bulen, 1969), 2-N,6-N-bis(2,3-dihydroxybenzoyl)-L-lysine and 2-N,6-N-bias(2,3-dihydroxybenzoyl)-L-lysine amide (Kunze et al., 1989), providing a growth advantage specifically to *S. enterica* subspecies I, II, IIIa, IIIb, IV, and VI in the gut microbiota. A stronger influence of the host on the IroN receptor compared to the FimA adhesin can be explained by the fact that the IroN receptor is under selective pressure of both, the host immune system and the host's gut microbiota, whereas FimA is under pressure of the host's immune system only. Taken together, nsSNPs identified among the IroN haplotypes of different NTS host-origin groups may represent an evolutionary response to both the immune selective pressure of the hosts and their unique microbiotas, as IroN is involved in acquisition of secondary metabolites produced by the host microbiota, which can explain the association between the IroN phylogeny and the host origin of NTS.

Evaluation of the genetic relatedness among 240 NTS isolates of *S.* Heidelberg, *S.* Dublin, *S.* Montevideo, *S.* Cerro *S.* Typhimurium var 5- and *S.* 4,5,12,:I:- by PFGE analysis revealed that the isolates of *S.* Heidelberg were the most heterogeneous group, composed by multiple closely and distantly related clones. In a sharp contrast to their overall genetic heterogeneity, *S.* Heidelberg isolates showed a remarkable genetic homogeneity of their two antigens, adhesin FimA and receptor IroN, with only one single allele in each protein. The similar agreement between the overall genetic makeup and the genetic diversity of FimA and IroN was observed for *S.* Typhimurium var 5- and S. 4,5,12,:i:- isolates. The data presented here show that the nsSNPs in two outer membrane proteins, FimA and IroN, are based on serovar identity, driven by host specificity which most likely play roles in the process of NTS adaptation to their specific hosts.

Both the Agresti-Coull method and Fisher's exact test strongly suggested that among all of the tested virulence factors, the prevalence of *pefA* was the most segregative characteristic of the tested NTS population. Although the bovine NTS isolates were composed of three serovars, Dublin, Montevideo and Cerro, not a single isolate was found to carry *pefA*, a gene encoding a major fimbrial antigen subunit (Woodward et al., 1996) whereas the same virulence factor was detected in NTS isolates from avian and porcine hosts. This virulence factor most likely has an impact on the ability of NTS isolates to form biofilms, an important phenotype in overall NTS pathogenicity, as the NTS isolates of bovine origin had a reduced ability to form biofilm compared to that of the other two NTS hosts. This significant difference in the presence/absence of the *pefA* virulence gene among the collection of NTS isolates was phenotypically confirmed. NTS isolates that did not produce biofilms were significantly associated with NTS isolates of the bovine origin.

In summary, the present study has determined a key amino acid substitution, A169, in FimA that shifted the affinity of this adhesin toward N-acetyl-D-glucosamine, a glucose derivative in the cell wall matrix of prokaryotic and eukaryotic organisms. This mutation was significantly associated with *S.* Heidelberg and *S.* Cerro, suggesting that A169 may play a role in seovar and host specificity of NTS. The results also indicate that there is a strong positive association between the phylogeny of the IroN receptor and different host origins of NTS isolates, indicating an influence of immune selection pressures imposed by a specific host on this unique receptor of *S. enterica*. Through screening some of the most important virulence factors among a large collection of NTS isolates, we found that *pefA* was the most significant determinant segregating NTS isolates from different hosts. This virulence factor, involved in NTS adherence and biofilm formation, was completely absent from NTS isolates of the bovine origin, which most likely resulted in the significant percentage of the population of bovine isolates being unable to produce biofilm.

AUTHOR CONTRIBUTIONS

SV and CC conceived the study. SA, SH, RA, JM, CF, and RN carried out experiments. DL and KO provided non-typhoidal *Salmonella* (NTS) isolates. SV, SA, RA, SH, and DB analyzed the data. SV and JA facilitated the collaborative project. SV drafted the manuscript. All authors read and approved the final version of the manuscript.

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A Novel pAA Virulence Plasmid Encoding Toxins and Two Distinct Variants of the Fimbriae of Enteroaggregative *Escherichia coli*

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Enteroaggregative *Escherichia coli* (EAEC) is an increasingly recognized pathogen associated with acute and persistent diarrhea worldwide. While EAEC strains are considered highly heterogeneous, aggregative adherence fimbriae (AAFs) are thought to play a pivotal role in pathogenicity by facilitating adherence to the intestinal mucosa. In this study, we optimized an existing multiplex PCR to target all known AAF variants, which are distinguished by differences in their pilin subunits. We applied the assay on a collection of 162 clinical Danish EAEC strains and interestingly found six, by SNP analysis phylogenetically distinct, strains harboring the major pilin subunits from both AAF/III and AAF/V. Whole-genome and plasmid sequencing revealed that in these six strains the *agg3A* and *agg5A* genes were located on a novel pAA plasmid variant. Moreover, the plasmid also encoded several other virulence genes including some not previously found on pAA plasmids. Thus, this plasmid endows the host strains with a remarkably high number of EAEC associated virulence genes hereby likely promoting strain pathogenicity.

Keywords: enteroaggregative *E. coli*, EAEC, diarrhea, virulence genes, fimbriae

INTRODUCTION

Enteroaggregative *Escherichia coli* (EAEC) is a common diarrheal organism and has been associated with acute and persistent diarrhea in a variety of settings (Cravioto et al., 1991; Cobeljic et al., 1996; Huppertz et al., 1997; Harrington et al., 2006; Scavia et al., 2008; Hebbelstrup Jensen et al., 2014). However, EAEC strains express a heterogeneous array of putative virulence factors, therefore, recognition of specific pathogenic factors within this pathotype remains challenging (Nataro et al., 1995a; Boisen et al., 2012; Estrada-Garcia and Navarro-Garcia, 2012; Lima et al., 2013).

Enteroaggregative *E. coli* was first defined by its distinctive "stacked-brick" pattern of aggregative adherence (AA) to HEp-2 cells, mediated by the aggregative adherence fimbriae (AAFs; Nataro et al., 1992). AAFs are believed to play a key role in EAEC pathogenicity by promoting host intestinal colonization via binding to the host intestinal mucosa (Yamamoto et al., 1991; Hicks et al., 1996).

The AAFs have been shown to promote biofilm formation and adhesion to various surfaces and are associated with inflammatory responses in the host (Sheikh et al., 2001; Harrington et al., 2005; Boisen et al., 2008; Farfan et al., 2008; Boll et al., 2012, 2013). Currently, five allelic variants are described (AAF/I-AAF/V), all sharing high level of conservation among the accessory genes whereas the pilin genes display much greater divergence (Savarino et al., 1994; Czeczulin et al., 1997; Bernier et al., 2002; Boisen et al., 2008; Jonsson et al., 2015; Nagy et al., 2016). The AAFs belong to the chaperone-usher family of adhesins, and consist of the pore-forming usher (encoded by *agg3C* in AAF/III), the chaperone (encoded by *agg3D* in AAF/III), the long fimbriae which consist of polymerized major pilin subunits (encoded by *agg3A* in AAF/III) and a minor pilin subunit at the tip (encoded by *agg3B* in AAF/III; Savarino et al., 1994; Berry et al., 2014).

The newest variant, AAF/V, was discovered just recently, and was revealed to be a chimeric variant of AAF/III, in which all accessory genes (*agg3CD*) as well as the minor pilin subunit gene (*agg3B*) are shared except of the major pilin subunit gene that is replaced by *agg5A* (Jonsson et al., 2015).

The expression of AAFs is regulated by an AraC-like transcriptional activator called AggR, which is shown to be an important virulence regulator in EAEC (Nataro et al., 1994; Morin et al., 2010). Most of the virulence genes of EAEC, including AggR, the surface protein dispersin and the AAFs, are located on a 72–120 kb plasmid, termed the pAA plasmid (Nataro et al., 1992; Johnson and Nolan, 2009). In addition to regulating the virulence factors on the plasmid, AggR also promote the expression of the AggR Activated Island (Aai), a type VI secretion system, encoded by a pathogenicity island (designated PAI-1) inserted at *pheU* on the chromosome in EAEC prototype strain 042 (Morin et al., 2013).

Currently, five pAA plasmids have been completely sequenced, each harboring the genes encoding for one of the five respective AAF variants. Although the pAA plasmids share common features including EAEC-associated virulence genes, the genetic composition and the synteny of the plasmids are quite different. Some of the *E. coli* pAA plasmids encode toxins, such as the autotransporter Pet and EAST1 in strain 042, SepA in the German O104:H4 outbreak strain C227-11, and EAST1 in strain 55989 (Eslava et al., 1998; Rasko et al., 2011).

Since AAF mediated adhesion is believed to play an essential role in EAEC pathogenicity, a better understanding of the prevalence of the different AAF variants and their relation to clinical outcome will contribute to the overall understanding of this important pathogen and potentially pave the way for future treatment strategies in the form of anti-adhesion therapy.

In this study, we optimized a previously described AAF multiplex PCR assay (Boisen et al., 2012) to include the recently identified AAF variant AAF/V and investigated the distribution of AAF variants in a collection of 162 clinical EAEC isolates. Interestingly, six isolates were found to harbor two AAF variants and these were revealed to be encoded by a novel pAA variant also encoding several other virulence factors, suggesting a key role of this plasmid in EAEC pathogenicity.

MATERIALS AND METHODS

Bacterial Strains

Escherichia coli strains were isolated from stool specimens of adults suffering from diarrhea, during a multicenter study with participants: the Departments of Clinical Microbiology at Hvidovre Hospital (HH) and Slagelse Hospital (SH), and the Department of Microbiology and Infection Control at Statens Serum Institut (SSI). EAEC strains were identified from the genes *aatA, aggR, aaiC* and *aap* targeted by multiplex PCR as previously described (Boisen et al., 2012). Patients with EAEC positive stool samples, without any co-infections were eligible for inclusion in this study. The bacterial strains used in this study are described in **Table 1**. Stock cultures were frozen at −80°C in Luria-Broth (LB) with 10% glycerol. All strains were grown at 37°C.

PCR

DNA templates for the multiplex PCR were prepared by boiling a suspension of five isolated colonies in 200 μL mQ water. The AAF multiplex was optimized from a previous study conducted by Boisen et al. (2012). The primers for the multiplex are listed in **Table 2**. The PCR was performed with the multiplex PCR kit according to the manufacturer's instructions (Qiagen inc., Valencia, CA, USA). The multiplex PCR cycles comprised (a) 15 min denaturation at 95°C, (b) 30 s denaturation at 94°C, (c) annealing for 1.5 min, and (d) extension 1.5 min at 72°C with 35 cycles from step (b). The final extension was 10 min at 72°C.

Plasmid Profiling

Plasmids were purified according to the method descried by Kado and Liu (1981), followed by separation on a 0.8% (w/v) agarose gel and stained with GelRed® (Biotium, Hayward, CA, USA). The approximate molecular weight of each plasmids were determined by comparing with the reference *E. coli* strain 39R861, harboring four plasmids of 6.9-, 36-, 63- and 147 kb (Macrina et al., 1978).

MinION Sequencing of the Plasmids of C700-09

The plasmid DNA of C700-09 was extracted using QIAGEN Plasmid Midi kit (QIAGEN, Copenhagen, Denmark) according to the manufacturer's instructions. The plasmids were sequenced commercially using the MinION sequencing platform (DNASense, Aalborg, Denmark). The fully assembled $pAA^{C700-09}$ is deposited in the EMBL-EBI repository[1] under project number PRJEB18579.

Whole-Genome-Sequencing

Genomic DNA was extracted from isolates using the DNeasy blood & tissue kit (QIAGEN, Copenhagen, Denmark) and fragment libraries were constructed using a NexteraTM kit (Illumina, Little Chesterford, UK) followed by 251-bp paired end sequencing (MiSeq; Illumina) according to the manufacturer's instructions. Reads were assembled *de novo* using CLC GenomicsWorkbench 7.5 (QIAGEN, Aarhus, Denmark). The

[1]https://www.ebi.ac.uk/ena

TABLE 1 | Strains used in this study.

Strains	Description	Reference
55989	Prototype EAEC strain expressing AAF/III	Bernier et al., 2002
C338-14	Prototype EAEC strain expressing AAF/V	Jonsson et al., 2015
C700-09	EAEC strain carrying *agg3A* and *agg5A* simultaneously	Boisen et al., 2012
C763-16	EAEC strain carrying *agg3A* and *agg5A* simultaneously	This study
C761-16	EAEC strain carrying *agg3A* and *agg5A* simultaneously	This study
C760-16	EAEC strain carrying *agg3A* and *agg5A* simultaneously	This study
C762-16	EAEC strain carrying *agg3A* and *agg5A* simultaneously	This study
C764-16	EAEC strain carrying *agg3A* and *agg5A* simultaneously	This study

TABLE 2 | Primers used in this study.

	Gene	Description	Primer sequence	Amplicon size (bp)	Annealing temperature
Optimized AAF multiplex	*agg4A*	Major pilin subunit of AAF/IV	F 5′-TGAGTTGTGGGGCTAYCTGGA-′3 R 5′-CACCATAAGCCGCCAAATAAGC-′3	169	57
	aggA	Major pilin subunit of AAF/I	F 5′-TCTATCTRGGGGGGCTAACG-′3 R 5′-ACCTGTTCCCCATAACCAGAC-′3	218	
	aafA	Major pilin subunit of AAF/II	F 5′-CTACTTTATTATCAAGTGGAGCCGCTA-′3 R 5′-TAGGAGAGGCCAGAGTGWATCC-′3	292	
	agg3A	Major pilin subunit of AAF/III	F 5′-AGCTAGTGCTACTGCAAAATTAAAGTT-′3 R 5′-CAGGTTTAATATATTGGTCTGGAATAAC–′3	359	
	agg5A	Major pilin subunit of AAF/V	F 5′-GTTTCATCAACTGGAACTATTACTATTT–′3 R 5′-TAATTTAAGCTGAAGAATCCAGTCAAT-′3	401	
	agg3/4C	Usher-encoding protein of three AAF variants; III, IV and V	F 5′-CATARTGAAGGTATAACATTTGGTCAGA-′3 R1 5′-GTCAGCATAACACTTACTGTTCATTC-′3 R2 5′-GTAGTTTGCATAGCAATGGCTATTCATT-′3	477	
	rpoA	qRT-PCR	5′-GAAGAGATGGATGACGACGAAGACG-′3 5′-GTACGCAGCTCGGCGAATTTCTCAC-3′	115	60
	Agg3A	qRT-PCR	F 5′-GCGTGGGAACAAATACTGGACA-′3 R 5′-TGGTCTGGAATAACAGCTTGAACTC-′3	81	60
	Agg5A	qRT-PCR	F 5′-TTTCCATCTTTAGCATCTGCCC-′3 R 5′-ATTAGTTCGCCTCCTCCTGT-′3	145	60

whole-genome sequencing data is deposited in the EMBL-EBI repository[2] under the primary identification number PRJEB18579.

Sequence Analysis

The pAA plasmid was annotated using the RAST annotating system (Aziz et al., 2008). Putative hypothetical genes identified by RAST annotation were manually curated using NCBI BLASTn and BLASTp searches. Putative EAEC genes were mapped to a panel of EAEC plasmids/genomes using CLC Main Workbench 7.5.1. BLASTn atlases of the pAA virulence plasmids were constructed using BLAST Ring Image Generator v0.95 (BRIG; Alikhan et al., 2011).

Serotyping

Serotyping was performed in silico by using serotype finder[3]. Phenotypical serotyping was performed at the International *Escherichia* and *Klebsiella* Centre (World Health Organization), Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark using methods described elsewhere (Orskov and Orskov, 1992).

RNA Extraction and qRT-PCR

For optimal expression of the AAFs, overnight cultures grown in LB-broth were diluted 1:100 in 20 ml Dulbecco's modified Eagle Medium (DMEM) supplemented with 0.45% glucose (DMEM-HG). The strains were incubated with shaking at 37°C until an optical density (OD_{600}) of 0.8. RNA was extracted with the RNase mini kit with the addition of an on-column digestion in order to remove contaminating DNA using the RNAse-Free DNase Set (Qiagen, Inc, Valencia, CA, USA). The RNA was quantified by Qbit analysis and cDNA was synthesized from 1 μg of bacterial RNA using random hexamer primers and the Thermoscript reverse transcriptase (RT) enzyme (Invitrogen, Carlsbad, CA, USA) for 10 min at 25°C, 1 h at 50°C, and 5 min at 85°C. As negative controls, all samples were tested without the addition of RT. The RNA was extracted from three independent experiments, and the qRT-PCR reactions were performed in duplicates on each sample. qRT-PCR was performed by using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and the expression level for each queried gene was normalized to

[2]https://www.ebi.ac.uk/ena

[3]https://cge.cbs.dtu.dk/services/SerotypeFinder/

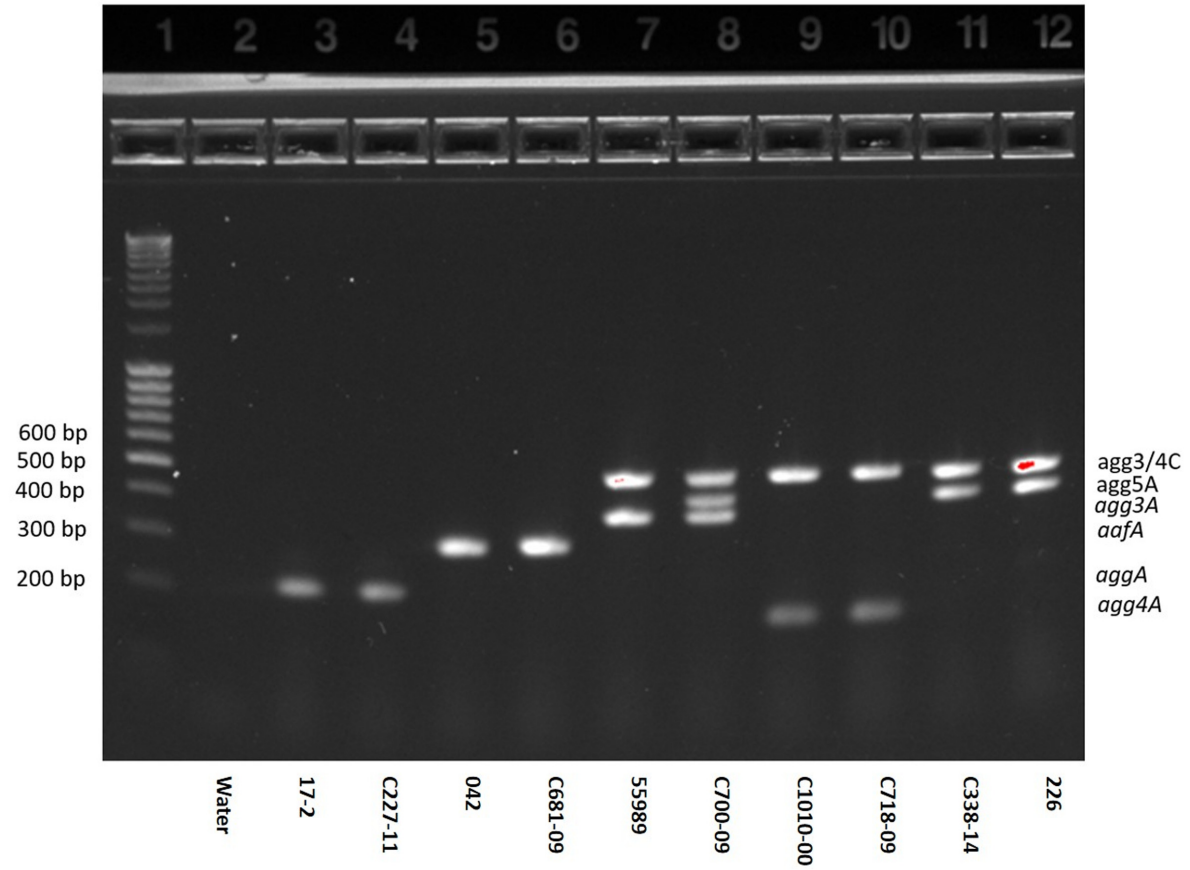

FIGURE 1 | Agarose gel of the optimized AAF multiplex demonstrated on 10 EAEC strains. PCR products representing genes for *aggA* (AAF/I), *aafA* (AAF/II), *agg3/4C* (usher of AAF/III, AAF/IV and V, all detected by the same primer set), *agg3A* (AAF/III), *agg4A* (AAF/IV) and *agg5A* (AAF/V) were separated on a 1.2% agarose gel. Lane 1: ladder, lane 2: negative control (water), lanes 3–4: EAEC strains 17-2 and C227-11 both harboring *aggA*, lanes 5–6: EAEC strains 042 and C681-09 both harboring *aafA*, lane 7: EAEC strain 55989 harboring *agg3A* and *agg3C*, lane 8: EAEC strain C700-09 harboring *agg3A, agg3C* and *agg5A,* lanes 9–10: EAEC strains C1010-00 and C718-09 both harboring *agg4A* and *agg4C,* lanes 11–12: EAEC strains C338-14 and 226 both harboring *agg5A* and *agg3C*. As shown in lane 8, the strain C700-09 tested positive for the usher of *agg3C* as well as the *agg3A* and *agg5A* gene.

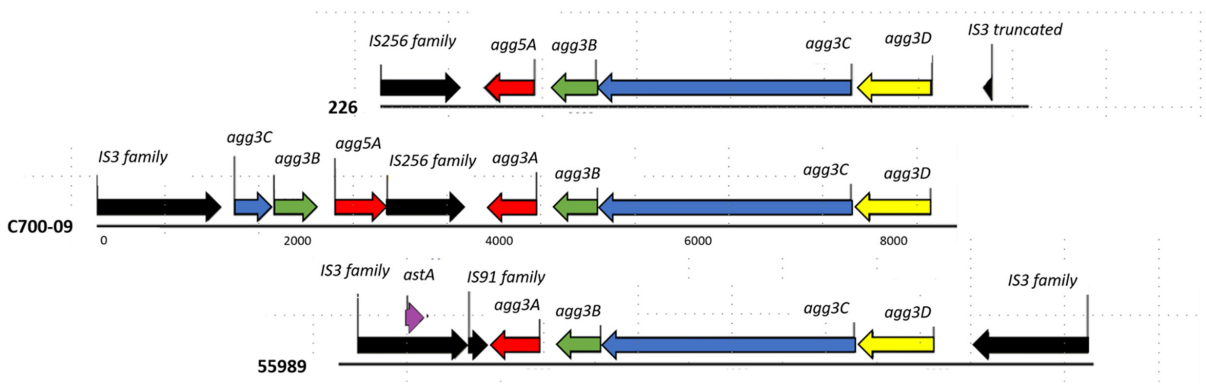

FIGURE 2 | Comparison of the genetic organization of the AAF cluster. Annotation of the chimeric cluster of C700-09 compared to the two AAF clusters (AAF/III from strain 55989 and AAF/V from strain 226). The genes encoding the assembly genes (*agg3DC*) as well as the minor pilin subunit (*agg3B*) are shared between AAF/III and AAF/V as previously described (Jonsson et al., 2015). Different colors represent different gene functions: black, mobile elements; purple, the gene encoding EAST-1; yellow, the chaperone-encoding gene; blue, the usher-encoding gene; green, the minor pilin subunit; and red, the major pilin subunit. IS elements are named according to ISfinder and the numbers below the lines corresponds to basepairs.

the constitutively expressed *rpoA* gene as previously described (Fujimitsu et al., 2008).

Statistical Analyses

Data were analyzed by performing two-tailed or, when appropriate, one-tailed Mann–Whitney tests using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). Each value used for statistical analyses is the mean of three replicates. Results were considered significant when $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

RESULTS

Optimization of an AAF PCR Multiplex Assay to Include AAF/V

A novel AAF variant, AAF/V, was recently identified. We here optimized a previously described multiplex PCR assay to detect AAF variants (Boisen et al., 2012) also to include AAF/V. Whereas, the previously described multiplex detected several of the usher genes from AAF/I-IV, we decided only to include the genes encoding the five major pilin subunits and the usher found in AAF/III, AAF/IV or AAF/V (*agg3/4C*) which can be detected by a single primer set. The multiplex PCR was validated by testing EAEC strains, with known AAF profiles. All strains exhibited the expected AAF gene profiles. However, surprisingly, we found that one strain (C700-09) harbored two distinct fimbrial genes; not only *agg3A* but also the gene encoding *agg5A* (**Figure 1**, Lane 8), along with the usher encoding gene (*agg3/4C*).

Prevalence of AAFs in a Collection of Clinical EAEC Isolates

We next used the optimized PCR multiplex assay to investigate the prevalence of the different AAF variants in a collection of 162 EAEC isolates isolated from Danish patients suffering from diarrhea. The most frequent gene encoding an AAF major pilin subunit was *aggA* (25.3%) encoding the AAF/I major pilin subunit, followed by *agg4A* (17.3%; AAF/IV), *agg5A* (14.2%; AAF/V), *agg3A* (13.6%; AAF/III) and *aafA* at 13.6% (AAF/II). Thus, there was a high degree of resemblance of the prevalence of the AAF variants to previous studies (Boisen et al., 2012; Lima et al., 2013; Jonsson et al., 2015). Twenty-one strains (13%) were negative for all five AAF variants.

Surprisingly, as observed for isolate C700-09 above, five (3.1%) of the tested strains were positive for two major pilin genes, *agg3A* and *agg5A* encoding the major pilin subunit of AAF/III and AAF/V, respectively.

Identification of a Novel pAA Plasmid Encoding Both AAF/III and AAF/V Genes

To further investigate the EAEC strains harboring two distinct AAF variants, we isolated plasmids from strain C700-09 and sequenced them using the Oxford Nanopore MinION technology. C700-09 was found to harbor three plasmids of 5.4, 72, and 120 kb, respectively. By BLAST analysis, the 120 kb plasmid was identified as the pAA plasmid ($pAA^{C700-09}$),

TABLE 3 | Comparison of *E. coli* C700-09 to the other completely sequenced pAA plasmids.

E. coli Strain	*aggR*	*aap*	*aatA-P*	AAF variant	Toxins present on pAA	Other virulence factors	Size of pAA (basepairs)	Plasmid replicons	Accession number	Reference
C700-09	+	+	+	*agg3A*, *agg5A*	*pet*, *pic*, *senB*	*orf3*, *orf4*, *aar*, *capU*, *virK*, *shf*, *aaiA-aaiP*	120.286	IncFII, Col156	PRJEB18579	Boisen et al., 2012
55989	+	+	+	*agg3A*	*astA*	*orf3*, *orf4*, *aar*	72.482	IncFIB	NC_011752	Bernier et al., 2002
226	+	+	+	*agg5A*	*astA*	*orf3*, *orf4*, *aar*	102.786	IncFIB, IncFII	SRA055981	Dallman et al., 2012
042	+	+	+	*aafA*	*pet*	*orf3*, *orf4*, *aar capU*, *virK*, *shf*,	113.346	IncFIC	FN554767	Chaudhuri et al., 2010
C227-11	+	+	+	*aggA*	–	*orf3*, *orf4*, *aar*	74.217	IncFII, IncFIB	NC_018666	Scheutz et al., 2011
PO86A1	+	+	+	*agg4A*	–	*orf3*, *orf4*, *capU*, *virK*, *shf*,	120.730	IncII, IncFIB	AB255435	Johnson and Nolan, 2009

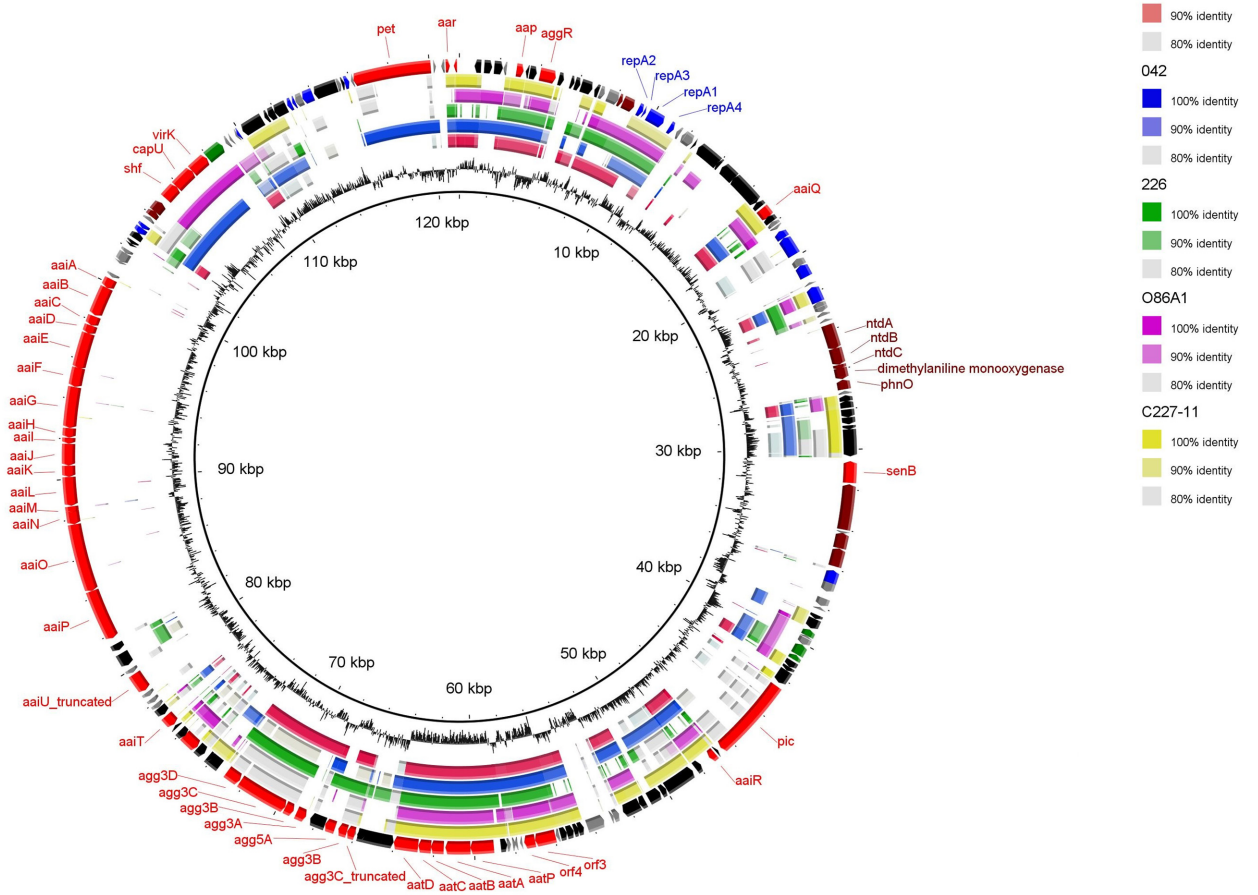

FIGURE 3 | Circular map of pAA$^{C700-09}$ compared to the other public available pAA plasmids each harboring a different AAF variant. The outer ring shows predicted ORFs. Different colors represent different putative functions: gray, hypothetical proteins; red, EAEC-specific virulence factors; blue, plasmid replication and maintenance; maroon, catabolism and metabolism; green, transmembrane proteins; and black, mobile elements. pAA$^{C700-09}$ is compared with the five pAA plasmids; 55989, 042, 226, O86A1 and C227-11. Inner ring, GC content. The circular plasmid diagram was generated using BRIG.

whereas the 72 kb plasmid was found to be a resistance plasmid encoding antibiotic resistance genes [*strA, strB, blaTEM-1B, sul2, tet(B), dfrA8*]. The smallest plasmid of 5.4 kb encoded 11 hypothetical proteins.

The sequencing data revealed that the pAA plasmid contained a region encoding the AAF/III-gene cluster followed by a truncated variant of the AAF/V cluster encoded by the opposite strand (**Figure 2**). The two AAF gene clusters were interspaced by a 1025 bp region encoding an insertion element. The truncated AAF/V gene-cluster lacked the entire *agg3D* gene and the majority of the *agg3C* gene was replaced by an insertion element (**Figure 2**). In addition to the fimbriae-encoding region, the pAA plasmid included 186 putative ORFs of which 50 encoded for hypothetical proteins with no match in the RAST database. Twelve putative ORFs were involved in catabolism and metabolism, four putative ORFs encoded for transmembrane proteins and 15 were involved in transfer, replication, or plasmid maintenance function. Sixty-two putative ORFs were mobile elements including integrases, transposases or phage related factors and the remaining 43 putative ORFs have been demonstrated or predicted to have a role in virulence.

BLAST comparison was performed on five completely sequenced pAA plasmids (**Table 3**) with pAA$^{C700-09}$ (**Figure 3**). Although common features were observed among the pAA plasmids, many of which correspond to EAEC virulence genes, substantial variation in the genetic composition was observed, as previously described (Johnson and Nolan, 2009). Several virulence genes previously described on pAA plasmids were also encoded by pAA$^{C700-09}$ including the cytotoxic SPATE protein Pet, the AggR transcriptional regulator, the AggR-activated regulator Aar, the surface protein dispersin and its secretion machinery Aat. Moreover, *senB* typically found on the Inv plasmid of *Shigella* and enteroinvasive *E. coli* encoding the shET2 toxin was located on the plasmid (Nataro et al., 1995b).

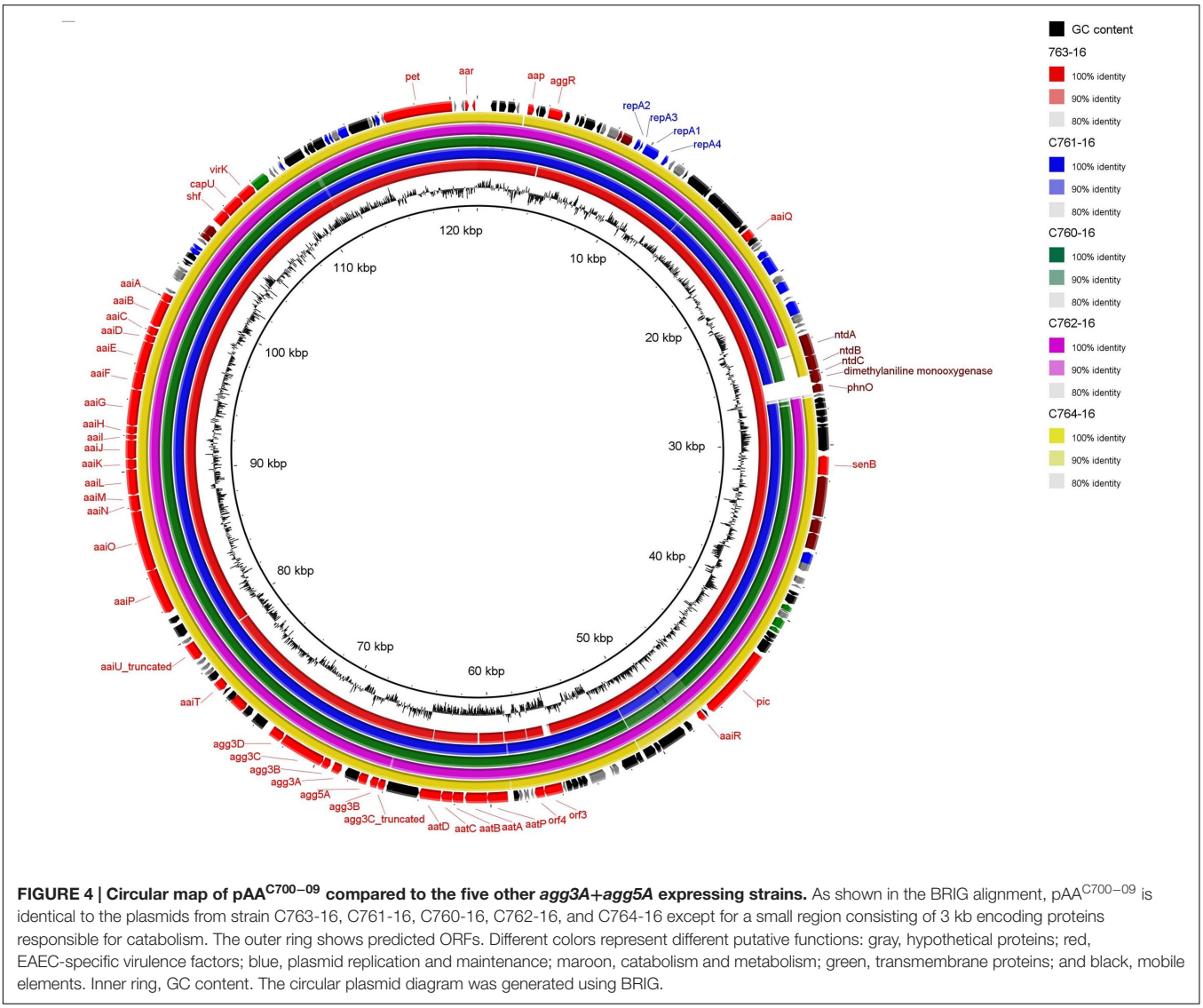

FIGURE 4 | Circular map of pAA$^{C700-09}$ compared to the five other *agg3A+agg5A* expressing strains. As shown in the BRIG alignment, pAA$^{C700-09}$ is identical to the plasmids from strain C763-16, C761-16, C760-16, C762-16, and C764-16 except for a small region consisting of 3 kb encoding proteins responsible for catabolism. The outer ring shows predicted ORFs. Different colors represent different putative functions: gray, hypothetical proteins; red, EAEC-specific virulence factors; blue, plasmid replication and maintenance; maroon, catabolism and metabolism; green, transmembrane proteins; and black, mobile elements. Inner ring, GC content. The circular plasmid diagram was generated using BRIG.

Like the other pAA plasmids, pAA$^{C700-09}$ was a F-type plasmid with stability, maintenance and transfer regions. Whereas pAA$^{C700-09}$ was of similar size as p042 and pO86A1, the F-transfer region was truncated, which was also observed in p55989 (Johnson and Nolan, 2009) indicating that pAA$^{C700-09}$ is likely defective in self transfer ability. The pAA$^{C700-09}$ shared the same replicon as the pAAs of the AAF/V expressing strain 226, the German outbreak strain C227-11 and O86A1 (IncFII), and not the pAA plasmid of 55989, which had the IncFIB replicon (**Table 3**).

Interestingly, using BLAST analysis, we found that unique regions of pAA$^{C700-09}$ encoded several virulence genes typically located on the chromosome of EAEC or *Shigella* spp. isolates. These included a type VI secretion apparatus (Aai) normally found on the chromosome of EAEC which appears to have been inserted into the pAA plasmid via transposable elements. The *aai* cluster was first observed in strain 042, encoded on a 117 kb pathogenicity island inserted at the *pheU* tRNA site (Dudley et al., 2006). The prototype strain 042 was shown to harbor 26 genes in the aai cluster (*aaiA-aaiY*), whereas in pAA$^{C700-09}$, the *aai* cluster comprised 16 genes *(aaiA-aaiP)* with 85% nucleotide identity to 042. Importantly, it has previously been shown that *aaiA-P* are sufficient for secretion of the effector protein AaiC (Dudley et al., 2006).

In addition to the *aai* cluster, the serine protease autotransporter Pic, normally located on the chromosome of EAEC and *Shigella* spp., was also located on pAA$^{C700-09}$ (**Figure 3**) (Henderson et al., 1999; Behrens et al., 2002).

The pAA$^{C700-09}$ Plasmid is Found in Strains Positive for both AAF/III and AAF/V

We next performed Illumina-based whole-genome sequencing of the five additional EAEC strains harboring *agg3A+agg5A* in order to establish if they harbor plasmids similar to that of C700-09. BLAST analysis clearly indicated that all strains harbored the

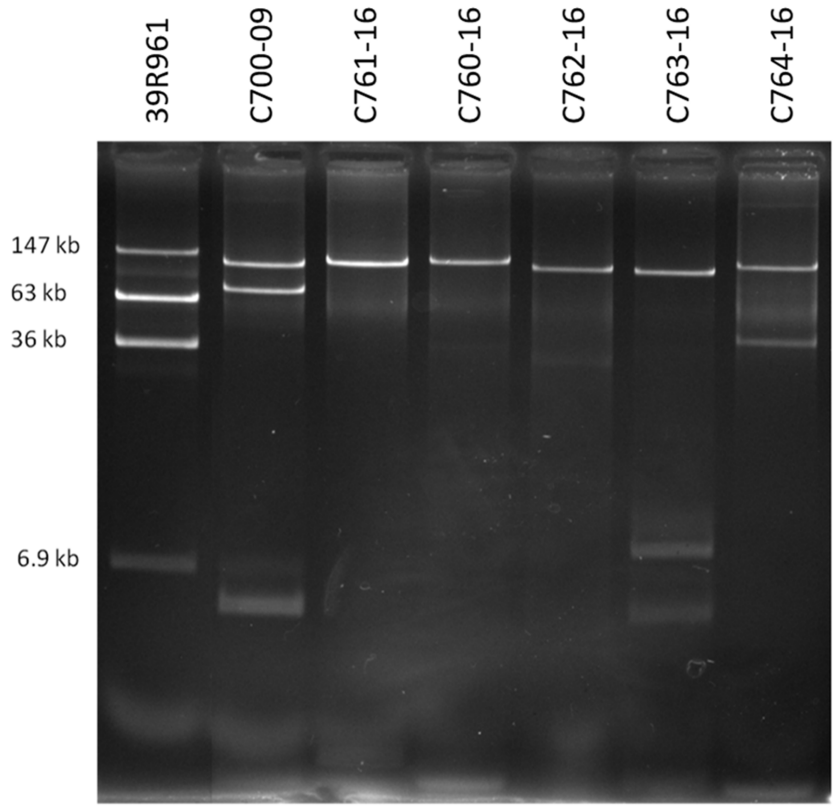

FIGURE 5 | Plasmid profiles of the six strains encoding *agg3A* and *agg5A.* Plasmid profiles with *E. coli* 39R861 as a marker (147, 63, 37, and 7 kb) in lane 1. The plasmid profile showed that all of the *agg3A+agg5A* strains harbored a plasmid of approximately 120 kb.

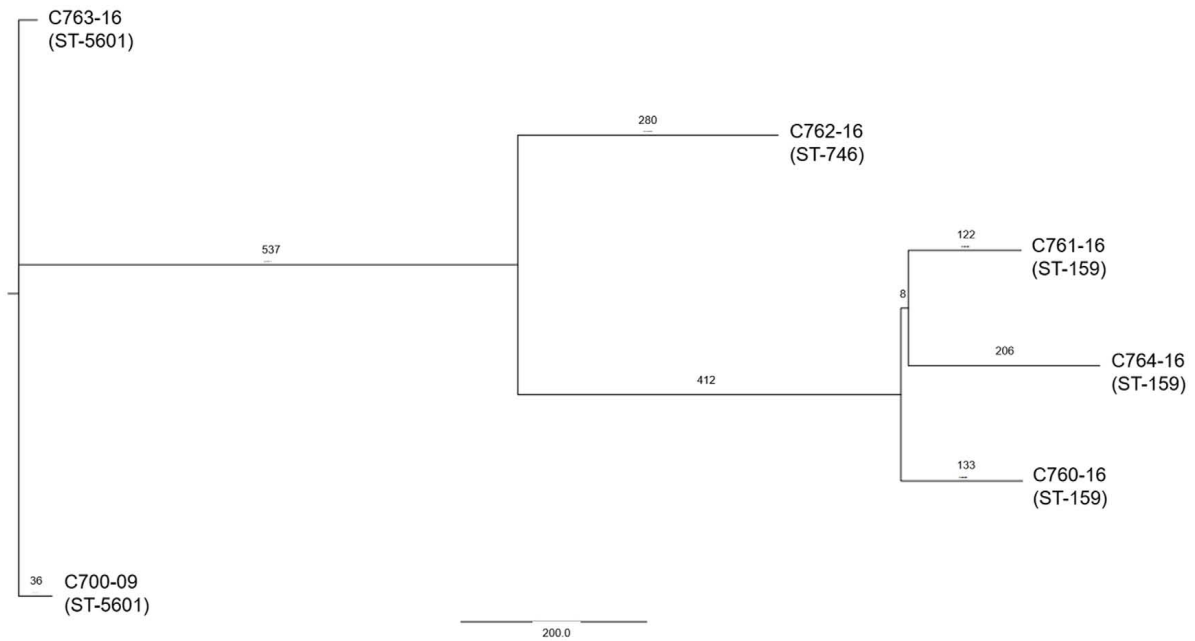

FIGURE 6 | A phylogenetic SNP tree of the six strains encoding *agg3A+agg5A*. The strains were clustered completely according to their MLST type. The tree was reconstructed using an in-house pipeline and the SNPs were extracted from the six genomes. The numbers above the nodes corresponds to the number of SNPs present between the strains.

same pAA plasmid as C700-09 (**Figure 4**), except for a small region consisting of approximately 3 kb which contained genes encoding proteins involved in kanosamine synthesis pathway (*ntdABC*; Vetter et al., 2013) as well as *phnO* encoding an aminoalkylphosphonate N-acetyltransferase (Hove-Jensen et al., 2012). This was further verified by plasmid profiling of the six strains. Whereas the pAA plasmid of 55989 consists of 72 kb, the six strains all harbored a large plasmid of approximately 120 kb, verifying that all six strains harbor pAA$^{C700-09}$ (**Figure 5**).

Clonality of Strains Harboring pAA$^{C700-09}$

We next investigated the phylogenetic relations of the six strains harboring pAA$^{C700-09}$. EAEC strains are known for their heterogeneity, and comprise many different serotypes as well as MLST types. The serotypes of the six strains were examined both *in silico* using the CGE pipeline[4] as well as phenotypically and all six strains were found to belong to the same serotype O181:H19. MLST analysis using the CGE pipeline revealed that the strains belonged to three MLST types. However, these MLST types are single locus variants at the *adk* locus and therefore belong to the same MLST complex (Wirth et al., 2006). To further elucidate the phylogenetic relationship, a single-nucleotide polymorphism (SNP)-based phylogenetic tree was constructed. As expected, the isolates clustered according to their respective MLST type into three clusters. Importantly, although the six strains all belonged to the same MLST subgroup, the SNP analysis clearly revealed that pAA$^{C700-09}$ is present in different strains (**Figure 6**).

Both *agg3A* and *agg5A* Fimbrial Subunit Genes Are Expressed from pAA$^{C700-09}$

To investigate if both *agg3A and agg5A* fimbrial subunit genes were expressed, mRNA was isolated from four strains C700-09, C761-16, C760-16 and C762-16 and grown to mid log-phase and cultivated under AAF-inducing conditions in DMEM/0.5 % glucose (Morin et al., 2010) followed by RT-qPCR analysis. Interestingly, both fimbrial subunits genes were found to be transcribed in all four strains (**Figure 7**). Although the strains were grown in the exact same conditions, variations were observed between the patterns of *agg3A/5A* mRNA expression between the four strains. Three of the strains (C700-09, C760-16 and C762-16) had similar expression patterns, expressing significantly more *agg3A* mRNA compared to *agg5A* ($P < 0.05$), whereas the pattern was completely the opposite for strain C761-16 expressing *agg5A* at the same level as the wild type AAF/V strain C338-14, and with almost no *agg3A expression* ($P < 0.05$).

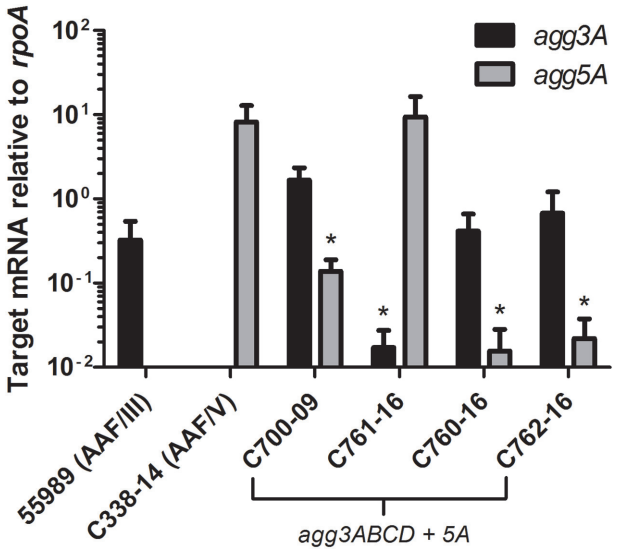

FIGURE 7 | qRT-PCR of four of the strains encoding *agg3A+agg5A* compared to the AAF/III and AAF/V wild type prototype strains. Transcript levels for *agg3A* (black) and *agg5A* (gray) were evaluated in the prototype AAF/III-expressing strain 55989, the prototype AAF/V-expressing strain C338-14 and four strains harboring both *agg3*A and *agg5A* genes by qRT-PCR. Transcripts were examined during mid-log phase and results are normalized to the constitutively expressed housekeeping gene *rpoA*. Each value used for statistical analyses is the mean of three independent experiments. Results were considered significant when $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

DISCUSSION

Enteroaggregative *E. coli* is an important diarrheal pathogen but its diagnosis and identification remains challenging due to the heterogeneity of the strains. Adherence to the intestinal mucosa is a crucial part of the pathogenesis, and is facilitated by AAFs. Most recently, a novel member of the AAF family was identified, thus bringing the number of AAF variants up to a total of five (Jonsson et al., 2015).

In order to detect all AAF variants, we optimized a previously described AAF multiplex PCR assay and screened a Danish collection of 162 clinical EAEC isolates. By use of the optimized assay, 87% of the isolates tested positive for an AAF variant, which is a significant improvement compared to previous studies, which only detected the presence of an AAF variant in approximately 50% of EAEC strains tested (Boisen et al., 2012; Lima et al., 2013). In general, we observed the same frequencies as previously described (Jonsson et al., 2015).

Interestingly, we found that 3% of the strains harbored genes encoding two of the pilin subunits *agg3A* and *agg5A*. These were revealed to be encoded by a novel pAA plasmid, pAA$^{C700-09}$, harboring a complete AAF/III gene-cluster as well as a truncated AAF/V gene-cluster. Notably, it was previously shown that AAF/V and AAF/III share the genes encoding for *agg3DCB*, whereas the major pilin subunits share only 32.2% identity (Jonsson et al., 2015).

Although EAEC pAA plasmids share a common plasmid backbone and core EAEC-associated virulence genes, the genetic compositions of the pAA plasmids of prototype EAEC strains are quite different from each other (Johnson and Nolan, 2009). Our study supports this, as pAA$^{C700-09}$ contains several virulence genes not found on the previously completely sequenced pAA plasmids. This includes a variant of the Aai cluster encoding a type VI secretion system as well as the gene encoding the

[4]https://cge.cbs.dtu.dk/services/

Pic toxin, which is typically chromosomally encoded in EAEC. Thus, acquisition of this plasmid endow the host strain with a remarkably high number of EAEC associated virulence genes hereby, likely promoting strain pathogenicity.

The population structure of EAEC is seen to be very heterogeneous (Okeke et al., 2010; Chattaway et al., 2014). Whole-genome SNP analysis of the six strains harboring $pAA^{C700-09}$, revealed that although the strains belonged to three related MLST types, $pAA^{C700-09}$ is found in clearly distinct strains. The phylogenetic resemblance of the strains may reflect that the plasmid was first obtained by a common ancestor, and has then due to fitness advantages been maintained in this clonal lineage. It is epidemiological interesting to clarify whether this plasmid is present in other clonal lineages.

Lastly, we wanted to investigate whether or not both variants were expressed. The levels of *agg3A* and *agg5A* mRNA expression was therefore quantified in four strains revealing that *agg5A* as well as *agg3A* were transcribed in all of the strains. Importantly, as AAF/III and AFF/V share accessory genes, it is likely that the Agg5A protein is assembled into functional fimbriae via expression of the accessory genes from the intact AFF/III gene cluster. Notably, variations were observed between the expression patterns between the four strains harboring $pAA^{C700-09}$. This could be related to the different genetic background of the strains but further studies are needed to clarify this.

The study provides valuable new information regarding the complexity of EAEC pathogenicity and the discovery of a novel pAA plasmid encoding a wide array of EAEC virulence genes and potentially enabling expression of two AAF variants are intriguing. However, several aspects remain to be elucidated. Although we establish that the genes encoding both types of major pilin subunits are transcribed, the fimbrial expression on the bacterial surface remains to be verified and characterized. Thus, further studies are needed to establish whether the fimbriae are expressed, and if so, whether they are composed of chimeric subunits of Agg3A and Agg5A polymerized into one long fimbria or two separate fimbriae. It would possibly be advantageous for the bacteria to express two separate fimbriae, as it would, not only enable binding to alternative host receptors but also potentially make the bacteria capable of exploiting different niches in the human gut. Additionally, AAFs have also previously been shown to cause inflammatory responses (Harrington et al., 2005; Boll et al., 2012), thereby being easily recognized by the host. Thus, the ability to switch between the expression of two fimbriae variants could be speculated to restrain the host immune response. Furthermore, as the two fimbriae variants have different binding specificities the individual variants could be more favorable to express in certain environments. This relationship has been shown for uropathogenic *E. coli* strains and their ability to switch between type 1 fimbriae and P-fimbriae expression when colonizing the lower and upper urinary tract, respectively (Melican et al., 2011).

More studies are needed to establish how the fimbriae are composed, the effect on pathogenicity as well as the impact of the additional genes inserted on this novel pAA plasmid.

AUTHOR CONTRIBUTIONS

KK and HJ obtained the funding and were responsible for the project. BJ collected the bacterial isolates used in the study. RJ, CS, CAS, and EB were responsible for the microbiological analyses performed in the study. FS, KJ, and NB were responsible for the serotyping and SNP analysis. RJ, CS, NB, EB, HJ, and KK were responsible for the interpretation of the research data and of the microbiological analysis. All authors have contributed to the writing of the manuscript and have all approved the final draft of the manuscript. All authors take responsibility for the accuracy and integrity of the research performed.

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Prevalence and Genetic Diversity of *Enterococcus faecalis* Isolates from Mineral Water and Spring Water in China

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Enterococcus faecalis is an important opportunistic pathogen which is frequently detected in mineral water and spring water for human consumption and causes human urinary tract infections, endocarditis and neonatal sepsis. The aim of this study was to determine the prevalence, virulence genes, antimicrobial resistance and genetic diversity of *E. faecalis* from mineral water and spring water in China. Of 314 water samples collected from January 2013 to January 2014, 48 samples (15.3%) were contaminated *E. faecalis*. The highest contamination rate occurred in activated carbon filtered water of spring water (34.5%), followed by source water of spring water (32.3%) and source water of mineral water (6.4%). The virulence gene test of 58 *E. faecalis* isolates showed that the detection rates of *asa1*, *ace*, *cylA*, *gelE* and *hyl* were 79.3, 39.7, 0, 100, 0%, respectively. All 58 *E. faecalis* isolates were not resistant to 12 kinds of antibiotics (penicillin, ampicillin, linezolid, quinupristin/dalfopristin, vancomycin, gentamicin, streptomycin, ciprofloxacin, levofloxacin, norfloxacin, nitrofurantoin, and tetracycline). Enterobacterial repetitive intergenic consensus-PCR classified 58 isolates and three reference strains into nine clusters with a similarity of 75%. This study is the first to investigate the prevalence of *E. faecalis* in mineral water and spring water in China. The results of this study suggested that spring water could be potential vehicles for transmission of *E. faecalis*.

Keywords: *Enterococcus faecalis*, mineral water, spring water, ERIC-PCR, virulence genes

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INTRODUCTION

Enterococci mainly inhabits in human and animal faces, ham sausage, pasteurized milk and drinking water (du Toit et al., 2000; Franz et al., 2003; Zou et al., 2011). Although some Enterococcal species are considered relevant for their technological properties (such as ripening, aroma development and inhibition of pathogens), they are not, unlike other lactic acid bacteria, recognized as probiotics (Kuriyama et al., 2003; Emaneini et al., 2008; Jamet et al., 2012). Indeed, enterococci are a major cause of nosocomial infections, such as urinary tract infections, endocarditis and neonatal sepsis (Franz et al., 2011; Sanchez Valenzuela et al., 2012; Werner et al., 2013). The main enterococcal isolates involved in nosocomial infections is *Enterococcus faecalis*

(Giraffa, 2002; Kayser, 2003). *E. faecalis* is an important opportunistic pathogen, which is frequently detected in mineral water and spring water for human consumption (Nicas et al., 1989; Martin-Platero et al., 2009; Buhnik-Rosenblau et al., 2013). Several studies have indicated that *E. faecalis* is a suitable indicator of the presence of pathogens in mineral water and spring water (Švec and Sedláček, 1999; Davis et al., 2005; Meng, 2007). The Natural Mineral Water National Standard GB 8537 (the National Food safety Standards of China) has short-listed *E. faecalis* as a microorganism indicator in mineral water and spring water factory in China.

For surveillance or tracing sources of *E. faecalis*, molecular typing methods such as pulsed field gel electrophoresis (PFGE) (Weng et al., 2013), amplified fragment length polymorphism (AFLP) (Bruinsma et al., 2002), random amplified polymorphic DNA (RAPD) (Martin-Platero et al., 2009), multi-locus sequence typing (MLST) (Homan et al., 2002; Werner et al., 2012) and enterobacterial repetitive intergenic consensus (ERIC-PCR) (Martin-Platero et al., 2009), can be used in *E. faecalis* isolates. Among these molecular typing approaches, PFGE is the most effective technology or typing of *E. faecalis* isolates due to its high reproducibility and discriminatory ability. However, PFGE is labor intensive and time-consuming (Weng et al., 2013). In contrast, ERIC-PCR is a relatively simple and cost-effective method, which has been successfully used for genotyping of different bacterial pathogens and for tracking the bacterial source of contaminated water products (Martin-Platero et al., 2009).

Enterococcus faecalis can produce dozens of virulence substances including hemolysin and surface adhesion substances (Aslam et al., 2012; Choi and Woo, 2013). The pathogenesis of five virulence genes including *asa1*, *ace*, *cylA*, *gelE* and *hyl* has been systematically studied. The *asa1*gene encodes surface adhesion substances by which *E. faecalis* can be fixed on a eukaryotic cell; the *ace* gene encodes surface adhesion proteins by which *E. faecalis* are resistant to immune function of the host cells; the *cylA* gene encodes hemolysin which can leads to the death of host cells; the *gelE* gene encodes gelatinase, which can hydrolyze gelatin leading to the proliferation of bacteria in the host cell; the *hyl* gene encodes hyaluronidase which can hydrolyze the tissue of host cell (Coque et al., 1995; Eaton and Gasson, 2001; Cosentino et al., 2010; Yang et al., 2015).

As shown in **Figure 1**, mineral water and spring water are generally produced using the same process, including three level filter (quartz sand filter, activated carbon filter, and fine filter), ozone sterilization, filling and capping, and light inspection of finished product. For manufacturers, the quality of source water, activated carbon filtered water and finished product are important. Source water reflects raw quality of mineral and spring water, and finished product is for human consumption. Activated carbon filter with a lot of pores and large surface area is a kind of common water treatment equipment, which processes a strong physical adsorption capacity to absorb organic pollutants and microbes. Recent studies have shown that activated carbon filter has become a gathering place for microbes and is the most serious in microbial contamination in whole production process of mineral water and spring water.

Systematic contamination survey of *E. faecalis* in mineral water and spring water has not yet been conducted. The aim of this study was for the first time to determine the prevalence, virulence genes, antimicrobial resistance and genetic diversity of *E. faecalis* from mineral water and spring water in China. The information generated in this study will provide insights into the prevalence and differentiation of *E. faecalis* isolates in mineral water and spring water.

MATERIALS AND METHODS

Sample Collection

From January 2013 to January 2014, a total of 314 water Samples were collected from 101 mineral water and spring water factories in 10 provinces of China (Guangdong, Guangxi, Fujian, Hainan, Hubei, Shanghai, Beijing, Yunnan, Guizhou and Sichuan). Samples of source water (112), activated carbon filtered water (101) and finished product (101) of spring water and mineral water were collected from each water factories. Samples of source water (112) include Surface water (14) and Groundwater (98). All water samples were maintained at 4°C during transportation and testing was conducted within 1 h after receiving the samples.

Isolation and Enumeration of *E. faecalis*

Briefly, 250 ml of water sample was filtered through a 0.45 μm membrane (Millipore Co., Billerica, MA, United States) in a stainless steel multi-line filter system (Huankai Co., Guangzhou, China). The membrane was placed on KF agar medium (Huankai Co., Guangzhou, China), a selective medium for *E. faecalis* and then cultured at 36°C for 2 days. Presumptive colonies with red color were selected for catalase test and cultured in brain heart infusion broth at 45°C for 2 days and Bile broth at 36°C for 3 days, respectively. Colonies positive for the three confirmation tests were considered presumed *E. faecalis*.

Enterococcus faecalis Identification

All the presumed *E. faecalis* were identified by the API 20 STREP biochemical identification system (Biomerieux Co., Lyon, France) and specific PCR for the *E. faecalis* species. According to seven code of API 20 Strep biochemical identification system, coincidence rate of *E. faecalis* isolates can be tested. Further identification of *E. faecalis* was determined by PCR using *sodA* species-specific primers. Genomic DNA was extracted from collected *E. faecalis* isolates by using a Bacterial Genomic DNA Purification kit (Dongsheng Biotech, Guangzhou, China) according to the manufacturer's instruction. *E. faecalis* were identified by amplification of 210 bp fragments with primer pairs EFS1 (5′ CTGTAGAAGACCTAATTTCA)/EFS2 (5′ CAGCTGTTTTGA AAGCAG) (Jamet et al., 2012).

ERIC-PCR Analysis

Total DNA from *E. faecalis* isolates was extracted as previously described. Genomic DNA concentration was determined

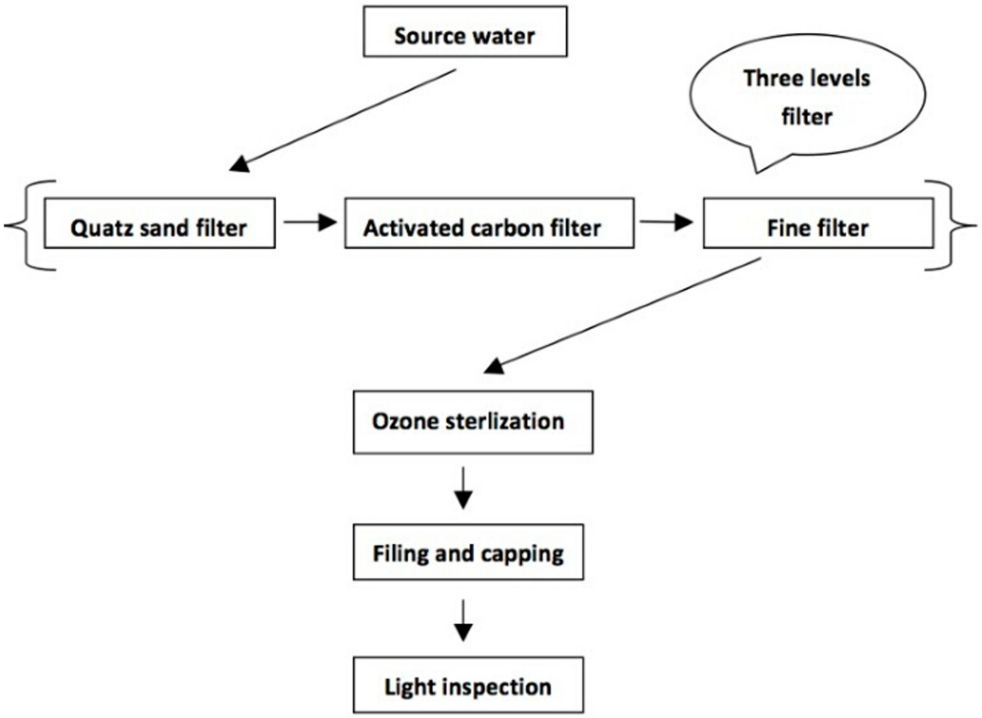

FIGURE 1 | Production flow chart of mineral water and spring water in China.

at 260 nm by using a Nano Drop ND 1000 UVe-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). The ERIC primers used were referred from the study of Versalovic et al. (1991). ERIC-PCR typing was performed for the collected *E. faecalis* isolates by using the protocol described by Li (2013) with some modifications. The PCR mixture (25 μl) contained 1.5 unit Hot start polymerase (Promega, Madison, WI, United States), 0.5 μmol/l each primer, 2.5 μmol/l $MgCl_2$, 200 μmol/l each dNTP, and 1 μl of the template genomic DNA (50 ng). Amplifications were performed with a DNA thermocycler (Applied Biosystems, Foster City, CA, United States) under the following temperature profiles: an initial denaturation at 95°C for 5 min; 35 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C; and a final extension at 72°C for 8 min (Martin-Platero et al., 2009; Li, 2013). The ERIC-PCR products were separated by electrophoresis in a 1.5% agarose gel with Goldview staining (0.005%, v/v) and then photographed using a UV Imaging System (GE Healthcare, Milwaukee, WI, United States). The images were captured in TIFF file format for further analysis.

Detection of Virulence Genes

Five virulence genes, *asa1*, *ace*, *cylA*, *gelE* and *hyl* were individually detected in all the collected *E. faecalis* isolates with the PCR technique (Eaton and Gasson, 2001; Zou et al., 2011). All primers were synthesized by Sangon Biotech company (Shanghai, China). The primers used to identify virulence genes are shown in **Table 1**. *E. faecalis* isolates CMCC 32219 (Guangdong culture collection centre) was used as positive control and distilled water was used as the negative control.

TABLE 1 | PCR primers for virulence gene detection.

No.	Primers	Sequence (5'→3')	bp	Function
1	asa1-F	CACGCTATTACGAACTATGA	375	Surface adhesion substances
	asa1-R	TAAGAAAGAACATCACCACGA		
2	ace-F	GGAATGACCGAGAACGATGGC	616	Surface adhesion proteins
	ace-R	GCTTGATGTTGGCCTGCTTCCG		
3	cylA-F	ACTCGGGGATTGATAGGC	688	Hemolysin
	cylA-R	GCTGCTAAAGCTGCGCTT		
4	gelE-F	TATGACAATGCTTTTTGGGAT	213	Gelatinase
	gelE-R	AGATGCACCCGAAATAATATA		
5	hyl-F	ACAGAAGAGCTGCAGGAAATG	276	Hyaluronidase
	hyl-R	GACTGACGTCCAAGTTTCCAA		

Antibiotic Resistance

According to the Clinical and Laboratory Standards Institute (CLSI) standards, all the collected *E. faecalis* isolates were tested by the disk diffusion method (Clinical and Laboratory Standards Institute [CLSI] (2006)). *E. faecalis* isolates CMCC 32219 (Guangdong culture collection centre) was used as positive control. A panel of antibiotics at the specific concentration per disk were tested: penicillin G (10 U), ampicillin (10 μg), linezolid (30 μg), quinupristin/dalfopristin

(15 μg), vancomycin (30 μg), gentamicin (120 μg), streptomycin (10 μg), ciprofloxacin (5 μg) levofloxacin (5 μg), norfloxacin (10 μg), nitrofurantoin (300 μg), tetracycline (30 μg) (Oxoid Co., Hampshire, United Kingdom) (Jamet et al., 2012; Sanchez Valenzuela et al., 2013). The isolates were classified as sensitive, intermediate, and resistant using the breakpoints specified by the CLSI.

Fingerprint Data Analysis

ERIC-PCR fingerprint patterns were analyzed using a Gel-Pro analyser (version 6.0) according to the manufacturer's instructions. The observed bands in the gels were evaluated based on the presence (code 1) or absence (code 0) of polymorphic fragments for the ERIC products. The cluster analysis was performed using NTSYSpc (version 2.10e), and similarities between ERIC-PCR profiles were calculated based on the simple-matching similarity matrix and unweighted pair group method with arithmetic.

RESULTS

Contamination of *E. faecalis* in Mineral Water and Spring Water

Of the 314 water samples tested, 48 (15.3%) were positive for *E. faecalis*, including 24 (21.4%) of 112 source water, 22 (21.8%) of 101 activated carbon filtered water samples and 2 (1.9%) of 101 finished product samples. From the contaminated samples, 58 *E. faecalis* isolates were obtained (Supplementary Table S1). As shown in **Table 2**, the rate of *E. faecalis* contamination in all mineral water samples was 3.8%, and the contamination rates of source water, activated carbon filtered water and finished product were 6.4, 4.7, and 0%, respectively. The rate of *E. faecalis* contamination in all spring water samples was 23.8%, and the contamination rates of source water, activated carbon filtered water and finished product were 32.3, 34.5, and 7.5%, respectively. In all the 48 contaminated samples, the contamination levels in spring water samples are significantly higher than those in mineral water samples. The contamination level of source water and activated carbon filtered water were 48.0 and 26.4 CFU/250 ml in spring water samples.

All the 112 source water Samples include 14 surface source water samples and 98 underground source water samples. As shown in **Table 3**, 16 (16.3%) samples of underground source water were positive in all 98 samples. Meanwhile, eight (57.1%) samples of surface source water were positive in all 14 samples.

TABLE 3 | Prevalence of *E. faecalis* in surface water and groundwater.

Samples	Positive amounts	Total amounts	Contamination rates%
Surface water	8	14	57.1
Groundwater	16	98	16.3

TABLE 4 | Virulence genes of 58 *E. faecalis* isolates.

Virulence gene	No. of positive sample (%)
asa1	46 (79.3)
ace	23 (39.7)
cylA	0 (0)
gelE	58 (58)
hyl	0 (0)

Detection of Virulence Genes in *E. faecalis* Isolates

In this study, the presence of *asa1*, *ace*, *cylA*, *gelE* and *hyl* genes was detected in 58 *E. faecalis* isolates. As shown in **Table 4**, all the 58 *E. faecalis* isolates (100%) harbored the *gelE* gene, among which 46 (79.3%) and 23 (39.7%) also had the *asa1* and *ace* genes, respectively. However, no *cylA* or *hyl* gene was detected from all the isolates.

Antibiotic Resistance

According the diameter of zone of inhibition, all the 58 *E. faecalis* isolates were classified as sensitive, intermediate, and resistant using the breakpoints specified by the CLSI. The result of antibiotic resistance showed that all 58 isolates were sensitive to 12 kinds of antibiotic (penicillin, ampicillin, linezolid, quinupristin/dalfopristin, vancomycin, gentamicin, streptomycin, ciprofloxacin levofloxacin, norfloxacin, nitrofurantoin, and tetracycline) which were selected according to the standard of CLSI. No resistant *E. faecalis* isolate was found.

Biochemical Identification

With *E. faecalis* ATCC 29212 (a), CMCC 32219 (b) and CMCC 32223 (c) (Guangdong culture collection centre) as positive controls, according to seven code of API 20 Strep biochemical identification system, biotypes of *E. faecalis* isolates can be tested. As shown in **Table 5**, biotypes of 44 *E. faecalis* isolates and two

TABLE 2 | Prevalence of *E. faecalis* from mineral water and spring water in China.

	Source water		Activated carbon filtered water		Finish product		Total	
Samples	CR (%)	CL (CFU/250 ml)	CR (%)	CL (CFU/250 ml)	CR (%)	CL (CFU/250 ml)	CR (%)	CL (CFU/250 ml)
M water	6.4	2.3	4.7	3.5	0	0	3.8	2.8
S water	32.3	48.0	34.5	26.4	3.4	7.5	23.8	36.0
Average	21.4	42.2	21.8	24.3	1.9	7.5	15.3	32.5

M and S water represent mineral and spring water, respectively; CR and CL represent contamination rate and level in positive samples, respectively.

TABLE 5 | Biochemical profiles of 58 *E. faecalis* isolates.

Biotypes	No. of isolates	7 code
A	a, b, 2∼5, 8∼11, 14∼36, 38∼41, 44, 46, 49∼52, 54, 56, 58	5143711
B	1, 6∼7, 37, 45, 47∼48, 53, 55, 57	7143711
C	c, 12∼13, 42∼43	5153711

a, b and c represent E. faecalis ATCC 29212, CMCC 32219 and CMCC 3222, respectively.

control isolates are 5143711. Biotypes of four *E. faecalis* isolates and one control isolate are 5153711, and biotypes of ten *E. faecalis* isolates are 7143711.

ERIC-PCR

The ERIC-PCR patterns of three control isolates (a, b, c) and 58 collected *E. faecalis* isolates are shown in **Supplementary Figure S1**. The ERIC of DNA profiles consisting of 5 to 15 bands ranged from 230 bp to approximately 4,000 bp. As shown in **Figure 2**, three control isolates (a, b, c) and 58 collected *E. faecalis* isolates were grouped into nine clusters at similarity coefficient of 75%. Control isolates ATCC 29212 (a), CMCC 32219 (b) and 21 collected *E. faecalis* were distributed among the cluster I, a prevalent cluster. Control isolate CMCC 32223 (c) and two collected *E. faecalis* isolates were distributed among cluster VIII. Cluster IV has only one isolate (NO.38) and Cluster VII has only one isolate (NO. 37).

DISCUSSION

In accordance with the Natural Mineral Water National Standard GB 8537, *E. faecalis* must be absent from 250 ml of water samples. In this study, 48 (15.3%) *E. faecalis*-positive samples from a total of 314 water samples were found. This result was consistent with previous investigations conducted in China (17.0%) (Li, 2013) and Greece (17.4%) (Grammenou et al., 2006). *E. faecalis* has not been systematically studied from mineral water and spring water, and our results were obtained from a large number of samples in China. Therefore, these nationwide data are more beneficial for risk assessment. The *E. faecalis* contamination rates in spring water samples were significantly higher than those in mineral water samples. The high prevalence of *E. faecalis* in the spring water indicated poor hygiene practices during manufacture process. The finished product of spring water presented a contamination rate of 3.4%, which can adversely affect the health of costumers. Therefore, the Chinese food safety management should implement further supervision for spring water products as well as implementation of Good Hygiene Practices (GHP). In addition, contamination rate of source water in surface was significantly higher than those in groundwater. Based on the contamination rate of *E. faecalis*, groundwater is better than surface water as source water.

In this study, *E. faecalis* contamination rate of activated carbon filtered in spring water reached 26.4%, which was the highest among all water samples tested. Activated carbon filter system is

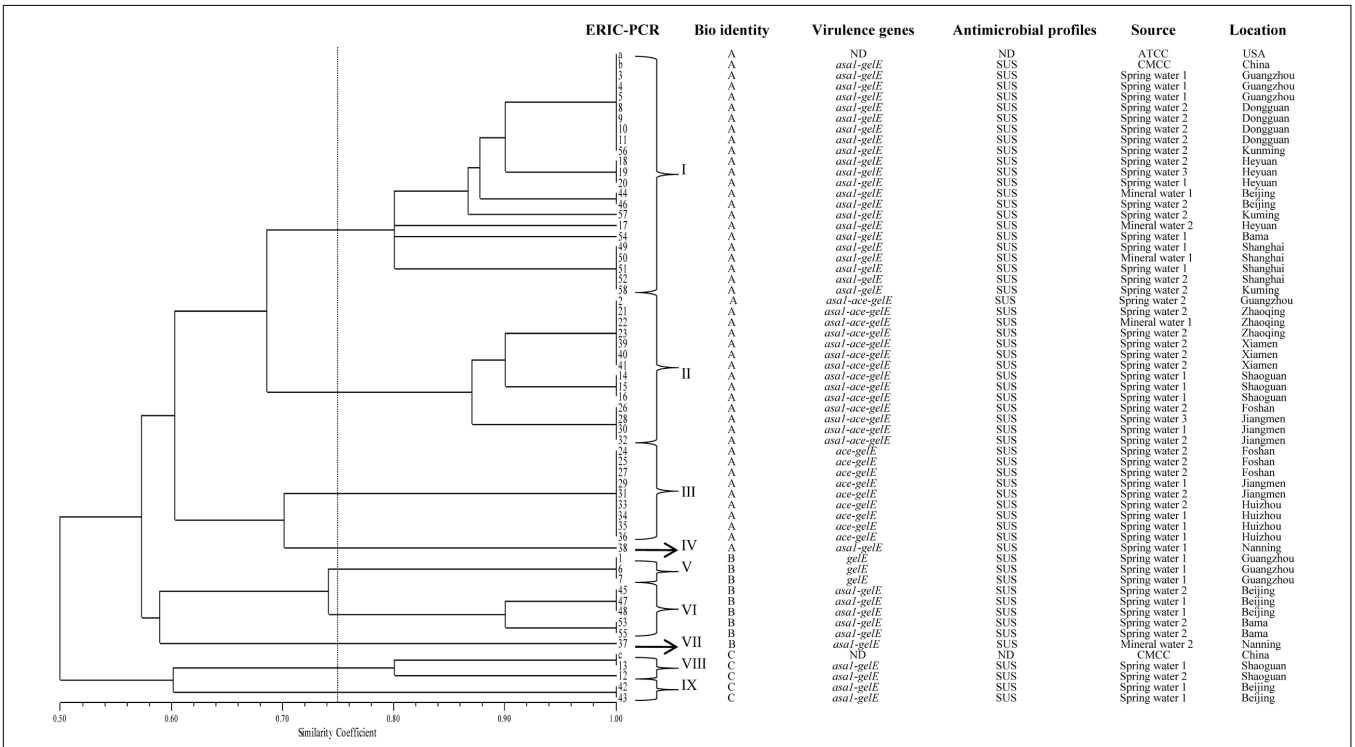

FIGURE 2 | ERIC-PCR DNA fingerprint analysis of *E. faecalis* isolates from mineral water and spring water in China. a, b and c represent *E. faecalis* ATCC 29212, CMCC 32219 and CMCC 32223, respectively; ND, not detect; SUS, susceptibility; 1, 2 and 3 represent source water, activated carbon filtered water and finish product, respectively.

commonly used for mineral water treatment to ensure equipment life, improve water quality and prevent pollution. Activated carbon filter which has a lot of pores and large surface area processes a strong physical adsorption capacity to absorb organic pollutants and microbes (Feng et al., 2013). Recent studies have shown that activated carbon filter has become a gathering place for microbes and is the most serious in microbial contamination in whole production process of mineral water and spring water (Camper et al., 1986). Hence, manufacturers of mineral water and spring water must establish measures for monitoring the activated carbon filtration system, as well as ensure timely cleaning and regular replacement of activated carbon (da Silva Fernandes et al., 2015; Fernandes et al., 2015).

Highly virulent *E. faecalis* isolates may cause diseases even at relatively low concentrations. In this study, up to 100% of *E. faecalis* isolates were *gelE*-positive and 79.3% were *asa1*-positive, which is consistent with previous studies and provides further evidence that these virulence genes are widely distributed among *E. faecalis* (Moraes et al., 2012; Anderson et al., 2015). The presence of these genes indicates pathogenic potential and probability to cause diseases. However, the expression of virulence genes is mainly related to quorum sensing. Further studies must be performed to examine whether the *E. faecalis* isolates in this study are pathogenic (Huebner et al., 1999; Delpech et al., 2012). All the 58 collected *E. faecalis* isolates were *cylA*-negative and *hyl*-negative, which is not consistent with previous studies (Creti et al., 2004; Medeiros et al., 2014). All the 58 collected *E. faecalis* isolates were sensitive to 12 kinds of antibiotic, which is different from previous investigation about drink water (Macedo et al., 2011), food (Gaglio et al., 2016) and clinical samples (Dahlén et al., 2012; Gilmore et al., 2013). The difference of the results may be due to different sources of the samples. The antibiotic resistance of *E. faecalis* come from different sources have huge difference (Abriouel et al., 2008). In this study, most of collected *E. faecalis* isolates derived from groundwater. So far, there is almost no research on antibiotic resistance of *E. faecalis* isolated from the groundwater.

ERIC-PCR is one of the most widely adopted PCR typing methods and is chosen for analyses of genetic diversity (Chen et al., 2014; Xie et al., 2015; Zhang et al., 2015). This method provides discriminatory value and is a rapid method for *E. faecalis* typing. In this study, the ERIC-PCR results provided a better overview of *E. faecalis* diversity. Most of collected *E. faecalis* isolates in the same area belong to the same cluster, which agrees with the results of previous studies (Martin-Platero et al., 2009). Three isolates (18, 19 and 20) obtained from spring water in Heyuan city showed 100% similarity. Two isolates (44 and 46) from spring water in Beijing city also showed identical ERIC patterns. Additionally, 10 isolates (a, b, 3, 4, 5, 8, 9, 10, 11 and 58) from different sources yielded an identical pattern, suggesting that they were highly homogenous and had a close genetic relationship. A correlation between the genomic profiles and the virulence genes was observed in these strains. Most of the isolates that carried *asa1*, *ace* and *gelE* were grouped in cluster B. In this cluster, a good correlation among ERIC patterns, virulence profiles, and the sample source was found in some isolates. In this study, no antibiotic resistance isolate was found, so no correlation was observed between the ERIC-PCR profiles and the antibiotic resistance profiles of the isolates.

In summary, our study for the first time revealed the high prevalence of *E. faecalis* from mineral water and spring water in China, which should have a potentially pathogenic effect on the health of consumers. The results of this study suggested that spring water product could be potential vehicles for transmission of *E. faecalis*. Meanwhile mineral water and spring water manufacturing factories must pay high attention to the contamination of activate carbon filters. These data may provide useful information for the development of public health policies and effective strategies to ensure the safety of our drinking water products.

ETHICS STATEMENT

All procedures performed in studies involving human participants were in accordance with the ethical standards.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: QW, JZ, and LW. Performed the experiments: LW and WG. Analyzed the data: LW, MC, and LX. Contributed reagents/materials/analysis tools: JW and LM. Contributed to the writing of the manuscript: LW and QW.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01109/full#supplementary-material

FIGURE S1 | Dendrogram of ERIC-PCR patterns of collected *E. faecalis* isolates. M and CK represents marker and control check, respectively; a, b and c represent *E. faecalis* ATCC 29212, CMCC 32219 and CMCC 3222, respectively.

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REVIEW
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Salmonella enterica Serovar Typhimurium Strategies for Host Adaptation

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Bacterial pathogens must sense and respond to newly encountered host environments to regulate the expression of critical virulence factors that allow for niche adaptation and successful colonization. Among bacterial pathogens, non-typhoidal serovars of *Salmonella enterica*, such as serovar Typhimurium (*S.* Tm), are a primary cause of foodborne illnesses that lead to hospitalizations and deaths worldwide. *S*. Tm causes acute inflammatory diarrhea that can progress to invasive systemic disease in susceptible patients. The gastrointestinal tract and intramacrophage environments are two critically important niches during *S*. Tm infection, and each presents unique challenges to limit *S*. Tm growth. The intestinal tract is home to billions of commensal microbes, termed the microbiota, which limits the amount of available nutrients for invading pathogens such as *S.* Tm. Therefore, *S.* Tm encodes strategies to manipulate the commensal population and side-step this nutritional competition. During subsequent stages of disease, *S.* Tm resists host immune cell mechanisms of killing. Host cells use antimicrobial peptides, acidification of vacuoles, and nutrient limitation to kill phagocytosed microbes, and yet *S.* Tm is able to subvert these defense systems. In this review, we discuss recently described molecular mechanisms that *S.* Tm uses to outcompete the resident microbiota within the gastrointestinal tract. *S.* Tm directly eliminates close competitors via bacterial cell-to-cell contact as well as by stimulating a host immune response to eliminate specific members of the microbiota. Additionally, *S.* Tm tightly regulates the expression of key virulence factors that enable *S.* Tm to withstand host immune defenses within macrophages. Additionally, we highlight the chemical and physical signals that *S*. Tm senses as cues to adapt to each of these environments. These strategies ultimately allow *S.* Tm to successfully adapt to these two disparate host environments. It is critical to better understand bacterial adaptation strategies because disruption of these pathways and mechanisms, especially those shared by multiple pathogens, may provide novel therapeutic intervention strategies.

Keywords: *Salmonella*, macrophages, signaling pathways, infection, microbiota

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Salmonella enterica SEROVAR TYPHIMURIUM INFECTION

Non-typhoidal serovars of *Salmonella enterica* (NTS) are leading causes of foodborne illness and diarrheal disease worldwide (Graham et al., 2000; Vojdani et al., 2008; Scallan et al., 2011; Ansari et al., 2012; Kabir et al., 2012; Kozak et al., 2013). In the United States, NTS infections result in more hospitalizations and deaths compared to infections caused by any other foodborne pathogen

(Scallan et al., 2011). Among NTS, serovar Typhimurium (*S.* Tm) is one of the most commonly isolated from patients around the globe (Galanis et al., 2006). NTS infections typically present as a self-limiting diarrheal disease (Acheson and Hohmann, 2001; Gordon, 2008); however, NTS gastrointestinal infections can develop into systemic disease in immunocompromised patients, as well as a small subset of immunocompetent patients (Acheson and Hohmann, 2001; Gordon, 2008). Currently, there are no effective vaccines against gastrointestinal infections. Additionally, treatment options are limited because antibiotics may lead to increased levels of *S.* Tm shedding and also because *S.* Tm is developing resistance to many antibiotics (Wiström et al., 1992; Martin, 2012; Diard et al., 2014; Gopinath et al., 2014; Strugnell et al., 2014). Accordingly, alternative therapeutic intervention strategies are needed.

S. Tm establishes infection in the gastrointestinal tract and causes acute gastroenteritis. A common feature of *S.* Tm disease is inflammatory diarrhea indicated by the presence of neutrophils in patient stool samples (Harris et al., 1972). Type III secretion systems (T3SSs) are molecular syringe-like structures that allow Gram-negative organisms to directly inject effector proteins into the cytosol of host cells (Deng et al., 2017). *S.* Tm uses a T3SS encoded within *Salmonella* Pathogenicity Island (SPI) 1 (T3SS-1) to actively invade epithelial cells, induce inflammation, and breach the epithelial barrier (Galán and Curtiss, 1989; Tsolis et al., 1999). After exiting the intestinal tract, *S.* Tm is phagocytosed by resident and recruited immune cells, including macrophages. *S.* Tm utilizes the SPI-2 encoded T3SS (T3SS-2) to survive and replicate within these phagocytes (Hensel et al., 1995; Ochman et al., 1996; Shea et al., 1996). The cumulative effects of T3SS-2 cause unchecked bacterial replication during systemic infection and lethal disease (Yoon et al., 2009).

Decades of research has identified a vast repertoire of *S.* Tm virulence determinants (extensively reviewed in Fàbrega and Vila, 2013). Recent studies have expanded our understanding of factors that influence virulence gene expression, including growth phase and environmental signals (Kröger et al., 2013; Srikumar et al., 2015); however, less is known about the signal transduction pathways that link environmental signals to virulence gene expression. In this review, we discuss recent findings concerning strategies that *S.* Tm uses to overcome microbiota- and host-derived obstacles within the intestinal and intramacrophage environments. Additionally, we highlight signals that *S.* Tm uses to coordinate expression of virulence genes required for adaptation to these distinct environments.

MAKING ROOM WITHIN THE CROWDED INTESTINAL TRACT

The gastrointestinal tract is home to billions of microbes termed the microbiota. Interactions between the host, the microbiota, and pathogens have profound impacts on infection (Yurist-Doutsch et al., 2014; McKenney and Pamer, 2015; Bäumler and Sperandio, 2016; Gart et al., 2016; Kendall and Sperandio, 2016; McKenney et al., 2016). The microbiota function as a barrier to limit pathogen colonization and shedding (Endt et al., 2010), an ability collectively referred to as colonization resistance. Colonization resistance is largely attributed to the ability of the microbiota to outcompete invading pathogens for nutrients; however, the microbiota can also modulate host mucosal immune responses important for clearing infection (Endt et al., 2010; Thiemann et al., 2017). Dysbiosis, or alterations to the microbiota, creates a non-competitive niche in which *S.* Tm is able to establish infection and rapidly replicate in the intestine. Antibiotic use results in disruptions to the microbiota and is a key risk factor associated with the *Salmonella*-associated diarrhea (Acheson and Hohmann, 2001; Gordon, 2008).

Although antibiotic-related dysbiosis provides an opening for *S.* Tm to establish infection, *S.* Tm also directly perturbs the microbiota to enhance and prolong infection (**Figure 1A**). *S.* Tm relies primarily on the T3SS-1, and to an extent the T3SS-2, to induce host inflammation, and the resulting innate immune response non-specifically targets the microbiota along with *S.* Tm (Barthel et al., 2003; Coburn et al., 2005; Stecher et al., 2007; Barman et al., 2008; Lawley et al., 2008; Sekirov et al., 2008; Juricova et al., 2013; Lam and Monack, 2014; Drumo et al., 2016; Rivera-Chávez et al., 2016b). As a result of T3SS-induced inflammation, a proportion of infecting *S.* Tm cells succumb to host immune responses; however, a sufficient amount of *S.* Tm cells survive to successfully establish infection (Raffatellu et al., 2009; Liu et al., 2012; Bogomolnaya et al., 2013; Maier et al., 2014; Diaz-Ochoa et al., 2016). Several environmental conditions contribute to T3SS-1 expression (Golubeva et al., 2012). For example, oxygen limitation and high salt concentrations enhance SPI-1 expression and epithelial cell invasion (Galán and Curtiss, 1990; Lee and Falkow, 1990; Bajaj et al., 1996; Mizusaki et al., 2008), whereas bile and some long and short chain fatty acids, such as a butyrate, oleate, myristate, and palmitate, repress T3SS-1 expression (Prouty and Gunn, 2000; Lawhon et al., 2002; Gantois et al., 2006; Eade et al., 2016; Golubeva et al., 2016). The balance of activating and repressing signals is thought to enrich *S.* Tm invasion in the ileum *in vivo* (Lawhon et al., 2002; Gantois et al., 2006; Eade et al., 2016). Expression of the T3SS-1 is regulated by a feed-forward loop in which the regulatory proteins HilD, HilC, and RtsA positively control expression of the master transcription factor HilA (Bajaj et al., 1995; Ellermeier et al., 2005). HilD, HilC, and RtsA are transcription factors that bind to the *hilA* promoter to induce *hilA* expression (Schechter and Lee, 2001; Olekhnovich and Kadner, 2002; Ellermeier and Slauch, 2004). HilA then activates the expression of the remaining transcription factors and structural components of the T3SS-1, as well as non-SPI-1-encoded effectors (Phoebe Lostroh and Lee, 2001). Expression of the T3SS-1 core regulatory system is in turn regulated by accessory factors that are presumed to respond to environmental signals (Golubeva et al., 2012), but how these signals are incorporated into the SPI-1 regulatory pathway has not been fully elucidated.

Besides causing unspecific disruption to the microbiota as a whole, *S.* Tm-induced inflammation impacts particular intestinal microbes that alter concentrations of metabolites and/or host responses that would otherwise limit *S.* Tm infection (**Figure 1B**). For example, *S.* Tm infection induces RegIIIβ expression, and RegIIIβ is directly bactericidal against *Bacteroides* sp. and

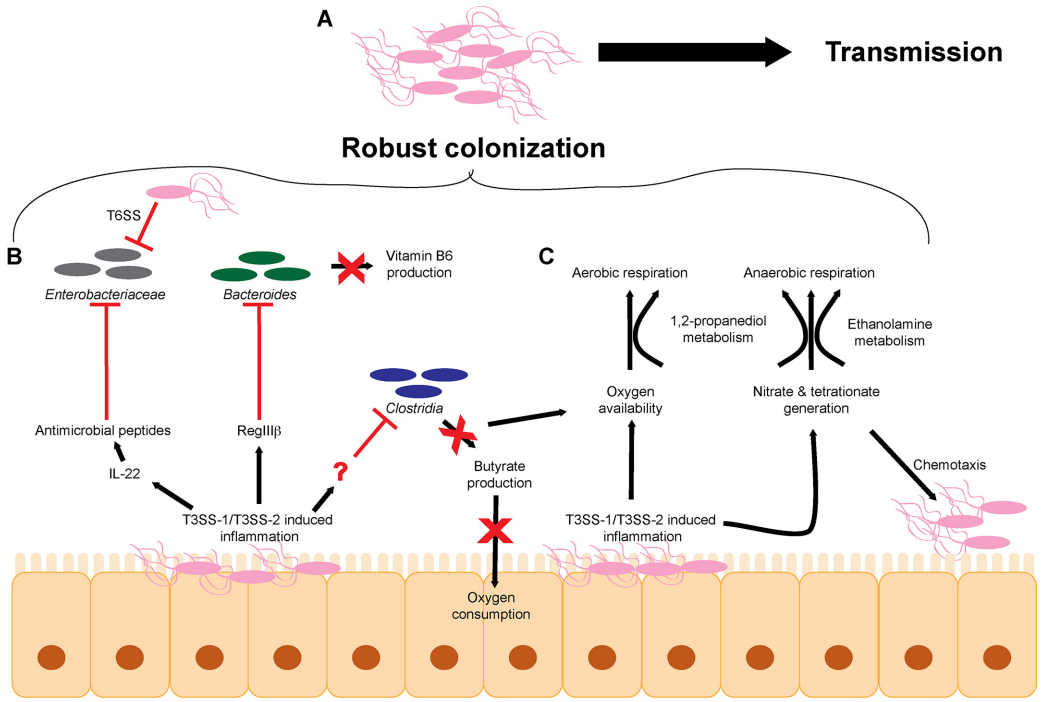

FIGURE 1 | *S.* Tm overcomes microbiota-mediated colonization resistance in the intestinal tract. **(A)** The cumulative effects of T3SS-1/T3SS-2-induced inflammation lead to robust colonization of the intestinal tract and efficient transmission to a new host. **(B)** *S.* Tm utilizes the T3SS-1 and T3SS-2 to generate a robust host immune response within the intestinal tract to eliminate members of the microbiota. **(C)** T3SS-1/T3SS-2-induced inflammation leads to the production of metabolites such as electron acceptors including oxygen, nitrate, and tetrathionate that promote aerobic and anaerobic respiration. T3SS, type III secretion system; T6SS, type VI secretion system.

Eubacterium rectale (Miki et al., 2017). RegIII lectin family proteins are expressed in the intestinal tract and are important for maintaining intestinal homeostasis and combating pathogens (Cash et al., 2006; Vaishnava et al., 2008; Miki et al., 2012). Suppression of *Bacteroides* sp. is associated with changes in metabolite availability in the gut, most notably a decrease in vitamin B6 concentrations (Miki et al., 2017). Significantly, reconstitution of *Bacteroides* or supplementation of vitamin B6 contributes to the resolution of *S.* Tm infection, further underscoring the complex interplay of host, bacteria, and metabolites (Miki et al., 2017). *S.* Tm T3SS-1 and T3SS-2-induced inflammation also leads to depletion of *Clostridia* sp. in the intestine, which enhances *S.* Tm colonization (Rivera-Chávez et al., 2016b) (detailed in the next section). The host factor that directly depletes *Clostridia* sp. has not been identified.

Additionally, commensal *Escherichia coli* represents a minor component of the microbiota during homeostasis, but during general dysbiosis, the proportion of *E. coli* within the bacterial community increases (Winter et al., 2013). *E. coli* and *S.* Tm compete for metabolites and deploy molecules to limit growth of each other (Raffatellu et al., 2009; Deriu et al., 2013; Sassone-Corsi et al., 2016). For example, iron is an essential nutrient for most microbes and is typically limited within the host; therefore, microbes have evolved mechanisms to scavenge iron (Behnsen and Raffatellu, 2016). *E. coli* and *S.* Tm produce and secrete siderophores, which are small molecules that chelate iron (Crouch et al., 2008; Behnsen and Raffatellu, 2016). *E. coli* siderophores can be conjugated to microcins, small antibacterial peptides that kill bacterial cells through an unknown mechanism (Rebuffat, 2012; Sassone-Corsi et al., 2016). To fight back, the *S.* Tm T3SS-1 and T3SS-2 promote expression of the chemokine IL-22, which results in the production of antimicrobials that kill *E. coli* but are ineffective against *S.* Tm (Godinez et al., 2008; Raffatellu et al., 2009; Stelter et al., 2011; Liu et al., 2012; Behnsen et al., 2014). By inducing IL-22, *S.* Tm eliminates Enterobacteriaceae species and thus direct nutritional competitors.

S. Tm also takes direct action against competitors using a type VI secretion system (T6SS). T6SSs are commonly found in the Proteobacteria and Bacteroidetes phyla and are structurally homologous to bacteriophage tail complexes (Bönemann et al., 2009; Russell et al., 2014; Hood et al., 2017). These dynamic structures contract to directly inject effector proteins into target cells (Basler et al., 2012). Although T6SS can inject effector proteins into host cells (Ma and Mekalanos, 2010; Schwarz et al., 2010), increasing evidence suggest that T6SSs primarily target and cause subsequent death of bacterial cells (Mougous et al., 2006; Pukatzki et al., 2006; Bingle et al., 2008; Cianfanelli et al., 2016). The T6SS encoded by *S.* Tm has selective bactericidal efficacy against commensal organisms *in vitro*, including other members of the Enterobacteriaceae, such as *E. coli* W3110, *Klebsiella oxytoca*, and *Klebsiella variicola* (Brunet et al., 2015;

Sana et al., 2016). However, *Enterobacter cloacae* and *E. coli* JB2 are resistant to *S.* Tm T6SS attack (Sana et al., 2016). It is currently unclear why different species are susceptible or resistant. The efficacy of the *S.* Tm T6SS against *K. oxytoca* data were validated *in vivo* during intestinal co-infection studies (Sana et al., 2016). These data support an important role for the *S.* Tm T6SS against other members of the microbiota; however, the benefits of selective killing remain to be defined. A better understanding of the commensal organisms that are directly targeted by *S.* Tm via the T6SS *in vivo* may reveal essential metabolites that are being differentially regulated that may impact *S.* Tm growth or virulence gene expression.

Collectively, these findings reveal that a central strategy *S.* Tm uses to colonize the host is to actively displace members of the microbiota. *S.* Tm achieves this through manipulation and exploitation of host responses (Stecher et al., 2007; Mooney et al., 2015). Moreover, *S.* Tm is capable of directly eliminating particular bacterial species. This transforms the intestinal tract into an environment that *S.* Tm is optimized to survive in and ultimately lowers the barrier of colonization resistance.

METABOLISM IN THE FACE OF INFLAMMATION

Dysbiosis not only results in changes in the bacterial populations but also changes the chemistry of the intestine (Zeng et al., 2017). Hence, a second aspect of *S.* Tm infection includes exploiting nutrients generated specifically during infection. The majority of bacteria that comprise the microbiota are obligate anaerobes that rely on fermentation for growth (Gibson and Roberfroid, 1995). Enterobacteriaceae, including *S.* Tm, are able to gain energy by respiration. Respiration generates higher amounts of ATP compared to fermentation and thereby enables *S.* Tm to outgrow many members of the microbiota (Wiles et al., 2006; Brochier-Armanet et al., 2009; Marteyn et al., 2010; Maier et al., 2013; Ng et al., 2013; Winter et al., 2013; Zeng et al., 2017) (**Figure 1C**). During homeostasis, host colonocytes consume oxygen, yielding a localized environment characterized by low oxygen partial pressure (Carreau et al., 2011; Espey, 2013). Colonocytes preferentially oxidize short-chain fatty acids, such as butyrate, to respire oxygen (Hamer et al., 2008; Donohoe et al., 2012; Kelly et al., 2015). Because *Clostridia* are the major producers of butyrate in the intestine, depletion of *Clostridia* with antibiotics or during *S.* Tm infection leads to a concomitant decrease in oxygen consumption by colonocytes (Sekirov et al., 2008; Louis and Flint, 2009; Gill et al., 2012; Vital et al., 2014; Rivera-Chávez et al., 2016b). *S.* Tm capitalizes on newly available oxygen to rapidly grow within the intestine (Rivera-Chávez et al., 2016b). These findings reveal a complex role for oxygen during infection. On the one hand, low levels of oxygen enhance SPI-1 expression, leading to increased inflammation and subsequent restriction of commensal organisms such as *Clostridia*. On the other hand, newly available oxygen promotes growth within the intestine. Oxygen limitation might therefore allow *S.* Tm to regulate the kinetics of SPI-1 expression within the intestine. In this proposed model, oxygen limitation early on during infection would promote SPI-1 expression, which would then in turn deplete commensal *Clostridia* via the host immune response, which would then result in an increase in oxygen availability leading to *S.* Tm outgrowth and subsequent reduction in SPI-1 expression. Additionally, the decrease in butyrate availability may also restrict the abundance of butyrate metabolizing members of the microbiota leading to even greater levels of dysbiosis.

The electron acceptors nitrate and tetrathionate also support *S.* Tm growth during infection (Winter et al., 2010; Lopez et al., 2012, 2015). The T3SS-1-secreted effector SopE is a bacteriophage-encoded activator of host Rho GTPases that results in host cytoskeletal rearrangements and activation of immune signaling pathways (Hardt et al., 1998). Additionally, SopE induces expression of host inducible nitric oxide synthase (iNOS) and generates nitrate (Lopez et al., 2012). Tetrathionate generation is a two-step process. Microbiota-produced hydrogen sulfide is converted by host colonocytes to thiosulfate, which reacts with oxygen radicals produced by host NADPH oxidase to generate tetrathionate (Winter et al., 2010). Tetrathionate reduction is coupled to ethanolamine, 1,2-propanediol, or fructose-asparagine oxidation (Price-Carter et al., 2001; Thiennimitr et al., 2011; Ali et al., 2014; Sabag-Daigle et al., 2016; Faber et al., 2017). Ethanolamine is a component of phosphatidylethanolamine, one of the most abundant phospholipids in host and microbial membranes (Randle et al., 1969; Dawaliby et al., 2015). The turnover of enterocytes and microbial cells as well as the diet provide a continuously replenished source of ethanolamine in the intestine (Cotton, 1972; Kawai et al., 1974; Dowhan, 1997, 2003; Snoeck et al., 2005; Bakovic et al., 2007). The accumulation of 1,2-propanediol depends primarily on the microbiota. It is thought that the ability of *Bacteroides* sp. to breakdown complex carbohydrates allows for the production of 1,2-propanediol as a byproduct of fermentation of methyl-pentoses (Faber et al., 2017), although experiments are needed to demonstrate this during the course of infection. This model is supported by the findings that 1,2-propanediol is nearly undetectable in germ-free mice; however, the presence of either *Bacteroides fragilis* or *Bacteroides thetaiotaomicron* is associated with 1,2-propanediol accumulation (Faber et al., 2017). Additionally, expression of *S.* Tm genes coding for 1,2-propanediol metabolism are induced in the presence of *B. thetaiotaomicron in vivo* (Ng et al., 2013; Faber et al., 2017). Furthermore, the presence of *B. thetaiotaomicron* within the intestinal tract enhances *S.* Tm expression of additional carbohydrate metabolism and transport genes, including sialic acid and fucose catabolic pathways (Ng et al., 2013). The *B. thetaiotaomicron*-encoded sialidase liberates sialic acid, which promotes *S.* Tm sialic acid metabolism and intestinal growth (Ng et al., 2013). These data indicate a somewhat paradoxical relationship between *S.* Tm and *Bacteroides* sp. As discussed above, *S.* Tm-induced inflammation restricts the levels of *Bacteroides* sp. (Miki et al., 2017) and yet *Bacteroides* sp. are critical for the accumulation of multiple metabolites that *S.* Tm utilizes during infection (Ng et al., 2013; Faber et al., 2017). These seemingly conflicting ideas suggest an even more complex relationship between pathogen and microbiota. Rather than presence or absence of commensals,

S. Tm may require a fine-tuned abundance of microbes to generate beneficial metabolites without these organisms directly competing with *S.* Tm.

S. Tm also exploits electron acceptors as spatiotemporal cues for colonization and tissue invasion. Nitrate and tetrathionate are indirect signals for *S.* Tm chemotaxis and thereby influence subsequent stages in *S.* Tm infection (Rivera-Chavez et al., 2013). The chemotaxis proteins Aer and Tsr sense changes in redox and proton motive force during tetrathionate and nitrate respiration, respectively (Edwards et al., 2006; Rivera-Chávez et al., 2016a). Both Aer and Tsr promote T3SS-1/T3SS-2-dependent intestinal colonization (Rivera-Chavez et al., 2013). Tsr-dependent chemotaxis correlates with localized host production of iNOS and enables *S.* Tm to invade ileal Peyer's patches (Rivera-Chávez et al., 2016a). Surprisingly, no additive effect is seen with Aer and Tsr-dependent chemotaxis (Rivera-Chavez et al., 2013). This suggests that these two sensing pathways may be interconnected and perhaps functionally redundant. Connectivity of these two pathways is further supported by the observation that the presence of nitrate reduces the expression of genes involved in tetrathionate respiration as well as the growth advantage conferred by tetrathionate respiration (Lopez et al., 2012). It is currently unclear how *S.* Tm balances the sensing and utilization of two signals that may restrict one another.

Much of our understanding of the host–microbiota–pathogen interplay has been generated through genetic manipulation of the pathogen, and alternative experimental approaches are shedding new light on this interaction. For example, defined commensal communities have been used to reconstitute the microbiota of germ-free mice and study the contributions of specific members of the microbiota necessary for effective colonization resistance (Dewhirst et al., 1999; Stecher et al., 2010; Brugiroux et al., 2016). Studies using defined microbial communities will also contribute to understanding how differences in microbial species between susceptible and resistant intestinal environments impact *S.* Tm virulence gene expression. Additionally, a recent RNA-seq study assessed gene expression in *S.* Tm grown under 22 different *in vitro* conditions that mimicked aspects of infection, which could reveal new signals important for controlling the expression of key metabolic and virulence determinants (Kröger et al., 2013). Future *in vivo* metabolomics studies may identify specific microbiota or diet-dependent molecules that impact *S.* Tm virulence gene expression and/or host response. In line with this, a high salt diet was recently identified as significantly altering the host response during *S.* Tm infection (Tubbs et al., 2017). Thus, the ability to manipulate commensal microbial communities and intestinal metabolite concentrations offers an exciting tool to further understand how microbiota and metabolites not only impact *S.* Tm growth but also impact *S.* Tm virulence.

Collectively, these findings highlight that *S.* Tm thrives during host dysbiosis, benefits from the host immune response, and utilizes virulence factors to directly and indirectly suppress members of the microbiota. However, *S.* Tm relies on members of the indigenous microbial community to expand its metabolic capabilities during infection that in turn promote outgrowth and transmission (Maier et al., 2013). Although some host factors and cytokines that are involved in the development of inflammation during infection are known (Behnsen et al., 2015), additional components are likely to contribute to *S.* Tm infection. A more comprehensive understanding of immune responses that contribute to dysbiosis and/or generate a nutritional niche for *S.* Tm is necessary to fully understand *S.* Tm infection strategies, and indeed, this remains an active area of research. Host inflammatory components could be enhancing intestinal pathology, restricting microbiota reconstitution, molding a new nutrient niche, or enhancing all aspects of infection.

INTRAMACROPHAGE ADAPTATION

After benefiting from components of the host immune response during intestinal colonization, *S.* Tm must withstand the bactericidal efforts of host phagocytes during systemic infection. *S.* Tm dissemination from the intestinal tract to systemic sites of infection largely depends on phagocytic cells (Vazquez-Torres et al., 1999). Of the host phagocytes, macrophages frequently interact with *S.* Tm during dissemination and within systemic sites, including the spleen and liver (Salcedo et al., 2001). Macrophages utilize several strategies such as acidification of phagosomes, generation of reactive oxygen and nitrogen species, and production of antimicrobial proteins and peptides to kill internalized pathogens (Flannagan et al., 2009); however, *S.* Tm is able to withstand these defense mechanisms through multiple molecular mechanisms. The T3SS-2 and associated secreted effectors create a replicative niche within macrophages termed the *Salmonella* containing vacuole (SCV) by modulating diverse host processes (Hensel et al., 1995; Ochman et al., 1996; Shea et al., 1996; Figueira and Holden, 2012). For example, these effectors inhibit SCV–lysosome fusion, modify host vesicle trafficking, localize the SCV within the cell, and evade the host autophagic response (Uchiya et al., 1999; Shotland et al., 2003; Guignot et al., 2004; Boucrot et al., 2005; Brumell and Scidmore, 2007; Jackson et al., 2008; Owen et al., 2014, 2016). Multiple environmental signals and bacterial regulators influence this critical adaptation step (discussed below).

COUNTERACTING HOST DEFENSE MECHANISMS

Pathogen recognition is the first line of defense for phagocytic cells such as macrophages. Host Toll-like receptors (TLRs) are transmembrane proteins located on the plasma membrane and endosomal membranes that recognize conserved molecular patterns associated with pathogens such as lipids, proteins, and nucleic acids (Kawai and Akira, 2010). TLRs have profound impacts on adaptive immunity and are thus broadly essential for host defense (Iwasaki and Medzhitov, 2004). Additionally, TLRs are critical for control of *S.* Tm replication as well as host survival during infection (Weiss et al., 2004). TLR activation and downstream signaling through the adapter proteins MyD88 and TRIF is linked to initial SCV acidification (Arpaia et al., 2011). The SCV acidifies rapidly following phagocytosis, dropping to a

pH between 4.0 and 5.0 within 60 min of formation (Rathman et al., 1996). Acidification of the SCV is essential for *S.* Tm expression and functional formation of the T3SS-2 as well as secretion of effectors (Rathman et al., 1996; Beuzón et al., 1999; Hansen-Wester et al., 2002; Rappl et al., 2003; Arpaia et al., 2011; Chakraborty et al., 2015). Thus, *S.* Tm co-opts part of the macrophage defense system to serve as an initiating signal that promotes bacterial survival.

In the host, concentrations of free iron are extremely low in part to limit growth of invading pathogens (Kühn, 2015; Behnsen and Raffatellu, 2016). For example, the host expresses iron regulatory proteins (IRPs) and lipocalin-2 to limit the amount of iron available within macrophages (Kühn, 2015; Nairz et al., 2015a,b). Host-mediated iron limitation significantly restricts *S.* Tm replication within macrophages and reduces lethal disease (Nairz et al., 2015a). Additionally, IRP and lipocalin-2 influence the immune response during *S.* Tm infection (Nairz et al., 2015a,b). Indeed, modulating the immune response, specifically interleukin 10, can rescue the bactericidal defects of lipocalin-2 deficient cells and mice (Nairz et al., 2015b). These results suggest that host iron concentrations indirectly affect *S.* Tm survival through alteration of the host immune response rather than by directly starving *S.* Tm of iron. Nonetheless, modulation of iron concentrations does influence *S.* Tm expression of virulence genes. For example, SPI-2 expression is reduced when *S.* Tm is grown in the presence of iron (Choi and Groisman, 2013; Choi et al., 2014), and the iron-sensing transcription factors Fur and PmrA limit SPI-2 expression during macrophage infection (Wösten et al., 2000; Choi and Groisman, 2013; Choi et al., 2014). However, SPI-2 expression has also been shown to be reduced when iron is chelated from cultures and during infection of macrophages that have low iron concentrations (Zaharik et al., 2002; Nairz et al., 2009). These contrasting findings require further investigation. Similarly, it is unclear what the iron availability is within the SCV with and without host iron acquisition mediators IRP and lipocalin-2.

Altogether, these findings highlight that *S.* Tm senses and responds to macrophage defense mechanisms, which impact *S.* Tm virulence and survival. By utilizing host defenses as signals, *S.* Tm incorporates antimicrobial processes into a bacterial signal transduction pathway that creates a suitable replication niche in an otherwise inhospitable environment.

INTRAMACROPHAGE SENSING AND SIGNALING

In addition to host defense-linked signals, *S.* Tm responds to concentrations of cations, nutrients, and ATP to activate expression of SPI-2 and other virulence factors to ensure survival (Valdivia and Falkow, 1997; Cirillo et al., 1998; Deiwick et al., 1999; Kim and Falkow, 2004; Löber et al., 2006; Osborne and Coombes, 2011; Lee and Groisman, 2012; Blair et al., 2013) (**Figure 2**). To sense these environmental signals and regulate SPI-2 expression, *S.* Tm uses several two-component systems (TCS) (Fass and Groisman, 2009). TCS are typically comprised of a sensor kinase that autophosphorylates upon sensing of a stimulus and then phosphorylates its paired response regulator, which in turn binds DNA to activate transcription of target genes (Stock et al., 2000). The PhoPQ TCS senses acidic pH, divalent cations, antimicrobial peptides, and potentially other signals to activate SPI-2 expression and modify components of the bacterial outer membrane (Bader et al., 2005; Prost et al., 2007; Dalebroux et al., 2014; Hicks et al., 2015). In addition to SPI-2, PhoP regulates other virulence factors, including the *mgtCBR* operon. The *mgtCBR* operon encodes an inner membrane protein, a Mg^{2+} transporter, and a regulator and is critical for intramacrophage survival (Soncini et al., 1996; Blanc-Potard and Groisman, 1997; Alix and Blanc-Potard, 2007; Lee and Groisman, 2010). It is still unclear what host or bacterial factors contribute to the presence of these SPI-2 activating signals within the SCV.

S. Tm also responds to signals that are not specific to the SCV, but rather are found in multiple environments throughout the host. For example, the host hormones epinephrine and norepinephrine (epi/NE) are ubiquitous throughout the body (Boyanova, 2017). The bacterial TCS QseBC and QseEF sense and respond to epi/NE during infection (Clarke et al., 2006; Reading et al., 2009). The sensor kinase QseC regulates the expression of genes encoded within SPI-1 and enhances epithelial cell invasion under conditions that promote SPI-1 expression (Moreira et al., 2010). Additionally, during macrophage infection, QseC activates SPI-2 expression to enhance intramacrophage survival (Moreira et al., 2010). Moreover, both epi/NE-responsive histidine sensor kinases, QseC and QseE, are required for systemic infection (Rasko et al., 2008; Moreira et al., 2010; Moreira and Sperandio, 2012). NE induces expression of both SPI-1 and SPI-2 associated genes, however, only expression of SPI-2 associated genes is QseC dependent (Moreira et al., 2010). These findings reveal that the same signal (epi or NE) enhances *S.* Tm virulence gene expression depending on the surrounding environment. The additional components of the intramacrophage environment that allow these epi/NE-dependent signaling pathways to distinguish between a SPI-1 or SPI-2 inducing condition warrant further studies.

Ethanolamine is another signal that plays environment-dependent roles in expression of *S.* Tm virulence traits (Anderson and Kendall, 2016). Ethanolamine is present in serum and is maintained intracellularly by host cells in part to recycle and produce phosphatidylethanolamine (Nikawa et al., 1986; Lipton et al., 1988, 1990; Sandra and Cai, 1991; Shiao and Vance, 1995). In the Enterobacteriaceae, including *S.* Tm, the transcription factor EutR directly senses ethanolamine (Roof and Roth, 1992; Luzader et al., 2013). EutR-dependent signaling promotes ethanolamine metabolism during intestinal infection (Anderson et al., 2015). As infection progresses, ethanolamine promotes *S.* Tm dissemination to systemic sites independently of metabolism (Anderson et al., 2015). Although ethanolamine metabolism does not provide a growth benefit for *S.* Tm during systemic infection (Stojiljkovic et al., 1995; Thiennimitr et al., 2011; Steeb et al., 2013; Anderson et al., 2015), EutR directly activates expression of SPI-2 within macrophages leading to increased survival and early dissemination (Anderson et al., 2015). It is

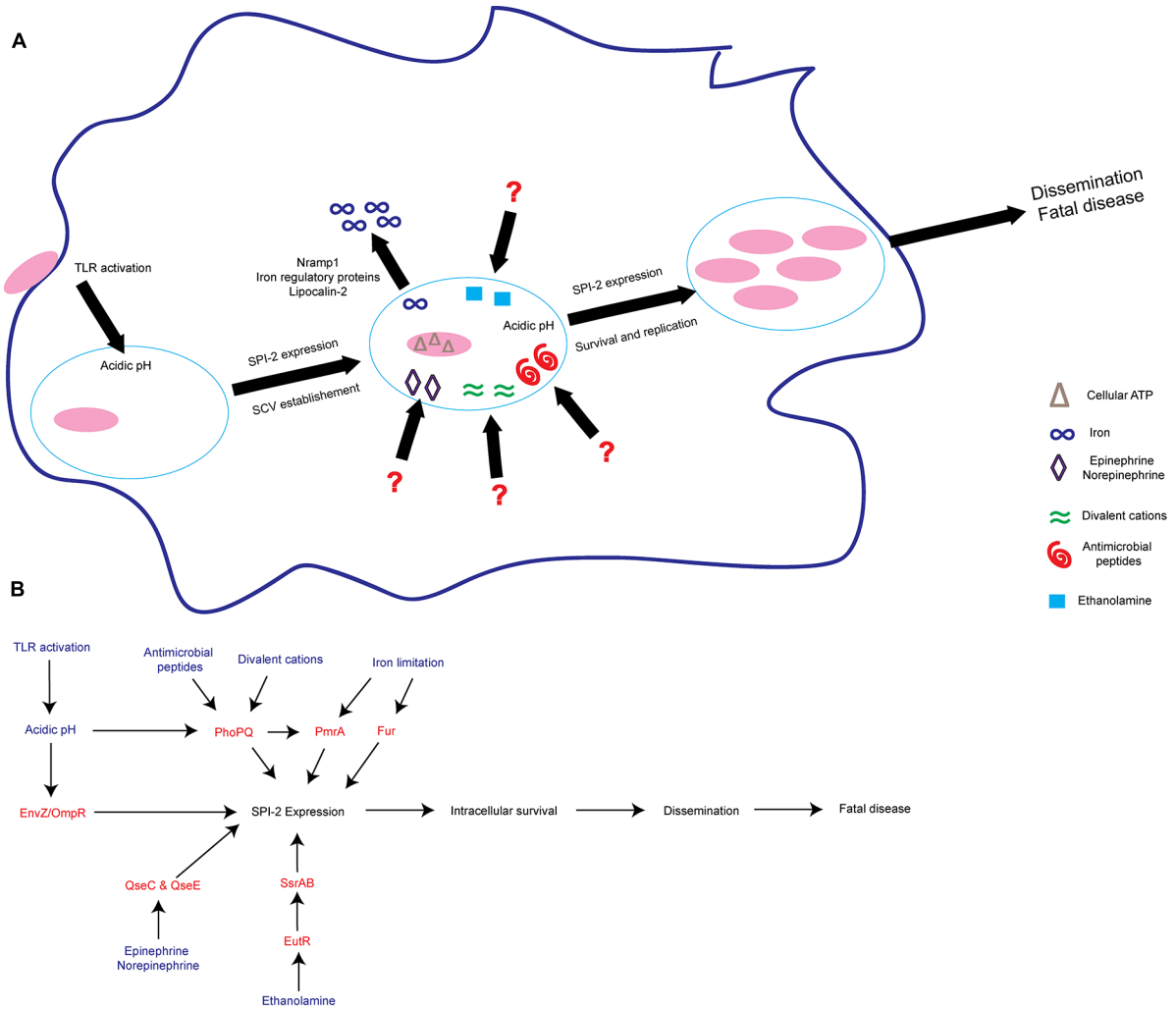

FIGURE 2 | *S.* Tm senses environmental cues to adapt to the intramacrophage environment. **(A)** *S.* Tm recognizes diverse signals within the macrophage. These include iron limitation, ethanolamine, acidic pH, antimicrobial peptides, divalent cations, epinephrine/norepinephrine, and cellular ATP. These signals act to induce SPI-2 expression and establish the SCV as a replicative niche. **(B)** Schematic of some of the regulatory proteins and associated signals important for activating SPI-2 expression. Signals are written blue; regulatory proteins and TCS are written in red. TLRs, Toll-like receptors; SCV, *Salmonella* containing vacuole.

currently unclear how ethanolamine can signal through the same receptor, EutR, to promote niche adaptation in distinct host environments. However, it is clear that ethanolamine plays a dual role in *S.* Tm infection by supporting growth in the inflamed intestine as well as enhancing subsequent stages of *S.* Tm disease.

The production of *Salmonella*-induced filaments (SIFs) is a critical component of *S.* Tm adaptation to intracellular environments (Garcia-del Portillo et al., 1993; Stein et al., 1996). This SIF network remodels the host endosomal network, allowing *S.* Tm to gain access to endocytosed molecules (Ohlson et al., 2008; Liss et al., 2017). The SCV/SIF continuum is required for efficient intracellular metabolism and promotes *S.* Tm replication (Liss et al., 2017). While the focus of this study was on access to metabolites (Liss et al., 2017), the same principle of incorporating extracellular molecules into the SCV may be true for signaling molecules that promote virulence. In such a model, signaling molecules present within host endosomes, or recently endocytosed molecules from the extracellular environment, would be shuttled through the SCV/SIF continuum and potentially impact *S.* Tm virulence gene expression. This revelation opens the possibility that large sets of molecules within various host tissues are able to reach the SCV within macrophages *in vivo*. Additionally, genes that have previously been identified as not being induced during macrophage infection *in vitro* may be the result of a relevant signal being absent from the culture conditions. Determining *in vivo* signals that *S.* Tm recognizes remains a daunting challenge but is necessary for a thorough understanding of the events that trigger intracellular adaptation.

The majority of work on environmental signals and sensing within macrophages has focused on transcriptional regulation; however, recent proteomic and RNA-seq approaches have shown

that regulation of gene expression is more complex. Post-translational modifications as well as small regulatory RNA (sRNA)-induced post-transcriptional changes regulate virulence during macrophage infection (Ansong et al., 2013; Westermann et al., 2016). sRNAs are non-coding 50–500 nucleotide transcripts that utilize base-pair interactions to post-transcriptionally regulate the expression of target mRNAs (Hébrard et al., 2012). *S.* Tm encodes approximately 300 unique sRNAs, and the expression of a subset of sRNAs is highly sensitive to signals encountered within the macrophage environment, such as nutrient starvation, and are controlled by key components of the SPI-2 regulatory system (Kröger et al., 2012, 2013; Amin et al., 2016; Colgan et al., 2016). Specifically, the sRNA PinT regulates SPI-2 expression and is critical for host adaptation *in vivo* (Chaudhuri et al., 2013; Westermann et al., 2016). It is possible that post-transcriptional regulation helps *S.* Tm incorporate environmental signals sensed within the SCV to modulate survival.

In addition to active intracellular replication, entering a non-replicating yet viable state is an alternative adaptation strategy. A portion of the infecting *S.* Tm population enters a persistent state within macrophages that is independent of SPI-2 (Helaine et al., 2010). These non-replicating *S.* Tm remain viable and are not killed by macrophage antimicrobial defenses (Helaine et al., 2010). This phenomenon has also been demonstrated within non-phagocytic cells *in vivo* (Núñez-Hernández et al., 2013). It is unclear how these persister cells impact disease progression *in vivo*, but perhaps persister cells promote asymptomatic carriage and transmission. Future studies are required to determine if there is transcriptional overlap between these persister cells and actively replicating *S.* Tm or if perhaps post-transcriptional regulation contributes to this phenotypic switch. Advancements in single cell expression techniques will allow replicating and dormant cells to be distinguished from one another. Similarly, it remains unclear what signals present within the SCV trigger the shift from replication to persistence. Although distinct signals may be responsible for transitioning to a persistent state, it is also possible that loss of the signals important for replication leads to dormancy.

Altogether, these studies reveal complex and dynamic regulatory circuits important for *S.* Tm survival within macrophages. *S.* Tm must appropriately repress and activate virulence gene expression to ensure adaptation to the SCV (Kato et al., 2003; Choi and Groisman, 2013; Westermann et al., 2016). Additionally, *S.* Tm must be able to initiate distinct regulatory pathways at different time points during infection (Moreira et al., 2010; Moreira and Sperandio, 2012; Anderson et al., 2015). Further understanding of these activating and suppressing cues, and how they are balanced with one another, will enhance our understanding of *S.* Tm pathogenesis.

HOST CELL DEATH

The replication-suitable SCV within macrophages is a temporary niche, as host cells die. The way in which cells die has a tremendous impact on host physiology and inflammation (Pasparakis and Vandenabeele, 2015; Medina and Ravichandran, 2016). Programmed host cell death can be classified based on the effector proteins required and the state of inflammation each type of death induces (Kroemer et al., 2009). Three of the major programmed cell death pathways are apoptosis, necroptosis, and pyroptosis (Kroemer et al., 2009). *S.* Tm infection induces apoptosis-like features in infected macrophages including chromatin condensation and caspase-3 and caspase-8 activity (Chen et al., 1996; Lindgren et al., 1996; Monack et al., 1996; van der Velden et al., 2000; Man et al., 2013; Günster et al., 2017). Additionally, *S.* Tm infection can induce RIPK3 and MLKL-dependent necroptosis (Robinson et al., 2012; Günster et al., 2017). The best-characterized form of *S.* Tm-induced cell death is caspase-1/caspase-11-dependent pyroptosis (Brennan and Cookson, 2000; Monack et al., 2001; Fink and Cookson, 2006; Fink et al., 2008). Host cells employ several means to recognize *S.* Tm virulence proteins and initiate regulated pyroptosis. For example, macrophage NLRC4 (Ipaf) recognizes *S.* Tm flagellin and components of T3SS-1 to activate caspase-1 (Franchi et al., 2006; Miao et al., 2006, 2010b; Zhao et al., 2011). Additionally, macrophage NAIP1 also recognizes components of T3SS-1 to initiate a pyroptotic death (Rayamajhi et al., 2013; Yang et al., 2013). Caspase-1 and caspase-11, as well as their activators NLRP3 and NLRC4, impact *S.* Tm pathogenesis and bacterial burden *in vivo* (Lara-Tejero et al., 2006; Raupach et al., 2006; Broz et al., 2010, 2012). Interestingly, the host is able to enhance clearance of *S.* Tm infection when pyroptosis is experimentally induced *in vivo* (Miao et al., 2010a; Stewart et al., 2011; Aachoui et al., 2013; Jorgensen et al., 2016). These findings suggest that *S.* Tm might try to evade inducing host cell death during some stages of infection. Although most commonly studied in macrophages, *S.* Tm-induced cell death also occurs in dendritic and epithelial cells (van der Velden et al., 2003; Sellin et al., 2014; Rauch et al., 2017). It remains uncertain if infected cells are simply overwhelmed by *S.* Tm over time *in vitro*, if *S.* Tm actively regulates virulence to promote or evade host cell death *in vivo*, if cell death is occurring throughout infection, and what the consequences of host cell death are during systemic disease.

CONCLUSION

S. Tm is able to recognize, adapt to, and survive within the intestinal tract and the intramacrophage environment during infection. These two environments present very different obstacles to infection, and *S.* Tm utilizes diverse strategies to overcome these distinct barriers. A better understanding of the signaling molecules, the signal transduction pathways, and the cross talk between signal transduction pathways that promote niche recognition may provide novel opportunities for therapeutic intervention. Importantly, several of the strategies used by *S.* Tm to adapt to host environments are conserved among several organisms such as pathogenic *E. coli*, *Listeria monocytogenes*, *Brucella abortus*, and *Staphylococcus aureus*. For

example, pathogens adapt to the host or regulate virulence by utilizing and/or sensing nitrate (Spees et al., 2013; Winter et al., 2013), epi/NE (Curtis et al., 2014; Halang et al., 2015; Moreira et al., 2016; Rooks et al., 2017), ethanolamine (Maadani et al., 2007; Kendall et al., 2012; DebRoy et al., 2014; Gonyar and Kendall, 2014; Mellin et al., 2014; Subashchandrabose et al., 2014), iron (Almirón et al., 2001; Zimbler et al., 2012; Hammer et al., 2013, 2014; Mashruwala et al., 2015; Palmer and Skaar, 2016), and pH (Heinzen et al., 1996; Sturgill-Koszycki and Swanson, 2000; Singh et al., 2008; Vandal et al., 2008). Additionally, T6SS are widely conserved, particularly within Proteobacteria and Bacteroidetes (Hood et al., 2017), and promote colonization of intestinal pathogens (Fu et al., 2013; Sana et al., 2017). Perhaps the fact that *S.* Tm utilizes all of these strategies, rather than a select few, makes *S.* Tm so successful and such a major burden on global healthcare (Scallan et al., 2011). Therefore, further study of these environmental adaptation strategies, using *S.* Tm as a model organism, will enhance our understanding of the host–microbiota–pathogen interface.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Microbiology

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A Negative Regulator of Cellulose Biosynthesis, *bcsR*, Affects Biofilm Formation, and Adhesion/Invasion Ability of *Cronobacter sakazakii*

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Cronobacter sakazakii is an important foodborne pathogen that causes neonatal meningitis and sepsis, with high mortality in neonates. However, very little information is available regarding the pathogenesis of *C. sakazakii* at the genetic level. In our previous study, a cellulose biosynthesis-related gene (*bcsR*) was shown to be involved in *C. sakazakii* adhesion/invasion into epithelial cells. In this study, the detailed functions of this gene were investigated using a gene knockout technique. A *bcsR* knockout mutant (*ΔbcsR*) of *C. sakazakii* ATCC BAA-894 showed decreased adhesion/invasion (3.9-fold) in human epithelial cell line HCT-8. Biofilm formation by the mutant was reduced to 50% of that exhibited by the wild-type (WT) strain. Raman spectrometry was used to detect variations in biofilm components caused by *bcsR* knockout, and certain components, including carotenoids, fatty acids, and amides, were significantly reduced. However, another biofilm component, cellulose, was increased in *ΔbcsR*, suggesting that *bcsR* negatively affects cellulose biosynthesis. This result was also verified via RT-PCR, which demonstrated up-regulation of five crucial cellulose synthesis genes (*bcsA, B, C, E, Q*) in *ΔbcsR*. Furthermore, the expression of other virulence or biofilm-related genes, including flagellar assembly genes (*fliA*, *C*, *D*) and toxicity-related genes (*ompA*, *ompX*, *hfq*), was studied. The expression of *fliC* and *ompA* in the *ΔbcsR* mutant was found to be remarkably reduced compared with that in the wild-type and the others were also affected excepted *ompX*. In summary, *bcsR* is a negative regulator of cellulose biosynthesis but positively regulates biofilm formation and the adhesion/invasion ability of *C. sakazakii*.

Keywords: *bcsR*, *Cronobacter sakazakii*, cellulose biosynthesis, biofilm formation, adhesion/invasion

INTRODUCTION

Cronobacter sakazakii is a Gram-negative, non-spore-forming, rod-shaped bacterium within the genus of *Cronobacter* spp., which is a group of emerging opportunistic pathogens associated with meningitis, septicemia, and necrotizing enterocolitis in neonates and infants. The mortality among infected infants is as high as 40–80% (Iversen and Forsythe, 2003). In addition, some patients who survive the disease suffer from mental or physical developmental delay and quadriplegia (Lai, 2001; Holy and Forsythe, 2014). *Cronobacter* spp. also infect elderly and immunocompromised

adults. Unlike neonatal infection, *Cronobacter* is associated with bacteremia, osteomyelitis, splenic abscesses, pneumonia, conjunctivitis, wound infections, and urinary tract infections in adults (Blackwood and Hunter, 2016).

C. sakazakii has the capacity to adhere to human intestinal epithelial and brain microvascular endothelial cells (Quintero et al., 2011). The first step before colonization and infection for most pathogens is adherence to host cell surfaces. Bacterial variants that do not express adherence factors are unable to adhere and initiate infections; thus, adherence is important for pathogenicity (Cleary et al., 2004). Adherence to intestinal epithelial cells is a key step that determines whether an infection becomes refractory to treatment because *C. sakazakii* entries into intestinal epithelial cells is followed by entrance into the bloodstream, invasion of brain microvascular endothelial cells, and survival in the cerebrospinal fluid (Mange et al., 2006; Nair et al., 2009).

The ability of bacteria to attach to a surface and form biofilms facilitates the development of a continuous source of contamination and maintains disease incidence (Kives et al., 2006). A biofilm is generally defined as a structured community of bacterial cells that are adherent to a zoetic or abiotic surface and acts as an adhesive foundation and defense barrier (Aparna and Yadav, 2008). *Cronobacter* spp. are capable of attaching to different surfaces to form biofilms to resist multiple stress conditions, improve adherence, and increase pathogenesis (Lehner et al., 2005; Kim et al., 2006). Therefore, the investigation of biofilm characteristics, such as formation capacity and biochemical components, is helpful to better understand the mechanisms underlying *C. sakazakii* adherence, invasion of epithelial cells, and pathogenesis.

Some virulence factors involved in the interaction between *C. sakazakii* and host cells have been reported, including outer membrane protein A (OmpA) (Nair and Venkitanarayanan, 2007) and extracellular polysaccharides (EPS) (Iversen et al., 2004). However, very little information is available regarding *C. sakazakii* adhesion pathogenesis at the genetic level. In our previous work, we demonstrated the involvement of a cellulose production-related gene, *bcsR*, in interactions between *C. sakazakii* and intestinal epithelial cells using a random transposon insertion mutant library (Du et al., 2016). Cellulose (poly-β-(1→4)-D-glucose) is the most abundant biopolymer in nature. Most bacterial cellulose-producing genes encoding proteins involved in the UDP-glucose polymerization process are organized in a bacterial cellulose synthesis operon (*bcs*) (Wong et al., 1990; Recouvreux et al., 2008). The cellulose gene cluster, comprising 9 genes (*bcsCZBAQEFG* and *bcsR*), is present in nearly all *Cronobacter* strains (Ogrodzki and Forsythe, 2015). Among these 9 genes, *bcsR* is a short gene encoding a 62-amino-acid protein. Some studies have reported a role for *bcsR* in cellulose production in certain bacteria (Grimm et al., 2008; Serra et al., 2013). However, the role of this gene in the adhesion process is unclear.

In this study, we investigated the function of *bcsR* in *C. sakazakii* ATCC BAA-894 by constructing a gene mutant. The ability of the mutant to invade/adhere to HCT-8 cells was investigated and compared with that of the wild-type (WT) strain. Additionally, we assessed biofilm formation ability and performed Raman spectroscopy to analyze differences between the biochemical components in the WT and *ΔbcsR* mutant biofilms. This work helps to elucidate the role of *bcsR* in cellulose biosynthesis and *C. sakazakii* pathogenesis.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

All bacterial strains and plasmids used in this study are listed in **Table 1**. *C. sakaza*kii ATCC BAA-894 (WT and mutant strains) was routinely grown at 37°C in Tryptic Soy Broth (TSB; Difco, MD, USA) and on Tryptic Soy Agar (TSA; Difco, MD, USA) under aerobic conditions with constant shaking, unless indicated otherwise. When necessary, ampicillin, kanamycin, and chloramphenicol were used at 100, 100, and 10 μg/ml, respectively.

Construction of a *bcsR* Gene Deletion Mutant

Site-specific mutation of *C. sakazakii* ATCC BAA-894 was performed using the Lambda-Red recombination method described by Geng et al. (2009). Briefly, the kanamycin resistance cassette from plasmid pET-26b was amplified using primers *kana*-F/*kana*-R. Two primer pairs, 202U-F/202U-R and 202D-F/202D-R, were used to amplify the upstream and downstream DNA sequences of the *bcsR* gene from the whole genome sequence of *C. sakazakii* ATCC BAA-894, obtained from GenBank (Grim et al., 2012). The amplified upstream and downstream DNA fragments of the *bcsR* gene and kanamycin resistance cassette were treated with appropriate restriction enzymes and subsequently cloned into the pMD18-T vector to produce pMD-*202* (*kana*), respectively, which was then transferred into *E. coli* DH5α. The segment consisting of *bcsR* upstream-*kana*-*bscR* downstream was cut from the vector and transformed into the wild-type (WT) strain *C. sakazakii* ATCC BAA-894

TABLE 1 | Bacterial strains and plasmids used in this study.

Strains or plasmids	Genotype or characteristics	Reference or source
Cronobacter sakazakii		
ATCC BAA-894	WT	ATCC
ΔbcsR	*ΔbcsR*::km^r	This study
cpbcsR	*ΔbcsR* with pACYC184-202	This study
E. coli		
DH5α	γ^- Φ80d*lacZ*ΔM15Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_k^- m_K^-) *supE44 thi-1 gyrA relA1*	38
PLASMIDS		
pKD46	*oriR101 repA101*(Ts) Amp^r *araADpgam-bet-exo*	39
pACYC184	p15A *ori* Cm^r Tet^r	39
pACYC184-202	pACYC184 with *bcsR*	This study

(harboring the pKD46 plasmid) via electroporation, and the kanamycin-resistant transformant, i.e., the *202::kana* mutant (*ΔbcsR*), was selected. All primer sequences are listed in **Table 2**.

Complementation Study

A complementation plasmid, containing the *bcsR*-coding sequence and its own promoter, was constructed. The *bcsR* gene was amplified by PCR using the primers 202 cp-F/202 cp-R (restriction sites were introduced into the primers) from *C. sakazakii* ATCC BAA-894 genomic DNA. The product was cloned into pACYC184 at appropriate restriction sites and then transferred into the mutant to generate *ΔbcsR* harboring pACYC184-202, *cpbcsR*. Nucleotide sequencing was performed to verify the sequence of the *bcsR*-coding region in the recombinant plasmid and complemented strain.

Growth Curves

C. sakazakii strain ATCC BAA-894 (WT) and the *ΔbcsR* and *cpbcsR* strains were cultured overnight in TSB medium at 37°C and subcultured as a 1% overnight culture in 100 ml of TSB medium. The cultures were incubated with shaking at 200 rpm per minute at 37°C for 14 h in a 100-ml conical flask. The optical density was measured at 600 nm (OD_{600}) per hour.

Scanning Electron Microscopy (SEM)

SEM was performed to examine the morphological differences between the *C. sakazakii* ATCC BAA-894 (WT), *ΔbcsR,* and *cpbcsR* strains (Wang et al., 2011). Bacterial strains were grown to the logarithmic phase and collected via centrifugation, and the pellets were fixed with 2.5% glutaraldehyde overnight at 4°C. The cells were washed three times with distilled water (5 min), followed by dehydration with an ethanol series (25, 50, 70, 80, 90, and 100%). The cells were further dried via vacuum freeze-drying for 1.5–2.5 h. The dehydrated bacterial powder was observed with a Hitachi SU1510 scanning electron microscope using an accelerating voltage of 5 kV (Hitachi, Tokyo, Japan).

Adhesion/Invasion Assay

HCT-8 cells, derived from adenocarcinomas in a human colon and rectum (Tompkins et al., 1974; White et al., 1996), have been successfully used as an infection model to investigate pathogenesis of bacteria in previous studies (Luck et al., 2005; Pradel et al., 2015; Zargar et al., 2015). To evaluate bacterial invasion into HCT-8 cells (ATCC CCL-244; American Type Culture Collection, Manassas, Virginia), an invasion assay was conducted as described by Rogers et al. (2012), with modifications. Briefly, bacteria were incubated overnight aerobically at 37°C, transferred into fresh TSB medium as 1% inoculum and grown to 1×10^8 CFU at 37°C with constant shaking. *C. sakazakii* cells were harvested by centrifugation at 3,000 g for 5 min, washed and resuspended in RPMI-1640 (Gibco, Invitrogen, USA). HCT-8 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA) in six-well tissue culture plates. The *C. sakazakii* cells were applied to the HCT-8 cell monolayer at a multiplicity of infection (MOI) of 100. After incubation for 1, 2, 3, or 4 h in the presence of 5% CO_2, the cells were washed three times with PBS and lysed in 1 ml of 0.1% Triton X-100 for 10 min. The bacteria were serially diluted in PBS and plated on TSA for quantification.

Analysis of Biofilm Formation Capacity

The experiment was performed as previously described (Du et al., 2012), with modifications. *C. sakazakii* was inoculated into 5 ml of TSB and incubated at 37°C with aeration until the cell density reached 10^7 CFU/ml. One hundred-microliter portions were loaded into a 96-well polystyrene plate and incubated at 37°C for 48 h without shaking. To fix the biofilms, 200 μl of 99% methanol was added for 15 min, the supernatants were removed, and the plates were air-dried. Subsequently, 200 μl of a 0.1% crystal violet (CV) solution was added. After 30 min, the excess CV was removed, and the plates were washed with normal saline (0.9% NaCl). Finally, the bound CV was released by adding 200 μl of 95% ethanol. The absorbance was measured at 570 nm using a Sunrise basic microplate reader (Tecan, Austria).

Raman Spectroscopy Analysis

Raman spectroscopy analyses were performed to examine the biochemical profiles of *C. sakazakii* biofilms according to methods described in a previous report (Du et al., 2016), with modifications. Briefly, 100 μl of each logarithmic phase bacterial culture was dropped onto a Memberance Filter (Millipore, Ireland), and the filter was placed on a TSA plate containing appropriate antibiotics. The plates were incubated at 37°C for 72 h, and the media were changed every 24 h. Spectroscopic analyses were performed using a Renishaw inVia Raman system (Renishaw, Gloucestershire, UK) equipped with a 785-nm near-infrared diode laser and a Leica microscope (Leica Biosystems, Wetzlar, Germany). Raman spectra were collected using a WITec alpha300 Raman microscope (WITec, Ulm, Germany) equipped with a UHTS-300 spectrometer (Lu et al., 2011a; Wang et al., 2015). Wavenumbers from 400 to 1,800 cm^{-1} were selected for Raman-based chemometric analyses. Peaks were assigned based on methods described in a previous study (Du et al., 2016). The area and height of each Raman band were calculated using MATLAB, and significant ($P < 0.05$) band variations were characterized and analyzed. Twenty spectra were collected for each strain in triplicate at minimum. Principal component analysis (PCA) was performed to segregate the biofilms obtained from the WT, mutant, and complementation *C. sakazakii* strains by creating a two- or three-dimensional image (Lu et al., 2012).

qRT-PCR

Quantitative RT-PCR assays were performed using SYBR® Premix Ex Taq II (Takara Bio Inc.), following the manufacturer's instructions, to investigate the transcriptional levels of various cellulose synthesis, biofilm-related, or virulence genes (Jing et al., 2016). Corresponding primers were designed based on the genome sequence of *C. sakazakii* ATCC BAA-894 (GenBank accession number CP000783.1; **Table 2**). Total bacterial RNA was isolated using an E.Z.N.A.™ Bacterial RNA Kit (Omega, USA).

TABLE 2 | Primers used in this study.

Gene amplified	Primers	Primer sequences (5′–3′)	Amplicon size (bp)	Note
MUTANT CONSTRUCTION				
kana-F	Km^r cassette	CG*GGATCC*GAGGTATGTAGGCGGTGC	26	BamHI
kana-R		CGC*GTCGAC*ATATGTATCCGCTCATGAATT	30	SalI
202U-F	Upstream of EAS_04202	CG*GAATTC*CTTGCCTTACGGGTCATCTC	28	EcoRI
202U-R		CG*GGATCC*GGTTTATTTCCTGGCTTTCG	28	BamHI
202D-F	Downstream of EAS_04202	CGC*GTCGAC*CACAACGTGAACAACTCGC	28	SalI
202D-R		AA*CTGCAG*CCACGCGTAGGTTTCC	24	PstI
COMPLEMENT-ATION				
cp202-F	*bcsR* gene sequence	CG*GGATCC*GCACAGCAGCACAATGAAATAG	30	BamHI
cp202-R		CGC*GTCGAC*GCGCACGCCCTGTAATG	26	SalI
qRT-PCR				
Control-RT-F	16S rRNA	GAGTGGCGGACGGGTGAGT	19	
Control-RT-R		GTCCGTAGACGTTATGCGGTATTAG	25	
bcsQ-RT-F	*bcsQ*	GTCACGCCCGCTTACATCAG	20	
bcsQ-RT-R		TGCGTTTGCAGCCAGAGC	18	
bcsR-RT-F	*bcsR*	CTGAGAATGACGCTAAGGC	19	
bcsR-RT-R		CATCCTTGCGCTCCTG	16	
bcsE-RT-F	*bcsE*	TAATGAATGTCACCGGCAACTG	22	
bcsE-RT-R		GCCGACCAGCAAACTTCCAT	20	
bcsA-RT-F	*bcsA*	TGGTGTTGATCAACCTGCTCG	21	
bcsA-RT-R		CGCCGAGGATAATCAGGTTGTAG	23	
bcsB-RT-F	*bcsB*	CGCGACGATAAAGATTTACTCC	22	
bcsB-RT-R		GGTTTGACCTCGCCCACTT	19	
bcsC-RT-F	*bcsC*	TACGCCTACGGGCTTTATCTC	21	
bcsC-RT-R		TAGCCGTCTCCATCAGTTCATT	22	
fliA-RT-F	*fliA*	TGGCAGCGTTATGTCCCG	18	
fliA-RT-R		GCGTTCTACAGCATTCAGCAAG	22	
fliC-RT-F	*fliC*	CAAACGACACCAACGGTTCTACG	23	
fliC-RT-R		TGCCGTTGAAGTTAGCACCACC	22	
fliD-RT-F	*fliD*	CCGTCGCCCACGAAGTAG	18	
fliD-RT-R		GGCACAGCTCGGCATCAC	18	
ompA-RT-F	*ompA*	TCCAAAGGTATCCCGTCCAAC	21	
ompA-RT-R		GAGCAGCGCGAGGTTTCAC	19	
hfq-RT-F	*hfq*	GTCTCGTCCGGTTTCTCACCATAG	24	
hfq-RT-R		GAGAGGCAGCGGAAGATGGC	20	
ompX-RT-F	ompX	CATAGGAGAAGCCGTAGTCGC	21	
ompX-RT-R		GGCTTACCGTATCAATGACTGG	22	

The $2^{-\Delta\Delta C}{}_{\mathrm{T}}$ value method was used to compare the expression of genes from different samples (Schmittgen and Livak, 2008). The 16S rRNA gene was used as an internal control for within-sample normalization of mRNA abundance (Choi et al., 2015). All real-time PCR reactions were performed using the Mastercycler ep gradient realplex system (Eppendorf, Germany). A reaction mixture lacking cDNA was used as the negative control.

Bacterial RNA was isolated from the WT strain (ATCC BAA-894), the *202* deletion mutant (*ΔbcsR*), or the complementation strain harboring the pACYC184-202 plasmid (*cpbcsR*). To obtain relative mRNA expression values on the y-axis, the mRNA level for each gene was divided by the mRNA level of the 16S rRNA-coding gene. The mRNA expression values were further normalized to the transcription levels exhibited by the WT strain. The means and standard deviations from three independent experiments are shown. Asterisks indicate significant differences ($^* P < 0.05$).

Statistical Analysis

Statistical analyses were carried out using Origin 8.0 (OriginLab Co., Northampton, MA) and MATLAB (MathWorks, Natick, MA, USA). Each experiment was independently repeated a minimum of three times to ensure reproducibility. All results were analyzed through the Duncan test and analysis of variance (ANOVA), a collection of statistical models used to analyze the differences among group means and their associated procedures (Tjur, 2005). The data are represented as the mean and standard deviation, and statistical analyses were performed using SPSS

19.0 (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Construction and Morphological Characteristics of the *ΔbcsR* Mutant in *C. sakazakii* ATCC BAA-894

The *C. sakazakii* gene (ESA_RS19400) homologous to the *bcsR* gene is located in a clockwise orientation in the genome of *C. sakazakii* ATCC BAA-894 and is also found in other *Cronobacter* spp. The gene (ESA_RS19400) exhibited high protein sequence similarity to the *bcsR* gene of *Kosakonia arachidis* (67%). However, the function of this gene is not yet known in *C. sakazakii*. To understand the roles of *bcsR* in pathogenesis of *C. sakazakii* ATCC BAA-894, we generated a mutant in which the entire *bcsR* gene was replaced by kanamycin resistance gene insertion using the λRed recombination technique. The *ΔbcsR* (*kana*) mutant was verified by PCR (**Figure 1**) and nucleotide sequencing. To further verify that the *bcsR* gene was knocked out, quantitative RT-PCR assays were performed, employing the bcsRY F/R primers and using WT, *ΔbcsR* or *cpbcsR* genomic DNA as the template. The results are shown in **Figure 6**. The expression of the *bcsR* gene was significantly reduced (26.57-fold change) in the *ΔbcsR* mutant when compared with that in the WT strain, which demonstrated that the *bcsR* gene was deleted.

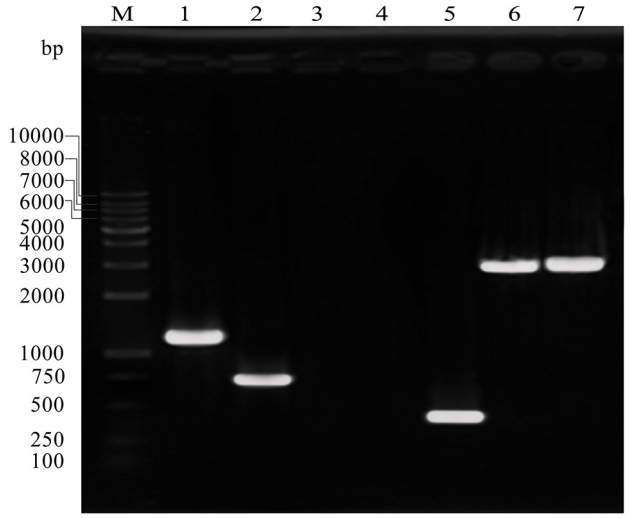

FIGURE 1 | PCR verification of the *ΔbcsR* mutant strain using specific primers targeting sequences outside of homologous fragments and within the resistance gene. Sample 1, amplified with Kan F and KanR, using *ΔbcsR* as the template, 1,174 bp; sample 2, amplified with cp202-VF and cp202-VR, using *cp202* as the template, 738 bp; sample 3, amplified with 202F and 202R, using *ΔbcsR* as the template, 0 bp; sample 4, amplified with KanF and KanR, using WT as the template, 0 bp; sample 5, amplified with 202F and 202R, using WT as the template, 275 bp; sample 6, amplified with 202-VF and Kan-VR, using *ΔbcsR* as the template, 2,989 bp; sample 7, amplified with Kan-VF and 202-VR, using *ΔbcsR* as the template, 3047 bp; M, DL10000 marker. Electrophoresis was performed using 1% agarose.

The deletion mutant showed a similar growth rate to that of the WT strain (**Figure 2A**). However, the complementation strain (*cpbcsR*) demonstrated a slightly lower growth rate than that of the WT in the early stages of growth. In addition, similar morphological characteristics were observed in SEM images of the WT, *ΔbcsR*, and *cpbcsR* strains (**Figure 2B**). Thus, *bcsR* gene knockout did not affect bacterial growth and morphology in TSB medium.

bcsR Affects Adhesion/Invasion

In our previous work, we showed that the *bcsR* gene in *C. sakazakii* ATCC BAA-894 strain was involved in interactions with intestinal epithelial cells (Du et al., 2016). To explore the virulence-related functions of the *bcsR* gene in *C. sakazakii* ATCC BAA-894, an adhesion/invasion assay was performed at different time points (1, 2, 3, or 4 h) using HCT-8 cells (**Figure 3**). The invasion rate of the *ΔbcsR* mutant was significantly lower than that of the WT at 1, 2, 3, and 4 h in the invasion assay. Although the adhesion/invasion ability of the complementation strain did not reach that of the wild-type strain, it showed a significantly higher adhesion/invasion ability than the *ΔbcsR* mutant strain. The results suggested that the phenotypic defect of the *ΔbcsR* mutant was indeed attributable to the lack of *bcsR*, which led to a decreased adhesion/invasion ability in HTC-8 cells (**Figure 3**). Thus, we speculated that *bcsR* might positively regulate *C. sakazakii* adhesion/invasion in HCT-8 cells.

Estimation of Biofilm Formation Ability

To investigate whether the *bcsR* gene contributes to biofilm formation of *C. sakazakii* ATCC BAA-894, a CV staining assay was carried out. The *ΔbcsR* mutant showed a significant decrease (1.5-fold change) in biofilm formation compared with that in the WT, and the complementation strain demonstrated similar biofilm formation to that in the WT strain (**Figure 4**). The results suggest the *bcsR* gene is a positive effector in biofilm formation.

Differences in Biofilm Composition between the *bcsR* Mutant and WT Strains

To further investigate variations in the biochemical components of the biofilms formed by the tested strains, Raman spectroscopic analyses were performed. A PCA model was established to differentiate between the WT, mutant, and complementation strain. There were clear segregations between the WT, mutant, and complementation strains (**Figure 5A**), whose biochemical components exhibited variations.

A comparison of Raman spectra indicated that two peaks were higher (1,287 and 1,367 cm^{-1}) and six peaks were lower (1,002, 1,157, 1,343, 1,450, 1,522, and 1,655 cm^{-1}) in the mutant than in the WT strain, and these differences were significant (**Table 3**). The 1,287 and 1,367 cm^{-1} peaks were assigned to cytosine and cellulose respectively (De Gelder et al., 2007). The 1,002 cm^{-1} peak reflected the characteristics of phenylalanine (Movasaghi et al., 2007). The 1,157 and 1,522 cm^{-1} peaks were assigned to carotenoids (Feng et al., 2014). The 1,450 cm^{-1} peak was assigned to fatty acids (De Gelder et al., 2007). And the 1,655 cm^{-1} peak was assigned to amide I and amide III (Lu et al., 2011b).

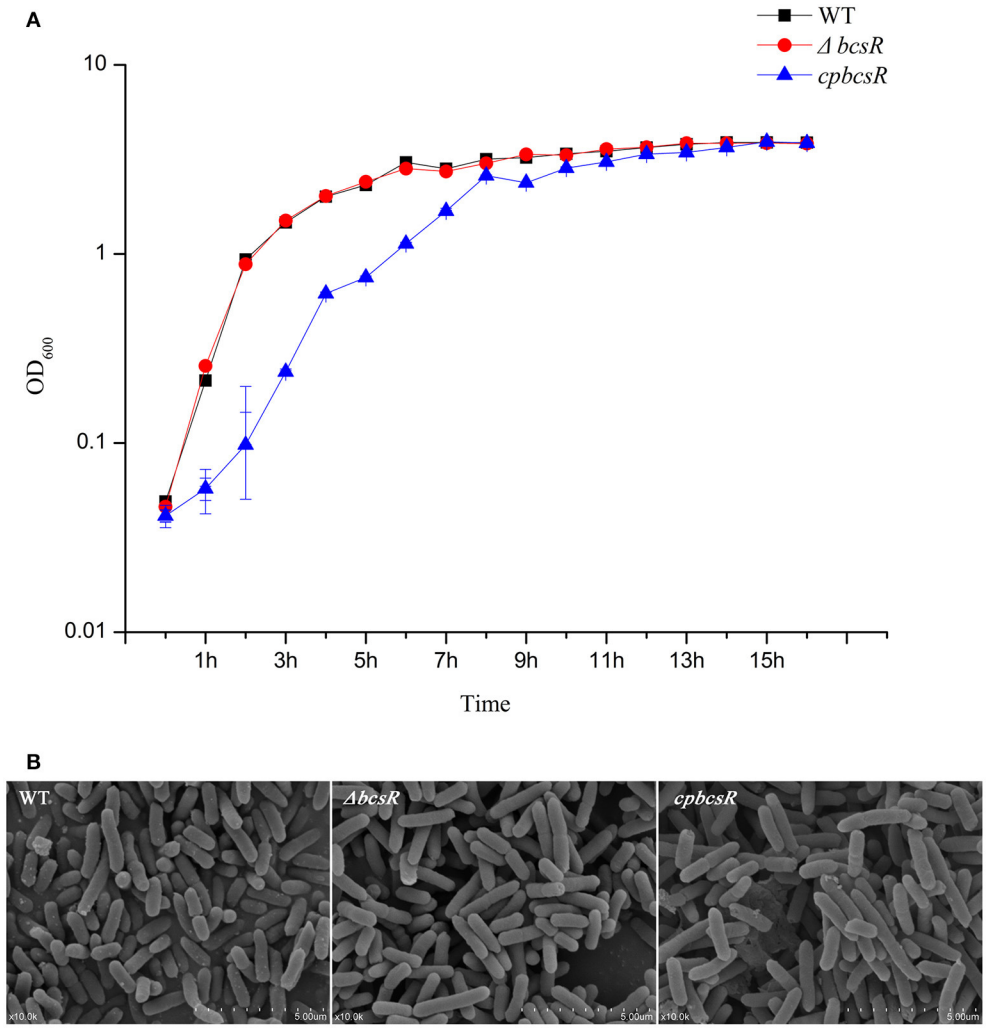

FIGURE 2 | (A) Growth curves of *C. sakazakii* WT, *ΔbcsR,* and *cpbcsR* strains grown in the TSB broth at 37°C. The OD_{600} of each strain was measured hourly. Error bars represent the standard error of the mean (SEM) from three independent biological replicates. **(B)** SEM of biofilms formed on glass by WT and mutant strains. WT: WT ATCC BAA-894; *ΔbcsR*: mutant strain; *cpbcsR*: complementation strain.

To clearly express the differences between the wild-type, mutant, and complementation strains at various points, we present the Raman intensities at each Raman wavenumber in **Table 3** and indicate the differences between each of the strains. The **Figure S1** shows the distribution of Raman peaks.

qRT-PCR Analysis

The expression of several genes (*bcsR, bcsQ, bcsE, bcsA*, *bcsB, bcsC, fliA*, *fliC, fliD, ompA, hfq,* and *ompX*) involved in cellulose synthesis, flagella, and toxicity, was detected in the *bcsR* mutant and WT strain by qRT-PCR.

The mRNA levels of the cellulose synthase operon genes *bcsQ, bcsE, bcsA*, *bcsB*, and *bcsC* increased by 2.68-, 1.41-, 3.91-, 3.27-, and 3.28-fold, respectively, in the *ΔbcsR* strain compared with those in the WT strain (**Figure 6**). The expression of *fliA*, *fliC,* and *fliD,* which are involved in regulating flagellar assembly, was reduced by 2.17-, 2.70-, and 1.79-fold, respectively, in the *bcsR* mutant compared with that in the WT strain (**Figure 6**). We also examined the expression levels of several toxicity-related genes, including *ompA, ompX,* and *hfq*, which have previously been associated with toxicity in *C. sakazakii*, in the *bcsR* mutant and WT strain. The mRNA levels of *ompA* and *hfq* genes in the *ΔbcsR* mutant decreased by 1.80- and 1.92-fold, respectively, compared with those in the WT strain (**Figure 6**). However, *ompX* transcription was not affected by the lack of *bcsR*. Thus, the *bcsR* gene down-regulated cellulosic synthesis in *C. sakazakii* ATCC BAA-894, in agreement with the Raman spectra data.

DISCUSSION

Bacterial pathogens must bind to epithelial cell surfaces prior to successful invasion. Therefore, adhesion and invasion into host tissue cells play essential roles in the

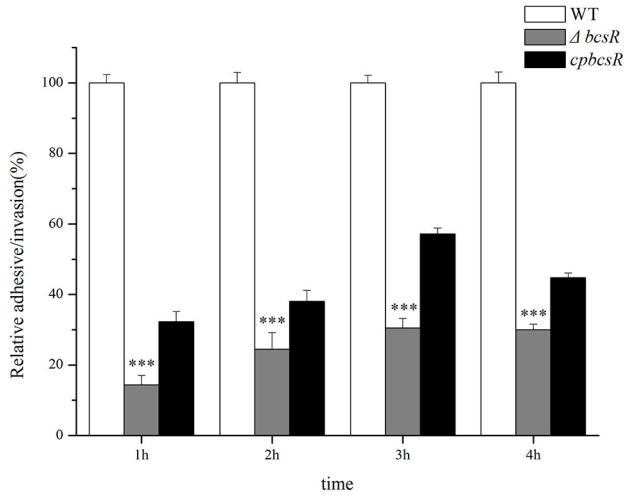

FIGURE 3 | Absence of the *bcsR* gene impairs epithelial cell invasion at different times. Percent invasion for the mutant and complementation strains relative to the WT strain; ***$p < 0.001$.

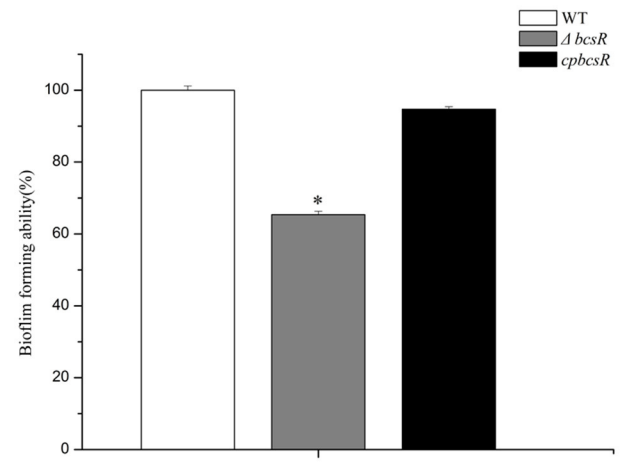

FIGURE 4 | Biofilm formation by the WT, *ΔbcsR,* and *cpbcsR* strains. Percentages of biofilm formation for the mutant and complementation strains relative to the WT strain are shown. The error bars represent the mean standard deviation; *$P < 0.05$.

virulence of most pathogenic bacteria. In our previous study, a cellulose biosynthesis-related gene was shown to be involved in *C. sakazakii* adhesion to epithelial cells. The *bcsR* gene was observed downstream of the *bcs* operon, which encodes enzymes or other proteins responsible for cellulose biosynthesis. However, the function of this gene in cellulose biosynthesis is unknown (Grimm et al., 2008). In this study, our data indicated that *bcsR* is a positive regulator of *C. sakazakii* adhesion/invasion into HCT-8 cells and biofilm formation.

Bacterial cells typically adhere to and interact with surfaces before eventually forming biofilms. Biofilms are defined as sessile communities embedded in polymeric substances produced by bacteria and are primarily composed of polysaccharides, proteins, and nucleic acids (Kim et al., 2006; Kolter and Greenberg, 2006; Serra et al., 2013). The ability to form a biofilm is a crucial trait that enhances bacterial resistance to environmental stresses and provides protection against drugs and disinfectants (Lehner et al., 2005; Furukawa et al., 2006). Acting as adhesive foundations and defense barriers, biofilms also play an important role in protecting embedded cells against detachment caused by flow shear. Potential bacterial toxicity may be enhanced when cells are present in biofilms, which are heterogeneously sessile bacterial communities that adhere to each other and to solid surfaces (Wang et al., 2012). Kunyanee et al. (2016) reported the important role of *Burkholderia pseudomallei* biofilms in bacterial pathogenesis in human epithelial cells with respect to initial attachment and invasion. In the *ΔbcsR* mutant of *C. sakazakii*, the decrease of biofilm formation suggests that this gene may affect adhesion/invasion by regulating biofilm synthesis.

According to the Raman spectra results, bands representing carotenoids, fatty acids and amides in the mutant exhibited significant decreases compared with those in the WT strain, indicating that *bcsR* may be positively associated with carotenoids, fatty acids and amides. The biofilms of certain *Cronobacter* strains contain high levels of carotenoids (Du et al., 2012). Therefore, *bcsR* may be positively associated with biofilm formation through the up-regulation of carotenoid, fatty acid, and amide biosynthesis in *C. sakazakii*.

The Raman spectra results also indicated that the band representing cellulose was greatly increased in the mutant compared with that in the WT strain, suggesting that *bcsR* may be a negative regulator of cellulose biosynthesis. Cellulose is an extracellular matrix component present in *C. sakazakii* biofilms (Grimm et al., 2008). Bacterial cellulose synthase is a multicomponent protein complex encoded in an operon containing at least three genes: *bcsA*, *bcsB*, and *bcsC* (Keiski et al., 2010). *bcsA*, *bcsB,* and *bcsC* encode for the cellulose synthase catalytic subunit, a cyclic-di-GMP binding protein and the cellulose oxidoreductase enzyme, respectively (Hu et al., 2015). The *bcsABC* genes in *Cronobacter* are necessary for cellulose production and are involved in biofilm formation and cell-cell aggregation (Hu et al., 2015). However, as a cellulose synthase operon gene, the function of *bcsR* remains even more elusive (Grimm et al., 2008). In this study, the decrease of biochemical components (including carotenoids, fatty acids, and amides) potentially explains the decline in biofilm formation after *bcsR* gene deletion in *C. sakazakii*. Interestingly, cellulose synthesis (**Figure 5B**) and cellulose synthase operon gene (*bcsABC*) expression levels significantly increased in the *bcsR* mutant (**Figure 6**). We hypothesize that *bcsR* affects the synthesis of other biofilm components during increased cellulose synthesis. In *Salmonella enteritidis*, the synthesis of colanic acid, lipopolysaccharide, enterobacterial common antigen, and cellulose are affected by the cellulose operon; these elements are highly important for biofilm formation (Solano et al.,

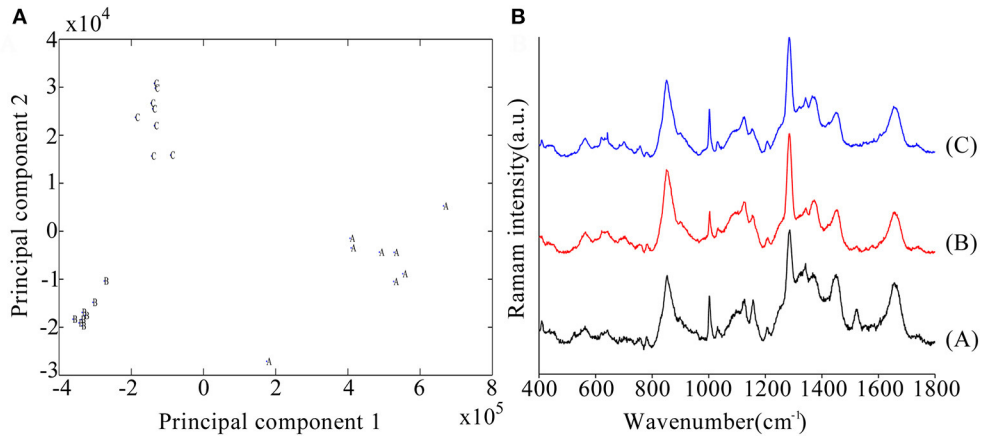

FIGURE 5 | (A) Principal component analysis (PCA) model based on Raman spectral features. A: Wild-type strain, B: mutant strains, C: complementation strains. **(B)** Comparison of *C. sakazakii* biofilm spectral features between the WT (A), mutant (B), and complementation strains (C) using Raman spectroscopy.

TABLE 3 | Raman intensities of different wavenumbers.

Wave numbers	Raman intensity (a.u.)		
	WT	***ΔbcsR***	***cpbcsR***
1002.84	2970.51a	1754.59b	2895.27a
1157.40	2759.70a	2396.31b	2230.74b
1287.64	3427.02a	6403.56b	5930.75b
1343.49	3464.45a	2982.50b	3168.39b
1367.63	2677.96a	4229.29b	3400.83c
1450.77	4399.85a	3326.01b	3033.83c
1522.21	2307.49a	473.93b	184.61c
1655.36	3875.54a	3470.61b	3375.95b

"a," "b," and "c" in the same raw with different letters indicate significant difference at the 0.01 level according to one-way ANOVA, while the same letters indicate that the difference is not significant.

2002). Cellulose is composed of β-D-1,4-glucan chains, and the precursor molecule is uridine diphosphate glucose (UDP-glucose) (Ross et al., 1991), which is also the substrate for the synthesis of colanic acid, lipopolysaccharide, and the enterobacterial common antigen. Excessive cellulose synthesis affects the synthesis of the other three components and thus affects biofilm synthesis (Solano et al., 2002). Channeling copious amounts of UDP-glucose to cellulose biosynthesis leads to the reprogramming of cellular metabolism to favor gluconeogenesis, which is a metabolic pathway that results in the generation of glucose from certain non-carbohydrate carbon substrates (Romling and Galperin, 2015). Furthermore, cellulose synthesis is energy-consuming and is affected by cellulose synthase activity. Cellulose synthase is specifically activated by the unique nucleotide cyclic diguanylic acid, which is synthesized from GTP by diguanylate cyclase. The synthesis of other biofilm components is also energy-consuming (Mika and Hengge, 2013). Therefore, excessive energy consumption for cellulose synthesis may not be conducive to the formation of other biofilm components. Consistent with our results, we speculate that excessive cellulose synthesis affects the synthesis of other components, thereby affecting biofilm formation.

Based on the qRT-PCR results, the expression of flagella (FliA, FliC, and FliD) and outer membrane proteins was reduced in the *bcsR* mutant. Therefore, we speculate that *bcsR* may be a global regulator of biofilm, flagella, and outer membrane protein biosynthesis, similar to CodY, which is a global transcriptional regulator that represses toxin gene expression by binding with high affinity to the *tcdR* promoter region (Girinathan et al., 2017). The *fliA*, *fliC,* and *fliD* genes regulate flagellar assembly (Chevance and Hughes, 2008). The *fliA* gene encodes an alternative sigma factor that regulates the transcription of class III flagellar genes, including filament structure genes and genes in the chemosensory pathway (Ohnishi et al., 1990; Chevance and Hughes, 2008; Choi et al., 2015). Because the *fliC* gene is a class III flagellar gene, and *fliD* encodes a late-secretion substrate of the filament-capping protein, free σ^{28} (a flagellar-specific sigma factor), which transcribes class III promoters, the enhanced expression of *fliC* and *fliD* may be attributable to large amounts of FliA, suggesting the *bcsR* gene affects the expression of class III flagellar genes via *fliA* (Chevance and Hughes, 2008). The three flagellar genes are reportedly involved in biofilm formation by *C. sakazakii* (Barken et al., 2008; Hartmann et al., 2010; Ye et al., 2016), consistent with our findings. Kim et al. (2010) reported important roles for OmpA and OmpX of *Cronobacter* in the invasion of Caco-2 and INT-407 cells. Hfq, oligomerized into a hexameric ring structure, is a posttranscriptional global regulator involved in the biosynthesis of OMPs, quorum sensing, stress responses, or metabolism and adhesin-mediating interactions with host tissue (Kakoschke et al., 2016). In our qTR-PCR analysis, the genes encoding OmpA, OmpX, and Hfq were all down-regulated in the mutant, suggesting their positive correlation with bacterial adhesion/invasion into epithelial cells. Based on the findings in this study, *bcsR* plays significant roles in biofilm formation and adhesion/invasion by regulating flagellar and toxicity-related genes.

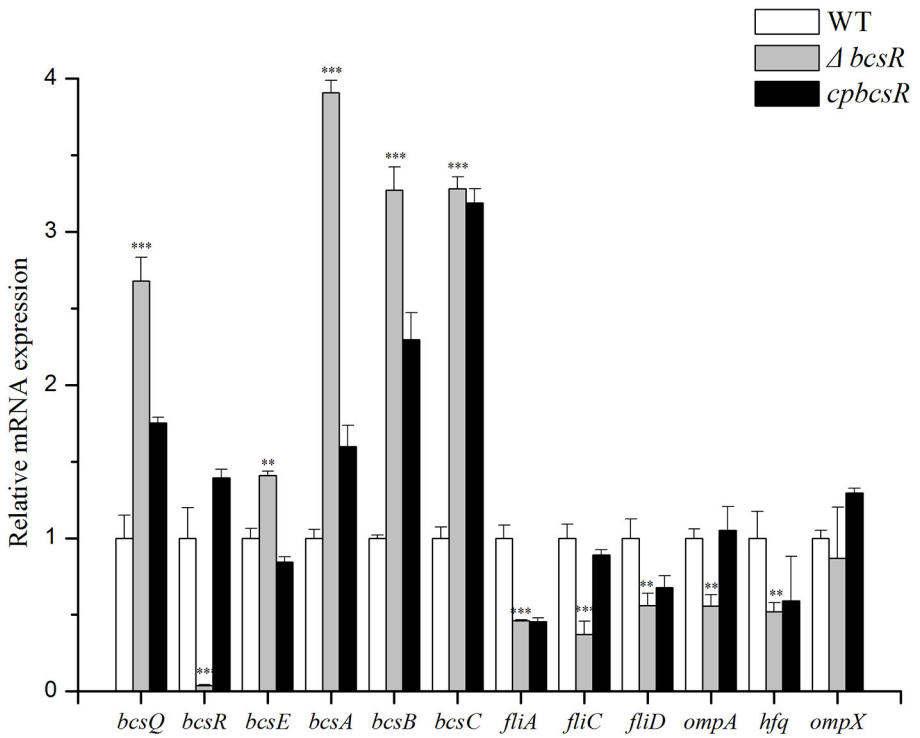

FIGURE 6 | Transcription levels of 9 genes in *C. sakazakii* were determined by qRT-PCR. Experiments were repeated three times. The error bars represent the mean standard deviation; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Interestingly, in *bcsR* complementation strains, certain functions were restored, but *bcsR* expression levels only barely reached that of the wild strain. To explore whether this result was due to the gene polarity effects, we investigated the expression of genes flanking *bscR* in the WT, mutant, and complementation strains through qRT-PCR analysis (**Figure 6**). The expression of *bcsQ* and *bcsE* was investigated. *bcsQ* and *bcsE*, are the cellulose synthase operon genes that are closest to *bcsR* in the downstream region and upstream region, respectively. In the complemented strain, although the expression of the flanking genes was not recovered to wild-type levels, no significant difference was observed in the wild-type, mutant, and complemented strains (fold change < 2). The lack of complete recovery of flanking gene expression may have been caused by the differences in the efficiency of *bcsR* gene expression in the complemented strain and the wild-type strain, considering that the *bcsR* gene was located in the pACYC184 plasmid in the complemented strain. This phenomenon was previously reported in studies performed by Kim et al. (2015) and Schilling and Gerischer (2009). Additionally, Kim et al. (2015) reported that failure of the pHFQ plasmid (a pACYC184 derivative with its own *hfq* promoter) to complement the hypermotility of the *hfq* strain might be caused by an imbalance of Hfq production from the low-copy-number pACYC184 plasmid. Therefore, we cannot completely exclude that the deletion of the *bcsR* gene did not affect the expression of other genes (*fliA*, *fliC, fliD, ompA,* and *hfq*) via polar effect. However, we are inclined to conclude that *bcsR* was a negative regulator for cellulose biosynthesis and we confirmed that cellulose biosynthesis was negatively related to biofilm formation and the epithelial cells adhesion/invasion capability of *C. sakazakii.*

CONCLUSION

In this study, a gene knockout technique was employed to demonstrate the positive role of the *bcsR* gene in *C. sakazakii* adhesion/invasion in epithelial cells and biofilm formation. However, *bcsR* was found to act as a negative regulator of cellulose biosynthesis. Raman spectrometry and qRT-PCR results verified positive regulation of pathogenesis and negative regulation of cellulose biosynthesis by *bcsR* in *C. sakazakii*. This study significantly contributes to our understanding of the detailed functions of the *bcsR* gene in bacteria.

AUTHOR CONTRIBUTIONS

JG contributed significantly to the conceived, designed, and performed the experiments, and the writing and editing of paper. PL, XD, and SW contributed significantly to conceived and designed of the work. ZH, RX, and BL mainly made a great contribution in performing the experiments. They all involved in the process of experimental design and the

writing of paper. All authors agree to be accountable for the content of the work contributed to the conception of the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01839/full#supplementary-material

Figure S1 | Raman peak distribution of *C. sakazakii* biofilm.

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ORIGINAL RESEARCH
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Hydrophobicity of Residue 128 of the Stress-Inducible Sigma Factor RpoS Is Critical for Its Activity

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RpoS is a key stress-inducible sigma factor that regulates stress resistance genes in *Escherichia coli*, such as the *katE* gene encoding catalase HPII and the *glg* genes encoding glycogen synthesis proteins. Monitoring RpoS activity can provide information on the stress sensitivity of *E. coli* isolates in clinical settings because the RpoS in these isolates is often mutated. In the present study, we found a novel, missense point mutation at RpoS residue 128 in a clinical Shiga toxin-producing *E. coli* (STEC) isolate. This mutation caused RpoS dysfunction and increased stress sensitivity. A mutant *rpoS* was cloned from a clinical STEC that is vulnerable to cold temperature and oxidative stresses. Mutant RpoS protein expression was detected in the clinical isolate, and this RpoS was non-functional according to HPII activity and glycogen levels, which are positively regulated by RpoS and thus are used as indicators for RpoS function. A reporter assay with β-galactosidase indicated that the dysfunction occurred at the transcriptional level of genes regulated by RpoS. Furthermore, substitution analysis indicated that the hydrophobicity of the amino acid at residue 128 was critical for RpoS activity; the simulation analysis indicated that the amino acids of RNA polymerase (RNAP) that interact with RpoS residue 128 are hydrophobic, suggesting that this hydrophobic interaction is critical for RpoS activity. In addition, substitution of Ile128 to Pro128 abolished RpoS activity, possibly as a result of disruption of the secondsary structure around residue 128, indicating that the structure is also a crucial factor for RpoS activity. These results indicate that only one point mutation at a hydrophobic residue of the complex formed during transcription leads to a critical change in RpoS regulation. Moreover, we found that Ile128 is widely conserved among various bacteria: several bacterial strains have Met128 or Leu128, which are hydrophobic residues, and these strains had similar or higher RpoS activity than that observed with Ile128 in this study. These data indicate that the hydrophobicity of the amino acid at residue 128 is critical for RpoS activity and is consequently important for bacterial survival. Taken together, these findings may contribute to a deeper understanding of protein functional mechanisms and bacterial stress responses.

Keywords: RpoS, missense mutation, stress response, Shiga toxin-producing *E. coli* (STEC), clinical isolates, food-borne pathogens

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INTRODUCTION

Organisms have stress response mechanisms to protect themselves from environmental stresses (Feder and Hofmann, 1999; Cabiscol et al., 2000). Shiga toxin-producing *Escherichia coli* (STEC) are found in the guts of cattle and they can survive under severe environmental stress conditions, including those in soil, river, and ground water, and they can infect humans (Rasmussen and Casey, 2001; Muniesa et al., 2006; van Elsas et al., 2011; van Overbeek et al., 2014). A greater understanding of the bacterial stress response can provide information for better control of bacterial infections.

RpoS is a key stress-inducible sigma factor (Hengge-Aronis, 1993; Klauck et al., 2007; Dong and Schellhorn, 2010; Battesti et al., 2011; Landini et al., 2014) that regulates stress resistance genes such as the *katE* gene encoding catalase HPII and the *glg* genes encoding glycogen synthesis proteins (Weichart et al., 1993; Tanaka et al., 1997) by binding RNA polymerase (RNAP) and the 5′ upstream region of the genes in *E. coli* (Hengge-Aronis, 2002; Mooney et al., 2005; Typas and Hengge, 2006; Typas et al., 2007). Recently, X-ray crystallographic analysis for the transcription initiation stage was reported, where the binding mechanism among RpoS, RNAP, and oligonucleotides was disclosed (Liu et al., 2016).

Mutated RpoS is often present in clinically isolated *E. coli* strains (Notley-McRobb et al., 2002; Dong et al., 2009), and strains with non-functional RpoS proteins are generally sensitive to stresses (Hengge-Aronis, 1993; Landini et al., 2014). However, RpoS dysfunction may be advantageous under certain conditions, such as those with scarcity of carbon sources (Ferenci, 2008; Chiang et al., 2011). The *rpoS* gene is considered as polymorphic (Jordan et al., 1999; Notley-McRobb et al., 2002; Martinez-Garcia et al., 2003), which influences the trade-off between self preservation and nutritional competence (SPANC; Ferenci, 2003; Ferenci and Spira, 2007). The phenotypic diversity observed in clinical isolates is at least partially attributable to diverse RpoS levels among isolates and the effect of these RpoS levels on SPANC (Levert et al., 2010). Because the presence of scarce carbon sources, readily selects for the loss of RpoS function in both laboratory (Chen et al., 2004) and pathogenic strains (Dong et al., 2009), stressful environmental conditions, such as scarce carbon and nutrient sources, may select for RpoS mutants in environmental *E. coli* populations. Once the RpoS protein is mutated, mutant RpoS is promptly degraded by proteinase owing to the strict regulation of the cellular RpoS level (Zhou and Gottesman, 1998; Becker et al., 2000; Klauck et al., 2001; Hengge, 2009; Battesti et al., 2015).

While surveying RpoS from clinical isolates to investigate the stress tolerance of these pathogens, we identified an STEC clinical strain (Kai1), isolated from a patient with STEC infection in Japan, that is highly sensitive to H_2O_2 oxidative stress but nonetheless expresses RpoS. In the present study, we cloned *rpoS* and sequenced it to identify the mutations, and investigated the mechanisms underlying RpoS dysfunction. As a result, we found that hydrophobicity and secondary structure preservation at Ile128 determine RpoS dysfunction.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The *E. coli* strain Kai1 is a clinical isolate in the strain collection of the Jikei University School of Medicine in Japan. The laboratory *E. coli* strains K−12 W32110 and K−12Δ*rpoS* were also employed in this study. In addition, K−12Δ*rpoS* with *rpoS* variants having amino acid substitutions, e.g., Ala, Arg, Asn, Asp, Gln, Glu, Leu, Lys, Met, Phe, and Pro, at residue 128 were generated. *E. coli* K−12 DH5α was used as a host for genetic manipulation. Strains harboring an *osmY* promoter-*lacZ* transcriptional fusion on a single copy λ-prophage and strains with a deficiency in RpoS were created by P1 transduction (Banta et al., 2013); these strains were a gift from Prof. Gourse. The bacterial strains used in this study are listed in Table S7.

Bacteria (10^6 CFU/ml) were inoculated into LB broth (BD Biosciences, USA) and cultured at 37°C; all experiments were carried out on stationary phase cultures (16 h of growth), except for the glycogen assay and reporter assay.

Cloning of *rpoS* with Its Intact Promoter

The *rpoS* sequence, which included its intact promoter (Takayanagi et al., 1994) was amplified by PCR with forward (5′-ACGAATTCTTAACATGGGTAGCACCGGAA-3′) and reverse (5′-GGAAGCTTTTACTCGCGGAACAGCG-3′) primers. The forward primer had an *Eco*RI recognition site at the 5′ end of the oligonucleotide, and the reverse primer had a *Hin*dIII recognition site at the 5′ end of the oligonucleotide. The amplicon and pSTV28, a low-copy-number plasmid with the chloramphenicol-resistant gene (Takara, Japan) and 15A origin as derived from p15A, were digested by *Eco*RI and *Hin*dIII and then ligated. The plasmid harboring *rpoS* was introduced in strain DH5α and then transferred into K−12Δ*rpoS*.

Generation of *rpoS* Variants with Point Mutations

To generate *rpoS* variants, $rpoS^{K-12}$ was used as a template, and point mutations were introduced using a PrimeSTAR Mutagenesis Basal Kit (Takara). The *rpoS* variants were introduced in strain DH5α and then transferred to K−12Δ*rpoS*. Primers used in this study are listed in Tables S8.

Measurement of Catalase HPII Activity

Bacteria were cultured on LB agar for 16 h. Twenty micrograms (wet weight) of bacterial cells were suspended in 100 μl of saline solution. Bacterial suspensions were heated at 55°C for 15 min. One hundred microliters of 1% Triton X-100 (Sigma) and 30% H_2O_2 solution was added to a test tube containing 1000 μl of bacterial suspensions, and catalase activity was measured as the height of the foam that formed (Iwase et al., 2013).

Glycogen Assay

Bacteria were cultured for 24 h at 37°C on Kornberg agar (1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract, 1.5% agar, 1% glucose, and 1.5% agar; Govons et al., 1969; Liu and Romeo, 1997; Wei et al., 2000; Iwase, under review). Ten micrograms of wet weight bacterial cells were suspended in

100 μl of saline solution and heated at 95°C for 15 min for enzyme denaturation. Following heat treatment, the samples were sonicated to disrupt the bacterial cells and then centrifuged at 10,000 *g* at 4°C for 30 min to remove bacterial debris. The resulting supernatants were further filtrated to obtain clarified samples. Three microliters of 2% iodine solution (2% iodine/1 M NaOH, Wako Pure Chemical Industries, Japan) were added to the filtrated supernatants (100 μl) and colorization was then measured spectrophotometrically within 5 min (492 nm).

Reporter Assay

β-Galactosidase assays were performed for evaluating RpoS activity using a strain harboring an *osmY* promoter-*lacZ* transcriptional fusion on a single copy λ-prophage and a strain with a deficiency in RpoS (Banta et al., 2013). Bacteria were cultured in LB broth with 2-nitrophenyl-β-D-galactopyranoside (ONPG; Thermo Fisher) at 37°C for 6 h, and color changes were measured using a spectrophotometer at 492 nm. Bacteria were also cultured in LB broth with X-gal (Sigma-Aldrich), which was used for qualitative analysis. In the presence of β-galactosidase activity, bacterial cells were stained blue.

mRNA Expression Analysis

mRNA expression in bacteria harvested at O/N culture (16 h) was analyzed using quantitative reverse transcriptional PCR (qRT-PCR) with gene-specific primers and SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen), according to manufacturer instructions. *rrsA* encoding 16S rRNA was used as a reference gene for normalization of qRT-PCR. Primers used in this experiment are shown in Table S9.

TABLE 1 | Missense point mutations in *rpoS* in Kai1 strain.

	Residue 33	Residue 128
$rpoS^{K-12}$	CAG (Gln)	ATC (Ile)
$rpoS^{Kai1}$	GAG (Glu)	AAC (Asn)

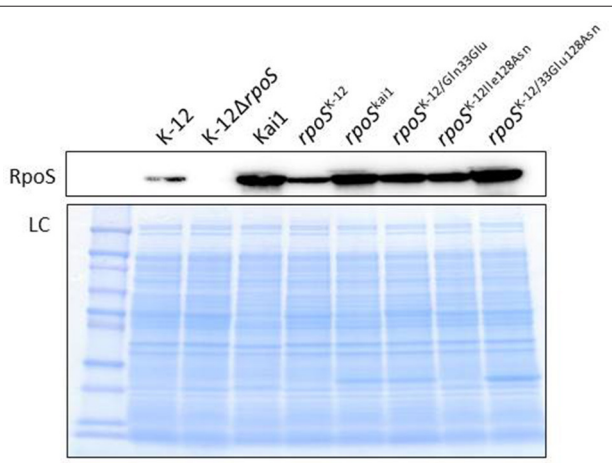

FIGURE 1 | Effect of substitution at residue 128 on RpoS expression. RpoS expression in *rpoS* variants harboring mutation(s) in $rpoS^{Kai1}$ ($rpoS^{K-12/Gln33Glu}$, $rpoS^{K-12/Ile128Asn}$, and $rpoS^{K-12/Glu33Asn128}$) and in the Kai1 strain. The experiment was conducted thrice, and representative images are shown. LC, loading control. The loading control image depicts a part of Coomassie stains of SDS-PAGE gels.

Oxidative Stress Test

Bacteria were cultured in LB broth at 37°C for 16 h. In the oxidative stress test, 20 mM H_2O_2 aqueous solutions were used as previously described with some modifications (Wang et al., 2011). Bacterial culture (10 μl) was added to 990 μl of 20 mM H_2O_2 aqueous solutions and was incubated at 25°C for 2 h. Fifty microliters of the suspension was plated onto LB agar and cultured at 37°C for 16 h for enumeration of bacterial counts to determine survival after H_2O_2 exposure.

Cold Stress Test

Bacteria were cultured in LB agar at 37°C for 16 h and incubated at 4°C during the study period. Cold-stressed bacteria on LB agar were cultured in LB broth at 37°C for 24–48 h for enumeration of bacterial counts to determine survival after cold- stress exposure.

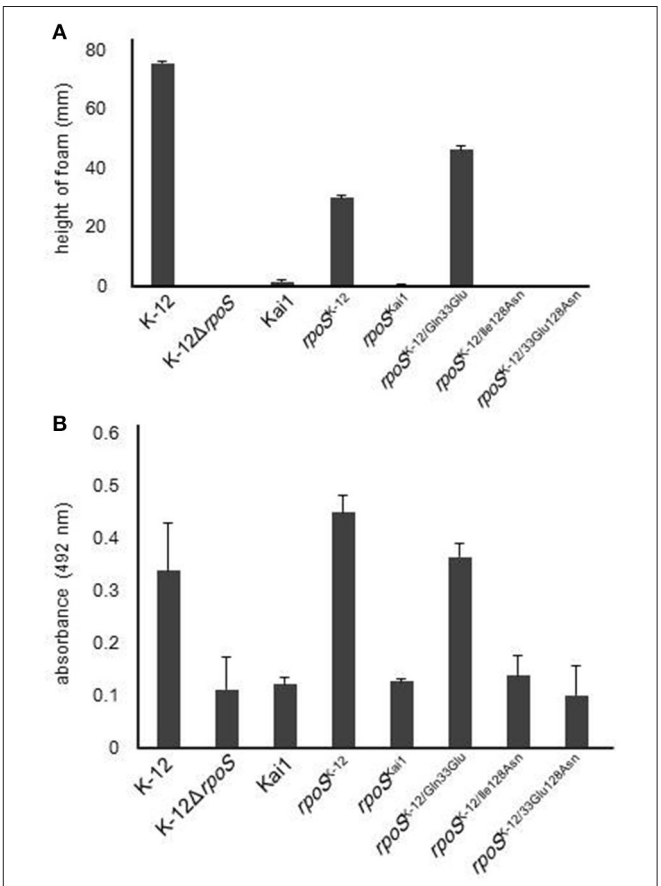

FIGURE 2 | Effect of substitution at residue 128 on RpoS activity. RpoS activity in *rpoS* variants was determined by HPII activity assays and analysis of glycogen levels, which are positively regulated by RpoS. **(A)** HPII activity in *rpoS* variants was measured by HPII assays. **(B)** Glycogen levels in *rpoS* variants were measured by glycogen assays. Mean values are shown ($n = 3$). Error bars represent standard deviations. The statistical significance of the differences is tabulated in Tables S1, S2.

Molecular Dynamics (MD) Calculation

MD calculations were conducted on the YASARA structure molecular modeling software package (Ver.14.4.15; Krieger et al., 2002). The calculations were run using AMBER 03 force field, the aqueous solution model with 0.9% NaClaq ion concentration, point charges assigned at $pH = 7.4$ and additional Na^+ or Cl^- for charge neutralization in a cubic cell boundary defined at 5 Å from the protein surface. Simulations were started from the structural data of fragment Leu114-Asp135 in RpoS taken from the previously reported structure of RpoS-RNAP-oligonulectotide complex (PDB: 5IPL) and conducted for the solution model at 310.15 K. Calculations were continued over 12 ns until Cα-RMSDs (root-mean-square deviations) reached at equilibrium. Snapshot figures every 25 psec were stored.

Conservation of Amino Acid at Residue 128 of RpoS among Various Bacteria

The *rpoS* sequences of gram-negative bacteria were randomly selected in the NCBI gene database and were analyzed by the ClustalW, a multiple sequence alignment program, (DNA Data Bank of Japan).

Statistical Analysis

For multiple group comparisons, analysis of variance (ANOVA) was performed, and the significance of differences was evaluated using the Scheffe's F test if the ANOVA results were significant. A value of $P < 0.05$ was considered to indicate statistical significance. Calculations were performed using Excel software (Microsoft, US) and Statcel (OMS, Japan).

RESULTS

Sequence Analysis of the *rpoS* Gene in Strain Kai1

To investigate the function of RpoS in strain Kai1, we compared the sequence of the Kai1 *rpoS* gene ($rpoS^{Kai1}$) with that of strain K−12 ($rpoS^{K-12}$), encoding a functional RpoS. We found two missense point mutations in $rpoS^{Kai1}$ compared to $rpoS^{K-12}$: an amino acid substitution at residue 33 (Gln to Glu) and one at residue 128 (Ile to Asn; **Table 1**). The substitution of residue 33 is often observed in clinical isolates with functional RpoS proteins (Atlung et al., 2002; Subbarayan and Sarkar, 2004). The substitution of residue 128, however, has not been previously reported. Therefore, it was possible that the RpoS dysfunction was due to the substitution of residue 128.

rpoS with Substitutions at Residues 33, 128, or 33 and 128 Expressed RpoS Protein

To determine whether the substitution of residues 33 or 128 affected RpoS protein expression, we first cloned $rpoS^{Kai1}$ and $rpoS^{K-12}$ into a low-copy plasmid pSTV28 and generated plasmids harboring $rpoS^{K-12}$ with the mutation(s) found in $rpoS^{Kai1}$ ($rpoS^{K-12/Gln33Glu}$, $rpoS^{K-12/Ile128Asn}$, and $rpoS^{K-12/Glu33Asn128}$). Subsequently, we introduced them into the K-12Δ*rpoS* strain and then performed western blotting for RpoS protein. All *rpoS* variants expressed RpoS (**Figure 1**). The data showed that *rpoS* with mutations observed in $rpoS^{Kai1}$ still expressed the RpoS protein (**Figure 1**), consistent with RpoS expression observed in strain Kai1 (**Figure 1**).

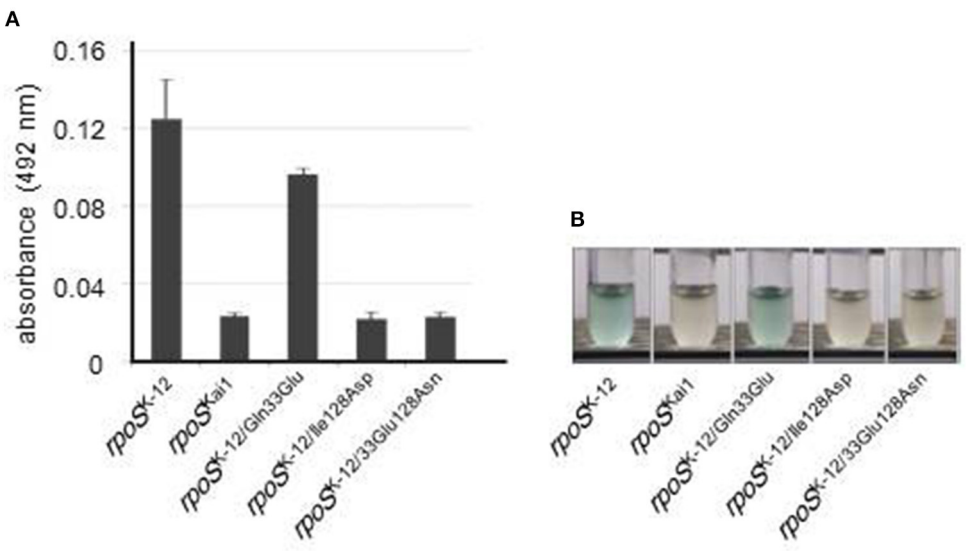

FIGURE 3 | RpoS dysfunction via substitution at residue 128. Reporter assays with β-galactosidase were performed for evaluation of RpoS function using a strain in which intact *rpoS* was deleted and an *osmY* promoter-*lacZ* transcriptional fusion was located on a single copy λ-prophage and using each *rpoS* variant. **(A)** β-Galactosidase activity was measured in the presence of ONPG, a substrate of β-galactosidase, using spectrophotometry. Mean values are shown ($n = 3$). Error bars represent standard deviations. **(B)** β-Galactosidase activity was visualized using X-gal, a substrate of β-galactosidase that produces the insoluble blue dye indigo. The experiment was conducted thrice, and representative images are shown. The statistical significance of the differences is tabulated in Table S3.

RpoS Activity Is Affected by the Residue 128 Substitution

Next, to investigate the effects of the substitution of residues 33 or 128 on the RpoS activity, we measured HPII activity and glycogen levels, which are positively regulated by RpoS and used as an indicator for RpoS activity (Weichart et al., 1993; Iwase et al., 2013). Both were low or undetectable in the *rpoS* variants containing the substitution at residue 128, while the substitution of residue 33 did not affect HPII activity (**Figure 2A**) or glycogen levels (**Figure 2B**).

RpoS Dysfunction via the Residue 128 Substitution Occurs at the Transcriptional Level of the RpoS-Regulated Genes

To investigate whether the residue 128 substitution affected in gene expression of RpoS-regulated genes, a reporter assay using β-galactosidase activity were performed. In the strains harboring *rpoS* with the residue 128 substitution, low β-galactosidase activity was observed (**Figure 3**). This result shows that the RpoS dysfunction resulted from the residue 128 substitution.

Taken together, the RpoS dysfunction observed in strain Kai1 is due to the substitution of residue 128 (Ile to Asn) in $rpoS^{Kai1}$.

RpoS Activity Is Affected by the Hydrophobicity of Residue 128

Next, to further investigate the significance of residue 128 on RpoS activity, we evaluated the effect of various amino acid substitutions at this position on RpoS activity using the HPII assay (**Figure 4**). The following amino acids were investigated: positively charged and hydrophilic amino acids (Arg and Lys), negatively charged and hydrophilic amino acids (Asp and Glu), an electrostatically neutral and hydrophilic amino acid (Gln), hydrophobic amino acids (Ala and Pro), a hydrophobic branched-chain amino acid (Leu), a hydrophobic

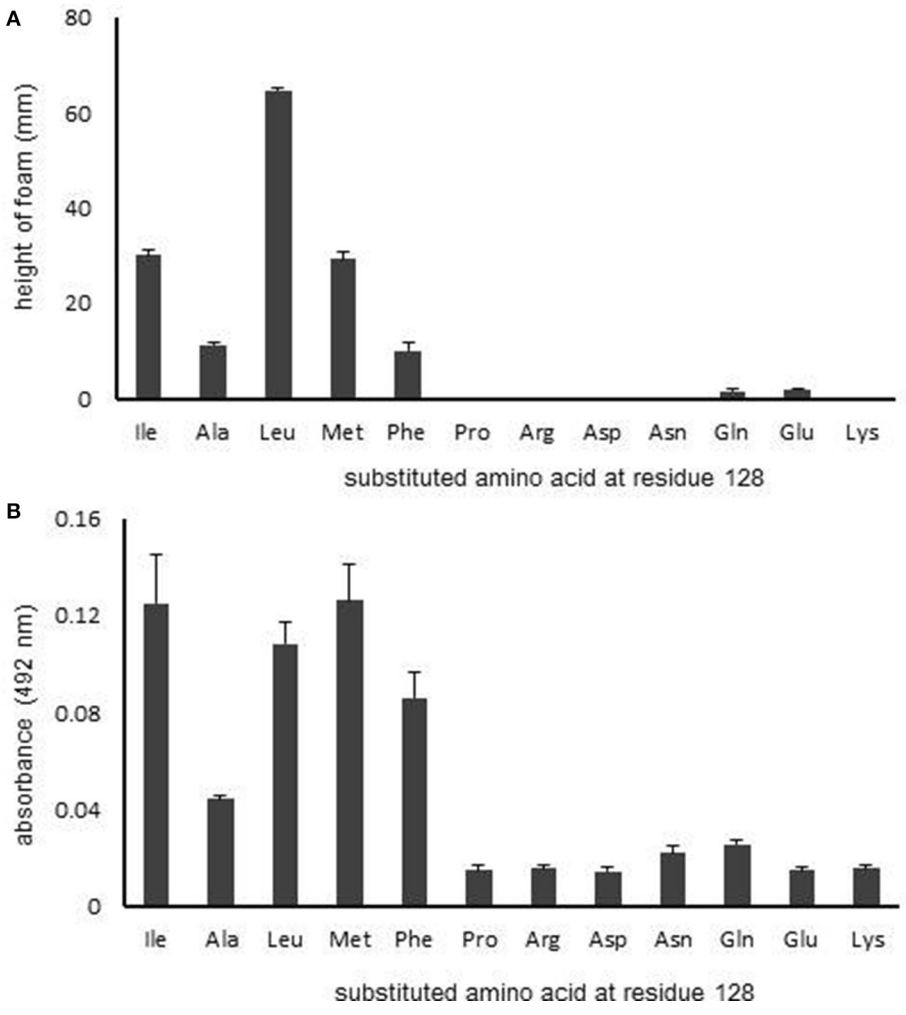

FIGURE 4 | Effect of the characteristics of the amino acid at residue 128 on RpoS activity. (A) HPII activity in *rpoS* variants was measured by HPII assays. **(B)** β-Galactosidase activity was measured using spectrophotometry. Mean values are shown ($n = 3$). Error bars represent standard deviations. The statistical significance of the differences is tabulated in Tables S4, S5.

amino acid containing sulfur (Met), and a hydrophobic amino acid containing an aromatic ring (Phe).

The *rpoS* variants that contained a hydrophobic amino acid, excepting for Pro, at the residue 128 retained RpoS activity (**Figure 4A**), whereas the RpoS activities of variants with hydrophilic amino acids at residue 128 were abolished (**Figure 4A**). We then conducted a reporter assay with β-galactosidase (**Figure 4B**). Similarly, the β-galactosidase activities of variants with hydrophilic amino acids at residue 128 were low (**Figure 4B**). These data indicate that the hydrophobicity of residue 128 is critical for RpoS function.

Alpha-Helix Structure Near Residue 128 Is a Key Factor for RpoS Activity

Despite the hydrophobicity of the proline residue, the proline substitution variant displayed no RpoS activity. Proline is generally known as an amino acid that destabilizes secondsary structures of proteins (Gray et al., 1996; Nilsson et al., 1998). The structural analysis for RpoS interacting with RNAP showed that residue 128 is on an α-helix (Liu et al., 2016). Assuming that secondsary structure preservation at residue 128 is another factor that affects RpoS activity, we evaluated the stability of the α-helix in partial structures around residue 128 for the wild-type protein and Asn128 and Pro128 variants using molecular dynamics (MD) simulation (Figure S1). For the calculations, we employed the fragment structure from residues 114 to 135 extracted from the X-ray crystal data of the RpoS-RNAP-4-nt nascent RNA ternary complex (PDB: 5IPL). The time courses of the average root mean squared deviation (RMSD) for the Cα-atoms are shown in Figure S2.

The obtained structures for the wild-type protein and Asn128 mutant indicated α-helicity in this region (Figures S1A,B). Contrarily, mutation of 128 to proline produced a bent structure (Figure S1C); Pro128 was the terminal of an α-helix unit and the secondsary structure from Gly124 to Leu127 was disrupted, and a salt bridge interaction between Arg127 and Glu132 may contribute to the production of such a structure. These data imply that the α-helix structure near residue 128 also plays an important role in RpoS function.

Residue 128 Substitution Affects External Stress Sensitivity

Additionally, we investigated the effect of the substitution at residue 128 on bacterial stress sensitivity (**Figure 5**). Bacteria expressing various RpoS which encoded by $rpoS^{Kai1}$, $rpoS^{K-12}$, $rpoS^{K-12/Gln33Glu}$, $rpoS^{K-12/Ile128Asn}$, or $rpoS^{K-12/Glu33Asn128}$ in addition to K−12 and Kai1 strains were exposed to H_2O_2 oxidative or cold stresses. Under oxidative stress conditions, *rpoS* variants with the residue 128 substitution ($rpoS^{Kai1}$, $rpoS^{K-12/Ile128Asn}$, and $rpoS^{K-12/Glu33Asn128}$) were more sensitive to oxidative stress than $rpoS^{K-12}$; there were no significant differences among the survival rates of $rpoS^{K-12}$, $rpoS^{K-12/Gln33Glu}$, or strain K−12 (**Figure 5A**). Of note, strain Kai1 was more resistant to stress than the *rpoS* variants carrying the residue 128 substitution ($rpoS^{Kai1}$, $rpoS^{K-12/Ile128Asn}$, and $rpoS^{K-12/Glu33Asn128}$), implying that strain Kai1 carried known or unknown RpoS-independent stress resistance mechanisms. Similar results were observed under cold stress conditions (**Figure 5B**); *rpoS* variants with the residue 128 substituted ($rpoS^{Kai1}$, $rpoS^{K-12/Ile128Asn}$, and $rpoS^{K-12/Glu33Asn128}$) were more sensitive to cold stress than $rpoS^{K-12}$.

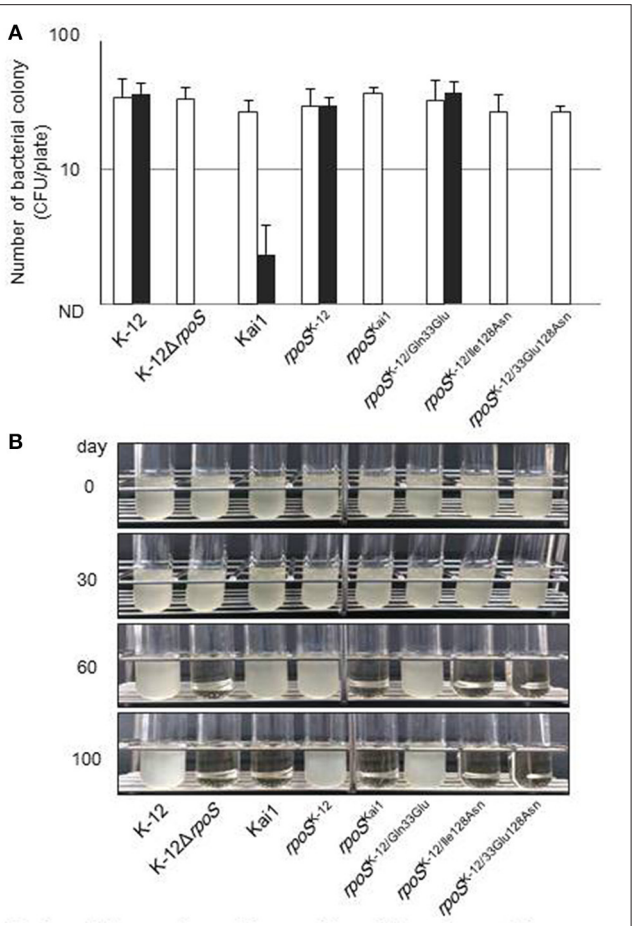

FIGURE 5 | Effect of substitution of the amino acid at residue 128 on bacterial stress sensitivity. (A) Bacteria were treated with 20 mM H_2O_2 aqueous solution, incubated at 25°C for 2 h, and then plated on LB agar for enumeration of bacterial counts to determine survival after H_2O_2 exposure. Filled columns, treated with H_2O_2; open columns, untreated control. Mean values are shown ($n = 3$). Error bars represent standard deviations. **(B)** Bacteria were cultured on LB agar at 37°C for 16 h and incubated at 4°C during the study period. Cold-stressed bacteria were cultured in LB broth at 37°C for 24–48 h to determine survival after cold-stress exposure. The experiment was conducted in triplicate, and typical images are shown. The statistical significance of the differences is tabulated in Table S6.

Ile128 or a Hydrophobic Amino Acid at Residue 128 Is Widely Conserved among Various Bacteria

Finally, to investigate importance of Ile128, we searched the *rpoS* sequence of various bacteria in the NCBI gene database (**Table 2**). *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2, *Pseudomonas aeruginosa* PAO1, *Yersinia pestis* CO92, and *Shigella dysenteriae* Sd197 contain Ile128 (**Table 2**), indicating

that Ile128 is widely conserved in Enterobacteriaceae. We further searched the RpoS sequences of *Legionella pneumophila*, *Coxiella burnetii*, and *Borrelia burgdorferi* B31, which have Met128, Leu128, and Ile128, respectively (**Table 2**). Interestingly, *L. pneumophila* and *C. burnetii* have Met and Leu which are hydrophobic; the activity of RpoS with Met128 was equal to that of RpoS with Ile128, and RpoS with Leu128 showed higher activity than that with Ile128 in our study. These data indicate that hydrophobicity of the amino acid at residue 128 is critical for RpoS activity and is consequently important for bacterial survival.

DISCUSSION

RpoS plays an important role in stress resistance (Klauck et al., 2007; Dong and Schellhorn, 2010; Battesti et al., 2011; Bleibtreu et al., 2014; Landini et al., 2014); therefore, many studies have investigated several *rpoS* mutations that modulate RpoS function and its regulation (Hengge-Aronis, 1993; Jishage and Ishihama, 1997; Chi et al., 2009; Dong et al., 2009; Carter et al., 2014; Maharjan and Ferenci, 2015). *rpoS* mutations of laboratory-stocked *E. coli* strains maintained in stab agar, leading to nutritional starvation, were reported to be associated with a high frequency of inactivating alleles (Bleibtreu et al., 2014). In a survey of *rpoS* among 2,040 environmental *E. coli* isolates, RpoS mutants were found to be present in the environment with a frequency of 0.003 among isolates (Chiang et al., 2011). These data indicate that RpoS mutants are generated under low nutrient conditions. The presence of mutant RpoS can be advantageous and disadvantageous: RpoS mutants showed faster growth in the presence of scarce carbon sources but also demonstrated lower stress resistance than strains containing RpoS positive strains (Chiang et al., 2011).

STEC can survive in various potentially stressful environments such as soil, river water, and vegetable surfaces, before infecting humans, and therefore, mutant RpoS is often present in clinically isolated strains. The presence of mutant RpoS may affect infection or pathogenicity of strains: mutant RpoS bearing strains are sensitive to acid and thus may find it difficult to pass the stomach. However, once these mutant RpoS-bearing pathogens reach the gut, they may cause severe damage to the host because wild-type RpoS suppresses STEC virulence factors (Iyoda and Watanabe, 2005; Dong et al., 2009; Dong and Schellhorn, 2010). This may be one reason for the high frequency of mutant RpoS in clinical isolates.

In the present study, we demonstrated that a point mutation in *rpoS* identified in a clinical STEC isolate affected RpoS activity with respect to the transcription of a gene regulated by RpoS; these results indicate that a single point mutation at a hydrophobic residue of the complex formed during transcription leads to a critical change in RpoS regulation.

The hydrophobicity of residue 128 was found to be critical for RpoS activity and stress resistance. Notably, the Pro128 variant had no RpoS activity, indicating that α-helicity is also a

TABLE 2 | Amino acid at residue 128 of RpoS among various bacteria.

Strain	Amino acid at residue 128
Escherichia coli MG1655	Ile
Salmonella enterica subsp. *enterica* serovar Typhimurium str. LT2	Ile
Pseudomonas aeruginosa PAO1	Ile
Yersinia pestis CO92	Ile
Shigella dysenteriae Sd197	Ile
Legionella pneumophila subsp. *pneumophila* str. Philadelphia 1	Met
Coxiella burnetii RSA 493	Leu
Borrelia burgdorferi B31	Ile

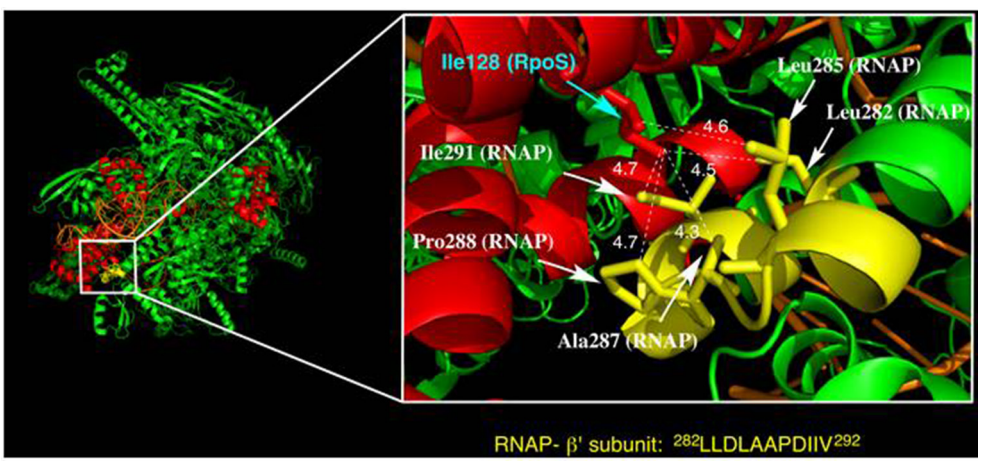

FIGURE 6 | Structure of a ternary complex of RpoS (red), RNAP (green and yellow), and synthetic oligonucleotide (orange) (from PDB: 5IPL). The magnified figure depicts the vicinity of Ile128 in RpoS. Amino acid residues in the RNAP b'-subunit near Ile128 of RpoS are marked in yellow. The moiety marked in yellow is a hydrophobic residue-rich region (see the amino acid sequence). Leu282, Leu285, Ala287, Pro288, and Ile291 in the RNAP b'-subunit face Ile128 of RpoS with distances of <5 Å.

crucial factor that determines the RpoS regulatory mechanism. According to the X-ray crystal structure of the RpoS-RNAP-4-nt nascent RNA ternary complex (PDB: 5IPL), Ile128 is present in the α-helix of Leu116-Glu132 (**Figure 6**), which is located at the complex surface. The helix faces two bundle moieties in the RNAP β' subunit (Ser263-Asn309). Several hydrophobic amino acid residues are located near Ile128 (Leu282, Leu285, Ala287, Pro288, and Ile291) with distances of <5 Å, suggesting that wide-range hydrophobic interactions in this area are an important factor in RpoS-RNAP binding. Introduction of a hydrophilic or helix-destroying amino acid residue into position 128 weakens RpoS-RNAP binding because of perturbation in the hydrophobicity of this region, resulting in significant RpoS dysfunction.

The binding ability of the mutant RpoS to RNAP and its detailed underlying mechanism should be assessed in future studies. In addition, a comparison of the crystal structures of mutant and functional RpoS proteins may offer further insight. These experiments can confirm our speculation that the mutation at residue 128 changing a hydrophobic amino acid to a hydrophilic amino acid weakens the hydrophobic interaction between RpoS and RNAP. Additionally, it would be interesting to investigate the effects of amino acid substitutions on RpoS expression because we observed mutations at residue 33 or at residues 33 and 128 to promote RpoS expression (**Figure 1**, Figure S3).

In conclusion, we found a novel, missense point mutation at RpoS residue 128. This point mutation results in the substitution of a hydrophobic amino acid with a hydrophilic or a helix-destroying amino acid at residue 128, leading to RpoS dysfunction. Remarkably, only one point mutation at a hydrophobic residue of the large macromolecular complex formed in transcription leads to a critical change in RpoS regulation. These findings provide insights on RpoS regulation and further bacterial stress responses.

AUTHOR CONTRIBUTIONS

TI designed the study. TI, TM, SN carried out experiments. TI, TM, SN, AT, YM discussed in detail about the obtained results. TI and TM wrote a draft, and TI wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00656/full#supplementary-material

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Escherichia coli O157:H7 Acid Sensitivity Correlates with Flocculation Phenotype during Nutrient Limitation

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Shiga toxin producing *Escherichia coli* (STEC) strains vary in acid resistance; however, little is known about the underlying mechanisms that result in strain specific differences. Among 25 STEC O157:H7 strains tested, 7 strains flocculated when grown statically for 18 h in minimal salts medium at 37°C, while 18 strains did not. Interestingly, the flocculation phenotype (cells came out of suspension) was found to correlate with degree of acid sensitivity in an assay with 400 mM acetic acid solution at pH 3.3 targeting acidified foods. Strains exhibiting flocculation were more acid sensitive and were designated FAS, for flocculation acid sensitive, while the acid resistant strain designated PAR for planktonic acid resistant. Flocculation was not observed for any strains during growth in complex medium (Luria Bertani broth). STEC strains B201 and B241 were chosen as representative FAS (2.4 log reduction) and PAR (0.15 log reduction) strains, respectively, due to differences in acid resistance and flocculation phenotype. Results from electron microscopy showed evidence of fimbriae production in B201, whereas fimbriae were not observed in B241.Curli fimbriae production was identified through plating on Congo red differential medium, and all FAS strains showed curli fimbriae production. Surprisingly, 5 PAR strains also had evidence of curli production. Transcriptomic and targeted gene expression data for B201 and B241indicated that *csg* and *hde* (curli and acid induced chaperone genes, respectively) expression positively correlated with the phenotypic differences observed for these strains. These data suggest that FAS strains grown in minimal medium express curli, resulting in a flocculation phenotype. This may be regulated by GcvB, which positively regulates curli fimbriae production and represses acid chaperone proteins. RpoS and other regulatory mechanisms may impact curli fimbriae production, as well. These findings may help elucidate mechanisms underlying differences among STEC strains in relating acid resistance and biofilm formation.

Keywords: STEC, acid resistance, nutrient limitation, curli, GcvB

INTRODUCTION

Shiga toxin producing *Escherichia coli* (STEC) O157:H7 and some related serotypes are of particular interest in food safety and public health due to their ability to colonize a human host with a low infectious dose, as little as 2–2,000 cells for foodborne outbreak strains, and cause severe illness (Buchanan and Doyle, 1997). *E. coli* O157:H7 and other STEC produce Shiga toxins, the most critical virulence factor involved in inducing gastrointestinal illness characterized by hemorrhagic colitis, which may develop into hemolytic uremic syndrome (HUS) and can ultimately result in death (Buchanan and Doyle, 1997; Park et al., 2001; Pennington, 2010). Previous work has identified STEC strains as acid resistant pathogens of concern for acidified foods (Breidt et al., 2007, 2013). Acid and acidified food products are defined as foods having a pH of 4.6 or lower, which prevents residual spores from germinating as mandated in the United States Code of Federal Regulations (21 CFR 114; US Food Drug Administration, 2012). These foods contain a variety of organic acids, which contribute to flavor and have antimicrobial effects (Breidt et al., 2004; Oh et al., 2009).

Weak acids, such as acetic acid and lactic acid, are able to diffuse across the bacterial cell membranes and dissociate intracellularly because the intracellular pH is higher than the environmental pH. The acidification of the cell cytoplasm and intracellular accumulation of acid anions can lead to cell death, but cell death due to acid stress is also dependent on other factors such as, acid type, pH, temperature, and growth phase (Lin et al., 1996; Diez-Gonzalez and Russell, 1997, 1999; Castanie-Cornet et al., 1999; Breidt et al., 2004). Disease outbreaks of *E. coli* O157:H7 from acid and acidified foods, such as the multi-state outbreak in apple cider, have made the relationship between the antimicrobial effects of weak acids and *E. coli*'s acid resistance systems an important concern (Miller and Kaspar, 1994; Buchanan and Doyle, 1997; Rangel et al., 2005).

Acid habituation, induced by growing cells in mildly acidic medium, results in higher survival rates in response to acid challenge compared to cells grown in neutral pH media (Buchanan and Edelson, 1996; Brudzinski and Harrison, 1998). There are multiple acid resistance systems (designated AR1 through AR4) in *E. coli*. AR1 was observed in stationary cells grown in pH 5.5 buffered Luria broth (Bearson et al., 2009). These cells were able to survive acid challenge whereas cells grown at higher pH could not. The RpoS sigma factor and the F_1F_0 ATPase are required for this system (Lin et al., 1996). Other acid resistance mechanisms require decarboxylase and antiporter systems, based on glutamate (AR2) or arginine (AR3) substrates, to effectively export protons out of the cell and raise the intracellular pH (Lin et al., 1995; Hersh et al., 1996; Castanie-Cornet et al., 1999; Iyer et al., 2003; Bearson et al., 2009). The activity of these acid resistance systems correlates with internal pH and the optimal pH function of the decarboxylases (Foster, 2004). A lysine decarboxylase system (AR4) has also been identified, but it is not as effective as AR2 or AR3 in raising intracellular pH (Iyer et al., 2003).

RpoS is activated during stationary phase or environmental stresses, such as, acid shock, to induce stress response genes. In *Salmonella enterica* serovar Typhimurium, it was found that RpoS expression affects the cell's acid stress response for all AR systems (Lin et al., 1995). Active RpoS has been found to be essential for AR1 in *E. coli,* and RpoS mutants appear to indirectly affect AR2 and AR3 decarboxylase activity (Lin et al., 1996; Castanie-Cornet et al., 1999). RpoS also plays an important role in the regulation of biofilm formation, including the formation of extracellular curli amyloid protein fibers (Uhlich et al., 2013).

Curli expression by STEC is characterized by adhesive fimbrial aggregates that can result in biofilm production, contributing to the transition of motile cells to the sessile phenotype during environmental stress. Regulation of curli is complex, mediated by RpoS, regulatory small RNAs (sRNA), cross-talk with other surface fimbriae, and other regulatory molecules acting at both the transcription and translational levels (Beloin et al., 2008; Pesavento et al., 2008; Boehm and Vogel, 2012; Lloyd et al., 2012a). Carbon source quality, osmotic imbalance, and nutrient deprivation activate regulatory small RNAs (McaS, OmpR, and GcvB) that target curli synthesis at the translational level (Vidal et al., 1998; Chen et al., 2002; Jorgensen et al., 2012). Of particular interest, GcvB acts as a global repressor of amino acid metabolism, and transcription of *gcvB* is dependent on the concentration of environmental glycine. GcvB is also known to be a regulator of acid induced chaperone proteins (Urbanowski et al., 2000). If the concentration of environmental glycine is high, *gcvB* transcription may be upregulated, repressing expression of *csgD* and the curli structural genes *csgBA*, as well as inducing acid stress chaperone proteins encoded by *hdeAB.* GcvB has also been found to increase expression of RpoS under acid stress (Jin et al., 2009; Stauffer and Stauffer, 2012a).

A correlation between the production of curli fimbriae and acid resistance has been observed previously in *E. coli* O157:H7 strains, but the regulatory mechanisms responsible for coordinate regulation and the relationship to nutrient limitation are not understood. We proposed a hypothesis that nutrient limitation influenced expression of GcvB, RpoS, and other regulatory molecules that control expression of CsgD (curli) and HdeAB (acid chaperones). Because the differences in regulation of curli fimbriae were only observed for more acid sensitive cells grown in a minimal medium, gene expression of carbon storage, curli fimbriae, and changes in global regulators for acid resistance were targeted. These data will contribute to our understanding of how STEC strains respond differently to nutrient limitation and acid stress.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

All *E. coli* O157:H7 and K-12 strains used were obtained from the USDA Food Science Research Unit, Raleigh, NC culture collection and are listed in **Table 1**. Bacteria were grown statically in either 10 ml of Luria Bertani broth supplemented with 1% glucose (LBG; LB, Becton Dickinson, Sparks, MD) or a defined minimal medium (5X M9 Minimal Salts, Sigma-Aldrich, St. Louis, MO) supplemented with 1% glucose and 0.005% thiamine (M9GT) at 37°C for 18 h to induce acid resistance. Glycine

TABLE 1 | *Escherichia coli* O157:H7 isolates, acid resistance, and designated flocculation and curli phenotypes used in this study.

Isolate		Source (reference)[a]	Reduction in log CFU/ml[b]	Phenotype[c]	Curli[d]
ID no.	Original ID no.				
B055	USDA-ARS K-12	Human isolate	N/A	FAS	–
B201	SRCC 1675	Apple cider, October 2002	2.42 ± 0.05	FAS	++
B202	SRCC 1486	Salami, October 2002	0.29 ± 0.01	PAR	–
B204	SRCC 1941	Pork, September 2002	0.17 ± 0.01	PAR	+
B241	28RCI	Bovine carcass	0.15 ± 0.02	PAR	–
B244	3014-93	Human outbreak	0.15 ± 0.01	PAR	+
B245	3055-93	Human outbreak	0.26 ± 0.08	PAR	–
B246	3139-98	Human outbreak	1.06 ± 0.18	FAS	+
B247	3159-98	Human outbreak	0.26 ± 0.08	PAR	+
B249	3187-95	Human outbreak	0.17 ± 0.03	PAR	–
B250	3261-98	Human outbreak	0.17 ± 0.03	FAR	++
B251	3361-91	Human outbreak	0.16 ± 0.03	PAR	–
B263	RM1242	Human, sporadic, 1997	1.04 ± 0.09	FAS	+
B264	RM1484	Apple juice, associated with 1996outbreak	0.21 ± 0.03	PAR	+
B265	RM1918	Human, outbreak, 1999, lettuce	0.27 ± 0.03	PAR	–
B266	RM2189	Human, outbreak, 1999, taco meat	0.16 ± 0.05	FAR	+
B269	RM4263	Human, outbreak, 2000, waterborne	0.09 ± 0.02	PAR	+
B271	RM4406	Human, outbreak, 2003, leafy vegetable	1.00 ± 0.05	FAS	++
B273	RM4688	Human, outbreak, 2002, leafy vegetable	0.25 ± 0.01	PAR	–
B296	RM5279	Human, outbreak, 2005, leafy vegetable	0.36 ± 0.12	PAR	–
B301	RM5630	Water	0.11 ± 0.01	PAR	–
B306	RM5850	Water	0.19 ± 0.05	PAR	–
B307	RM5875	Water	1.03 ± 0.11	FAS	++
B309	RM5714	Water	0.49 ± 0.07	PAR	–
B311	RM6011	Human, outbreak, 2006, leafy vegetable	0.31 ± 0.03	PAR	–
B349	NMSLD-1	Spinach	0.28 ± 0.06	PAR	–

Identification of E. coli O157:H7 isolates; a, reference source (Oh et al., 2009); b, log reduction with standard error after acid challenge (400 mM acetic acid, pH 3.3, 30°C) after growth in LBG; c, acid sensitivity and flocculation phenotypes FAR (flocculating - acid resistant), PAR (planktonic - acid resistant) and FAS (flocculating - acid sensitive); d, curli fimbriae production denoted by (–) indicating no curli production, (+) some curli expression, and (++) maximal expression of curli via colorimetric assay[d].

(5 mM) was added to M9GT as indicated below. Cultures were harvested by centrifugation (5,000 × g, 10 min, 10°C, Sorvall Superspeed Centrifuge, SS-34 rotor, DuPont Instruments, Newton, CT), decanted, and re-suspended in 10 ml sterile saline [0.85% sodium chloride (NaCl)] twice prior to use.

For acid challenge experiments, initial cell concentrations were approximately 10^9 CFU/ml. After acid challenge (see below) cells were diluted 1:10 in 0.1 M 3 N-morpholino propane sulfonic acid buffer (MOPS; pH 7.2, Sigma Aldrich) to relieve acid stress, followed by serial dilution in sterile saline. Cells were enumerated on LB agar (Becton Dickinson) using a spiral plater (Autoplate 4000, Spiral Biotech, Inc., Norwood, MA) and an automated plate reader (QCount, Spiral Biotech), or by plating 10 µl onto 5 ml of LB agar in 24-well microtiter plate wells to estimate counted colony forming units (CFU).

For gene deletion experiments, bacterial strains were grown in LBG broth or on LB agar supplemented with antibiotics at the following concentrations, 50 µg/ml ampicillin and 25 µg/ml chloramphenicol. For gene deletion experiments, bacteria were grown aerobically in 1.0 ml of LBG at 37°C overnight at 1200 RPM in the Eppendorf Thermomixer R (Hamburg, Germany). Overnight cultures (30 µL) were re-inoculated in 1.0 ml fresh LBG and grown for 3 h at 37°C before electroporation.

Curli and Motility Assays

Curli phenotype was determined for all strains of *E. coli* O157:H7 and a K-12 strain. Cultures were grown in LBG or M9GT broth at 37°C for 18 h, and then plated onto Congo red indicator (CRI) agar composed of 10 g/L casamino acids (Becton Dickinson), 1 g/L yeast extract (Becton Dickinson), and 20 g/L agar (Thermo Fisher Scientific, Fair Lawn, NJ) with 20 mg/L of Congo red (Polysciences, Inc., Warrington, PA) with and without 10 mg/L Coomassie Brilliant Blue G-250 (MP Biomedicals, Inc., Solon, OH; Uhlich et al., 2006). LB agar supplemented with 200 mg/L calcoflour white stain (Fluka Analytical), a UV fluorescent stain that binds to cellulose and chitin, plates were used to identify cellulose production (Hammar et al., 1995; Uhlich et al., 2006). Motility was determined using motility test medium (MTM; 3 g/L beef extract (Becton Dickinson), 10 g/L pancreatic digest of casein (Becton Dickinson), 5 g/L NaCl, and 4 g/L agar) stab cultures grown 18 h at 37°C (Atlas and Parks, 1993).

Acid Resistance Assay

To determine acid resistance of STEC strains, acetic acid solutions were prepared as described previously (Oh et al., 2009). The pH of the glacial acetic acid solutions (400 mM) was adjusted to 3.3 with the addition of sodium hydroxide (1N). Cell suspensions were added (200 μl) into 1.8 ml of acetic acid solution in 12-well tissue culture plates. The 12-well tissue culture plate was incubated aerobically at 30°C for 25 min (Oh et al., 2009). After incubation, 20 μl of the cell suspension was transferred into 180 μl of 0.1 M MOPS followed by serial 10-fold dilutions and plating onto LB agar to determine CFU/ml as described above.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed to confirm the presence or absence of flagella in acid resistant and sensitive strains. Cultures were grown statically in 10 ml of M9GT or LBG at 37°C for 18 h, harvested by centrifugation (5,000 × g, 10 min, 10°C), and then re-suspended in 1 ml sterile saline and serially diluted into 3% glutaraldehyde fixative at 4°C for 4 h. Cells were harvested in a microcentrifuge (10,000 × g, 10 min, 22°C, Spectrafuge 24D Digital Microcentrifuge, Labnet International, Inc., Edison, NJ), re-suspended in 0.1 M sodium acetate buffer at pH 7.0, and transported to North Carolina State University's Center for Electron Microscopy for processing. Cells were stained with 1 and 0.5% phosphotungstic acid (PTA; Ernest F. Fullan, Inc., Schenectady, NY) on 400-mesh carbon grids (Ladd Research Industries, Williston, VT). Cells on the grids were imaged at a magnification of either 12,000X or 15,000X on a JEOL JEM 1200 EX (JEOL U.S.A., Peabody, MA) transmission electron microscope at 80 kV.

RNA Extraction

Two volumes of RNA-protect Bacterial Reagent (Qiagen Sciences, Inc., Germantown, MD) were added to one volume (1.5 ml) of culture (approximately 10^9 CFU/ml) grown statically in M9GT ± 1% glycine at 37°C for 18 h. Cells were incubated at room temperature for 5 min and harvested by centrifugation (5,000 × g, 10 min, 10°C). Total RNA was extracted using the RNeasy Mini Kit (Qiagen Sciences) with an additional on-column DNaseI digestion following the manufacturer's protocol. For transcriptomic analysis, a higher yield of total RNA was required, and thus the RNeasy Midi Kit (Qiagen Sciences) was used. DNA and ribosomal RNA (rRNA) were removed from the extracted RNA using the TURBO DNA-free kit (Thermo Fisher Scientific) and Ribo-Zero rRNA Removal Gram-Negative kit (Illumina, Inc., San Diego, CA). The rRNA-depleted RNA samples were concentrated and purified using Zymo RNA Clean & Concentrator columns (Zymo Research, Irvine, CA). Concentration and quality of RNA were determined using the RNA Nano-chip and the RNA Pico-chip on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) for transcriptomic analysis, or a Nanodrop ND-1000 UV-visible wavelength spectrophotometer (NanoDrop Technologies, Wilmington, DE) for RT-qPCR experiments.

TABLE 2 | The primer sequences for RT-qPCR and chloramphenicol linear cassette.

Target gene	Primer code	Sequence	Amplicon length[a]
Tus	tus_For1	5′-CACAGAACGCGAAGTTA-3′	115
	tus_Rev1	5′-GCAATCAGTGGTGTAGG-3′	
csgA	csgA_For1	5′-GCTGATGCTCGTAACTC-3′	152
	csgA_Rev1	5′-GAGTCTTTACCGTTCCAC-3′	
csgB	csgB_For1	5′-GCCAATGATGCCAGTAT-3′	156
	csgB_Rev1	5′-TTGTGTCACGCGAATAG-3′	
csgD	csgD_For1	5′-AGTAAGGAGGGCTGATT-3′	143
	csgD_Rev1	5′-CCATGGAGGATCAAGAAC-3′	
	csgDKO_ChlorF50	5′-CGAACAGAAATTCTGCCGC CACAATCCAGCGTAAATAACG TTTCATGGCTTGTGACGGAA GATCACTTCG-3′	1,111
	csgDKO_ChlorR50	5′-TGCTTCTATTTTAGAGGCAG CTGTCAGGTGTGCGATCAATA AAAAAAGCGACCAGCAATAGAC ATAAGCG-3′	
	csgDSeq_For1	5′-ACTTCTACCTCAACGGCGTG-3′	776, 1,136
	csgDSeq_Rev1	5′-GCTGTCAGGTGTGCGATCA-3′	
csrA	csrA_For1	5′-CAGCCTGGATACGCTGGTAG-3′	147
	csrA_Rev1	5′-CTCGTCGAGTTGGTGAGACC-3′	
csrB	csrB_For1	5′-CAGGATGGAGAATGAGAAC-3′	106
	csrB_Rev1	5′-CTATTGCTTCCTGCTCAC-3′	
csrC	csrC_For1	5′-GCGAAGACAGAGGATTG-3′	149
	csrC_Rev1	5′-CCTGACTCATAACCCTTAAC-3′	
csrD	csrD_For1	5′-CTGGTTCTCCGTTCGCTTCT-3′	206
	csrD_Rev1	5′-TTGAACTTGCAGAGGCCGAT-3′	
cycA	cycA_For1	5′-AACAGCGTCCTCATCTA-3′	121
	cycA_Rev1	5′-GTGTCATCTTCCAGTGTC-3′	
flhC	flhC_For1	5′-GTGCGGTTTGTTGAAAG-3′	121
	flhC_Rev1	5′-ATGGCGGTTGACATAAG-3′	
flhD	flhD_For1	5′-CCGCTATGTTTCGTCTC-3′	100
	flhD_Rev1	5′-ACCAGCTGATTGGTTTC-3′	
fliC	fliC_ForA	5′-CGCGGAGTTCACATTTA-3′	140
	fliC_RevA	5′-CTAACGTTGCCGACTATAC-3′	
gcvA	gcvA_For1	5′-GAACACACCGGCAATAA-3′	119
	gcvA_Rev1	5′-GATCGTCAGGAAGATAAGC-3′	
gcvB	gcvB_For1	5′-CCTGAGCCGGAACGAAA-3′	106
	gcvB_Rev1	5′-GTCTGAATCGCAGACCAA-3′	
gcvR	gcvR_For1	5′-CCGTCATGTCAGTAGTTG−3′	100
	gcvR_Rev1	5′-ATTCCATGAACCGGAAAG-3′	
glyA	glyA_For1	5′-CCAGGAACAGATGGTTATC-3′	136
	glyA_Rev1	5′-CTGAAAGAAGCGATGGAG-3′	
hdeA	hdeA_For1	5′-GTACAAGCCTGAACGATAG-3′	154
	hdeA_Rev1	5′-GGACCTGTGAAGATTTCC-3′	

(Continued)

TABLE 2 | Continued

Target gene	Primer code	Sequence	Amplicon length[a]
hdeB	hdeB_For1	5′-ATTCCTGGCAGGTCATA-3′	117
	hdeB_Rev1	5′-TTTCATCTCTCCGTAAAGC-3′	
hfq	hfq_For1	5′-GGGCAAATCGAGTCTTT-3′	126
	hfq_Rev1	5′-GGCGTTGTTACTGTGAT-3′	
mlrA	mlrA_For1	5′-CGAACGTGGATCAAAGAG-3′	162
	mlrA_Rev1	5′-CAGACAAATGGCGATGTA-3′	
pgaA	pgaA_For2	5′-GGTCAGACTGTCGTTTATG-3′	100
	pgaA_Rev2	5′-AGTACGGTCTGGGTTATC-3′	
pgaB	pgaB_For2	5′-GTGGATGCCGGTATTAAG-3′	113
	pgaB_Rev2	5′-GAGAGAGACGGTGATATTG-3′	
pgaC	pgaC_For2	5′-CGTCTATTTCTGGGTCTATC-3′	141
	pgaC_Rev2	5′-GCGTGTATGGTTTCCTC-3′	
pgaD	pgaD_For2	5′-GGGCGCTGTACAATAAG-3′	104
	pgaD_Rev2	5′-GAGCTCATCAGGTATTGC-3′	
rpoS	rpoS_For1	5′-CGAATAGTACGGGTTTGG-3′	154
	rpoS_Rev1	5′-CGTTGCTGGACCTTATC-3′	

List of primers designed for RT-qPCR and amplification of the chloramphenicol linear cassette, with 50 nt homology up and downstream of target genes as well as defined cat cassette homology (indicated by underlined nts) to T-SACK, needed for gene deletion mutants. Corresponding amplicon length for RT-qPCR targets, linear cassette, and sequencing primers are listed; a, where sequencing primers have two amplicon lengths derived from the wild-type and respective gene deletion mutant strains.

RNA Sequencing and Transcriptomic Analysis

One library for each strain (B201, B241, and B250) was prepared using the Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) following the manufacturer's instructions with one exception: incubation time for RNA shearing was reduced to 2.5 min. The final cDNA library was quantified with a 2100 Bioanalyzer using a High Sensitivity DNA Chip (Agilent). Library enrichment was carried out with an Ion PGM™ Template OT2 200 kit and Ion OneTouch™ 2 system (Thermo Fisher Scientific). Each library was sequenced with an Ion 318 Chip using an Ion PGM 200 Sequencing kit (Thermo Fisher Scientific) on the Ion Personal Genome Machine (Thermo Fisher Scientific). RNA-Seq data were analyzed by ProteinCT Biotechnologies (Madison, WI, USA). Briefly, raw reads were mapped to the *E. coli* O157 B201 and B241 genome sequences (Baranzoni et al., 2016) using the Subjunc program from the Subread-1.4.6 package (http://bioinf.wehi.edu.au/subread). Raw gene counts were generated by FeatureCounts from the Subread package, and differential expression was calculated using the Limma package (https://bioconductor.org/packages/release/bioc/html/limma.html). Transcriptomics data are available under NCBI BioProject number PRJNA381969.

Quantitative Real-Time Reverse Transcriptase-PCR (RT-qPCR)

For quantitative analysis of gene expression, the Bio-Rad One-Step RT-qPCR kit (Bio-Rad, Hercules, CA) was used to synthesize cDNA from cellular RNA (2 μl template) extracted from *E. coli* B201, B241, and B250. Primers for selected transcripts (**Table 2**) were designed with the IDT Real Time PCR Tool. Gene expression analysis was performed on the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The Bio-Rad One-Step RT-qPCR kit (Bio-Rad) was tested without RNA, without primers, and without reverse transcriptase as negative controls to determine the efficacy of the kit, the presence of additional genomic DNA, and potential primer-dimer interactions, respectively. Target genes were normalized using the *tus* gene as an endogenous control (Crépin et al., 2012; Porcheron et al., 2014). Cycle threshold (C_T) values and relative fold change were calculated as ($2^{-\Delta\Delta C_T}$), using an average of three independent replications (Livak and Schmittgen, 2001).

Generation of a Functional Chloramphenicol Cassette

Primers for gene deletion mutants (**Table 2**) were designed to produce a functional chloramphenicol linear cassette (1,011 bp). Linear cassette primers were designed with 50 bp homology directly up and downstream of the target insertion site and with flanking homology arms to a *cat* (CmR) region in T-SACK (Li et al., 2013). T-SACK, an *E. coli* strain carrying amplifiable drug resistance cassettes [*tetA-sacB*, *amp*, *cat*, and *kan*], was generously provided to us by Dr. Donald Court at the National Cancer Institute (Frederick, MD). Additional primers for gene sequencing were designed with the IDT Real Time PCR Tool (**Table 2**). Platinum PCR Supermix High Fidelity (Invitrogen, Carlsbad, CA) was used for amplification of the functional chloramphenical cassette. T-SACK DNA template was used for the initial amplification of the chloramphenicol cassette, but secondary amplification was performed with PCR product template for increased DNA concentrations (approximately 400 ng/μl). PCR products were purified to eliminate unincorporated primers, primer-dimers, and non-specific amplification products with the QIAquick PCR Purification Kit (Qiagen Sciences, Inc., Germantown, MD). DNA concentration and quality was determined using the Nanodrop ND-100 UV-visible wavelength spectrophotometer (NanoDrop Technologies, Wilmington, DE). Lastly, the chloramphenicol cassette was visualized by confirming the correct band size, 1,111 bp, through gel electrophoresis.

Gene Deletion Mutants

The Quick & Easy *E. coli* Gene Deletion Kit (Gene Bridges, Heidelberg, Germany) was used according to manufacturer's protocol, with some exceptions, to produce gene deletion mutants. *E. coli* strains B201 and B241 were grown as stated for gene deletion experiments and transformed with 5 μl Red/ET expression plasmid carrying a functional *amp* region (pRedET, 20 ng/μl) by electroporation (Eppendorf 2510 Electroporator) at 1350 V, 10μF, 600 Ohms with a 5 ms pulse rate. Transformants (B201+pRedET and B241+pRedET) were selected on LB-ampicillin agar grown at 30°C overnight. Transformant colonies were grown as stated for gene deletion experiments, but at 30°C in LBG supplemented with ampicillin. Overnight cultures (30 μl) were re-inoculated in fresh media conditioned with ampicillin at 30°C for 2 h before inducing pRedET with 50 μl 10% L-arabinose and incubating at 37°C

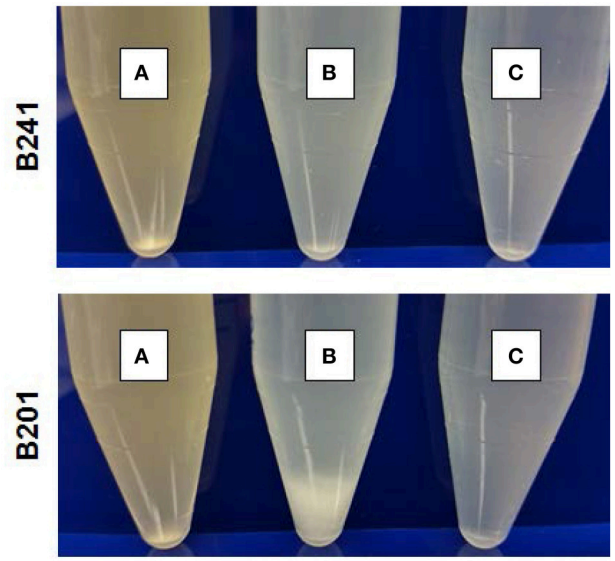

FIGURE 1 | Growth of *E. coli* O157:H7 PAR strain B241 **(top)** and FAS strain B201 **(bottom)** in (A) LBG, (B) M9GT, and (C) M9GT supplemented with 1% glycine. Representative flocculation phenotype is displayed by FAS strain B201 in M9GT whereas flocculation is repressed with glycine supplementation.

for 1 h. Induced cells were harvested in a cooled benchtop microcentrifuge (13,000 × g, 1 min, 4°C, Spectrafuge 24D Digital Microcentrifuge, Labnet International, Inc., Edison, NJ), decanted, and re-suspended in sterile ice cold de-ionized water three times before electroporation with the chloramphenicol cassette (1 μg/μl). Electroporated transformants were added to 1.0 ml LBG without antibiotics and incubated at 37°C for 3 h for recombineering to occur. Recombinant colonies were grown on LB agar conditioned with chloramphenicol at 37°C overnight.

Gel Electrophoresis and Sequencing of Gene Deletion Mutants

Gene deletion mutants were confirmed through colony PCR. Briefly, recombinant and wild-type colonies were re-suspended in 30 μl of de-ionized water and incubated at 98°C for 5 min to lyse cells. PCR was performed with Platinum PCR Supermix High Fidelity recombinant and wild-type DNA templates (2 μl), linear cassette primers, and sequencing primers. PCR products were visualized by agarose gel electrophoresis, ran at 65V for 100 min, and confirmed by band size by comparison with standards (accession number CP015020 and CP015023 for B201 and B241, respectively). Additionally, gene deletion mutants and wild-type target genes were confirmed through PCR product sequencing by Eton Biosciences, Inc. (Durham, NC). PCR product chromatographs were analyzed through FinchTV software (Perkin Elmer, Waltham, MA) and the corresponding sequences were aligned with B201 (accession number CP015020) and B241 (accession number CP015023) sequences through GenBank Blastn software (National Center for Biotechnology Information, Bethesda, MD; Baranzoni et al., 2016).

Statistical Analysis

All experiments were done with three or more independent replications. Statistical analysis for acid challenge and C_T data was performed in SAS (version 9.4 software, SAS Institute, Inc., Cary, NC). Differences ($p < 0.05$) in acid resistance between bacterial strains were determined using the student *t*-test. Distribution and variance was determined ($p < 0.05$) through the Shapiro-wilk and Fligner-Killeen tests, respectively. Differences ($p < 0.05$) in acid resistance between the presence or absence of flocculation or curli fimbriae production were determined using the Welch *t*-test, due to the data not being distributed normally and having heterogeneous variance. Differences ($p < 0.05$) between strain gene expression were analyzed with both ΔC_T and linearized ($2^{-\Delta C_T}$) values by student *t*-test controlling the False Discovery Rate using Benjamini-Hochberg correction.

RESULTS

Characterization of Flocculating and Planktonic STEC Strains

When grown in M9GT, the flocculation phenotype was found to correlate to acid sensitivity ($p < 0.05$). Planktonic strains were in general more acid resistant than flocculating strains, typically having less than a 1 log reduction in cell number in the acid resistance assay (**Table 1**). These strains were designated PAR (planktonic acid resistant). Flocculating strains had greater acid sensitivity, and were designated FAS (flocculating acid sensitive). Of the 26 *E. coli* strains tested 6 were defined as FAS, including a K-12 strain, while 18 were classified as PAR (**Table 1**). Two remaining strains (B250 and B266) had an intermediate phenotype and were designated FAR (flocculating acid resistant). B241 and B201 were chosen as representative PAR and FAS strains because of contrasting acid resistance, with log reduction values of 0.15 and 2.42, respectively, in the acetic acid assay (**Table 1**). B201 was isolated from apple cider, and B241 was a bovine isolate. These two O157:H7 strains have been sequenced (Baranzoni et al., 2016) and both STEC strains had similar genome size and content, and each strain harbored a large 81 kb (B241) or 95 kb (B201) plasmid.

Figure 1 shows the flocculation phenotype for representative strains B201 (FAS, 2.42 log reduction) and suspended phenotype for B241 (PAR, 0.15 log reduction) grown in M9GT and LBG. Additionally, acid resistance of 3 of the 26 strains B201, B241, and B250 (FAR, 0.17 log reduction) was tested when the strains were grown in M9GT with and without supplemental glycine. No significant difference was seen in viability between strains grown in M9GT and LBG. Flocculation was not apparent regardless of acid resistance phenotype in complex medium or M9GT with glycine supplementation (**Figure 1**).

Curli Expression for Acid Sensitive and Resistant STEC

Strains B201 and B241 were grown in both M9GT and LBG and subjected to TEM. We found that B201 cells grown in M9GT appeared in clumps with a network of fimbriae, and

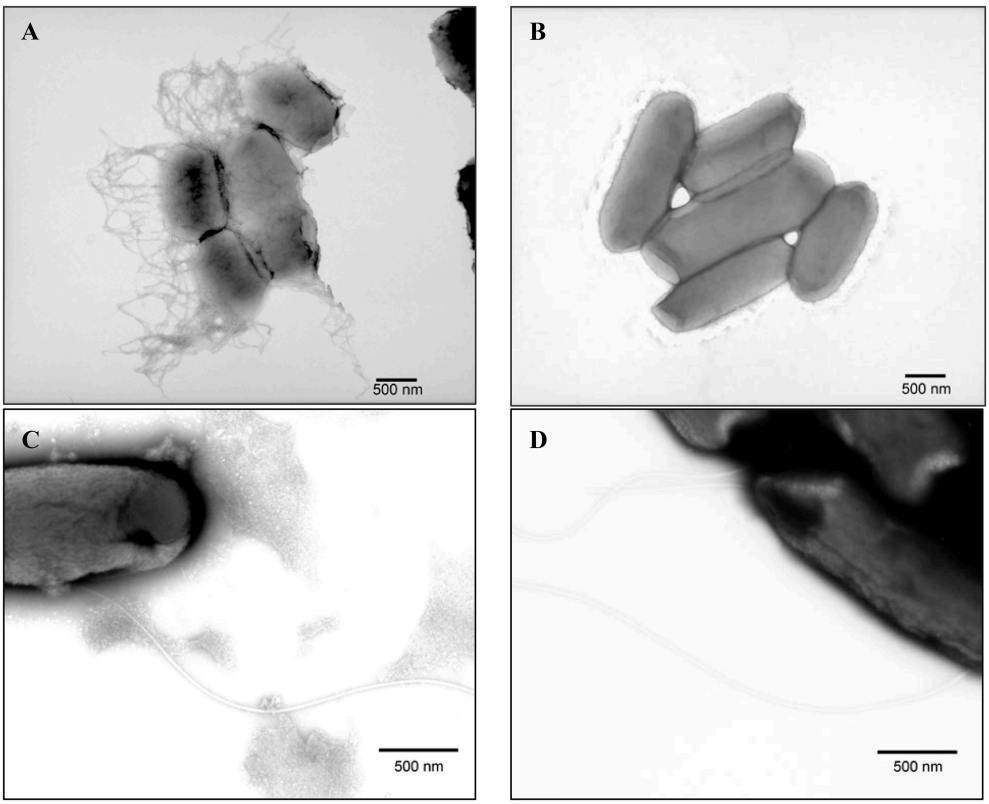

FIGURE 2 | Transmission electron microscopy images of PTA stained **(A)** acid sensitive (B201) grown in M9GT exhibiting curli, **(B)** acid resistant (B241) grown in M9GT without curli, **(C)** acid sensitive (B201) grown in LBG exhibiting flagella, and **(D)** acid resistant (B241) grown in LBG exhibiting flagella.

flagella were not observed (**Figure 2A**). These data indicated that B201 should be non-motile when grown in M9GT. Data from a motility test were inconclusive; however, B241 cells grown under the same conditions were not observed to have fimbriae or flagella (**Figure 2B**). However, B201 and B241 cells grown in LBG displayed flagella with no apparent fimbriae (**Figures 2C,D**, respectively). All strains were screened for the curli phenotype using CRI agar assay (**Table 1**). FAS strains all produced curli, and the curli phenotype was statistically associated ($p < 0.05$) with lower levels of acid resistance when compared to non-curli producing strains. Of the 20 acid resistant strains, 13 strains did not produce curli. Notably, acid resistant strain B250 exhibited high levels of curli production when compared to other resistant strains (**Table 1**). Interestingly, the calcofluor assay indicated that cellulose, which may be present during biofilm formation and associated with curli production, was not being produced by any of the strains (data not shown). Constructed curli deletion mutant B201Δ*csgD*, which did not express the curli regulatory protein CsgD, did not flocculate when grown in M9GT. Unsurprisingly, no difference in phenotype was observed for B241Δ*csgD* when grown in M9GT. Additional studies with B201Δ*csgD* and B241Δ*csgD* will be the subject to future work. These data support the hypothesis that curli fimbriae production was responsible for the flocculation seen in FAS STEC strains.

The Carbon Storage, Flagella, and Chaperone Proteins

To determine if carbon storage gene regulation correlated with curli fimbriae and flagella expression, we examined the expression of target genes for carbon storage regulation *csrABCD* (Dubey et al., 2003; Suzuki et al., 2006; Jonas and Melefors, 2009), flagella, and biofilm production *flhDC* (Wei et al., 2001; Yakhnin et al., 2013), *fliC* (Ogasawara et al., 2011), *mlrA* (Uhlich et al., 2013), and *pgaABCD* (Wang et al., 2005) in B201 and B241 grown in M9GT using a transcriptomic approach. We found that *csrB* and *csrC* (and other genes encoding sRNAs, including the regulatory sRNA *gcvB*, see below) were not present in the Limma package output, and quantitative expression studies for these genes were done using RT-qPCR. Expression of *csrA, flhC, flhD, mlrA,* and *pgaAB* was significantly different ($p < 0.05$) between B201 and B241, where *csrA* was down-regulated (−5.43 fold change) for B201 in comparison to B241 when grown in minimal medium and *flhC, flhD, mlrA,* and *pgaAB* were up-regulated (+1.76, +1.56, +1.27, +2.53, and +1.67, respectively). Fold-change values were determined for carbon storage, *csrBC*, and biofilm, *pgaCD,* genes (−1.33, −1.41, +1.85 and +1.76, respectively) although no statistical significance ($p > 0.05$) between mRNA levels was seen. Average C_T values and associated fold change are listed on **Table 3**. Transcriptomic analysis showed that some flagella genes, including the flagellar

TABLE 3 | Transcriptomics data for selected genes from strains B201 and B241.

Gene	B201 ($2^{-\Delta C_T}$)			B241 ($2^{-\Delta C_T}$)			Fold change	*p*-values ($2^{-\Delta C_T}$)	*p*-values (ΔC_T)
	Mean	*SD*	CV (%)	Mean	*SD*	CV (%)			
csgA	194.02	84.02	43	17.31	2.03	12	11.21	**0.0479***	**0.008***
csgB	74.56	58.06	78	11.10	1.70	15	6.71	0.2102	0.184
csgD	33.91	14.95	44	7.99	1.76	22	4.25	0.0822	**0.025***
csrA	42.11	16.95	40	228.67	79.32	35	−5.43	**0.0417***	**0.025***
csrB	93,040.36	73,676.54	79	123,916.42	93,430.23	75	−1.33	0.7058	0.735
csrC	34.94	45.76	131	49.36	74.84	152	−1.41	0.7893	0.961
csrD	19.20	3.80	20	14.96	2.53	17	1.28	0.2449	0.241
cycA	25.32	19.33	76	41.45	22.31	54	−1.64	0.5049	0.438
flhC	13.24	0.90	7	8.48	0.59	7	1.56	**0.0196***	**0.011***
flhD	16.06	1.95	12	9.12	0.54	6	1.76	**0.0225***	**0.011***
fliC	18.82	1.77	9	14.88	3.68	25	1.26	0.2449	0.241
gcvA	13.20	6.55	50	17.74	8.46	48	−1.34	0.5929	0.642
gcvB	3.33	1.34	40	8.19	3.95	48	−2.46	0.1955	0.115
gcvR	53.51	40.37	75	37.90	5.91	16	1.41	0.5929	0.735
glyA	12.67	9.91	78	76.65	13.48	18	−6.05	**0.0215***	**0.032***
hdeA	17.50	10.54	59	979.68	209.54	21	−55.02	**0.0196***	**0.006***
hdeB	13.15	8.84	67	52.00	12.80	25	−3.95	**0.0373***	**0.040***
hfq	72.72	37.24	51	317.29	256.31	81	−4.36	0.2449	0.105
mlrA	13.56	0.68	5	10.70	0.74	7	1.27	**0.0370***	**0.025***
pgaA	26.03	6.09	23	10.27	0.18	2	2.53	**0.0373***	**0.011***
pgaB	14.61	2.45	17	8.74	0.83	9	1.67	**0.0417***	**0.029***
pgaC	47.74	51.68	108	25.85	22.18	86	1.85	0.5929	0.642
pgaD	16.41	4.55	28	9.30	2.38	26	1.76	0.1369	0.098
rpoS	54.68	25.33	46	162.95	30.76	19	−2.98	**0.0370***	**0.036***

Data include: mean $2^{-\Delta C_T}$, standard deviation (SD), and coefficient of variation (CV). The fold-change values were normalization by tus gene expression using the $\Delta\Delta C_T$ method for comparison of B201 vs. B241. The bold values and asterisks denote statistically significant differences ($p < 0.05$) between strains for both $2^{-\Delta C_T}$ and ΔC_T-values.

assembly protein FliH and the flagellar transcription regulatory protein FlhD were upregulated in B241 compared to B201, along with DnaK, ClpB, HtpG, and FimC precursor (Supplementary Table 1).

The Curli Regulatory Network

To determine whether curli gene expression correlated with *gcvB* expression, we examined target genes *csgBA, csgD*, *gcvAB, gcvR* (Urbanowski et al., 2000), *glyA* (Ghaz-Jahanian et al., 2013), and *hfq* (Mika and Hengge, 2013) in B201 and B241. In addition to curli expression, we examined targets regulated by GcvB, such as, acid stress response (*hdeAB* and *rpoS*) and amino acid transport (*cycA*) genes. Expression of *csgA, glyA, hdeAB,* and *rpoS* was significantly different ($p < 0.05$) between B201 and B241, as measured by RT-qPCR by the $2^{-\Delta C_T}$ transformation (**Table 3**). However, *csgD* was statistically significant ($p < 0.05$) when using the ΔC_T method as well. The results supported the conclusions from the CRI assays that the genes required for the curli phenotype, *csgA* and *csgD*, were up-regulated (+11.21 and +4.25 fold change, respectively) for B201 in comparison to B241. Transcription levels for *glyA* (involved in glycine production and transport) were down-regulated (−6.05 fold change) in B201. Expression of *rpoS* was also down-regulated (−2.98 fold change) for B201 in comparison to B241. Furthermore, expression of *hdeAB* was down-regulated (−55.02 and −3.95 fold change, respectively). Fold-change values were determined for *csgB*, *gcvAB*, *gcvR*, and *hfq* genes (+6.71, −1.34, −2.46, +1.41 and −4.36 fold change, respectively), yet statistical analysis indicated that there was no significance ($p > 0.05$) between treatments. RNA-Seq transcriptomic data showed that *csgBA* were up-regulated, whereas *hdeAB* were down-regulated for B201 in comparison to B241. In addition, the glutamate decarboxylase (AR2) genes were upregulated in B241 (Supplementary Tables 1, 2). As expected, the genes showing the largest decrease in expression in B241 compared to B201 included *csgA* and *csgB* (Supplementary Table 2).

Effect of Glycine on Curli Expression

The glycine cleavage system, dependent on glycine concentrations in the environment, is known to regulate both curli and acid chaperone protein expression (Urbanowski et al., 2000; Jin et al., 2009; Stauffer and Stauffer, 2012a), thus addition of glycine to FAS strains was performed to determine if curli production persisted. The curli phenotype for B201 was not observed when glycine was added to M9GT medium (**Figure 1**), and similar results were observed for FAR strain B250 which had

TABLE 4 | Transcriptomics data for selected genes from strain B201 grown in M9GT and M9GT + glycine.

Gene	B201 M9GT ($2^{-\Delta C_T}$)			B201 M9GT + glycine ($2^{-\Delta C_T}$)			Fold-change	*p*-value ($2^{-\Delta C_T}$)	*p*-value (ΔC_T)
	Mean	*SD*	CV (%)	Mean	*SD*	CV (%)			
csgA	217.77	28.53	13	204.24	256.18	125	1.07	0.932	0.640
csgB	74.26	51.13	69	103.17	135.82	132	−1.39	0.892	0.983
csgD	37.82	4.19	11	26.91	15.03	56	1.41	0.578	0.615
cycA	26.45	11.30	43	34.79	13.07	38	−1.32	0.651	0.640
gcvA	14.60	5.43	37	8.81	4.69	53	1.66	0.578	0.614
gcvB	3.78	1.03	27	36.48	22.28	61	−9.66	0.416	0.117
gcvR	54.45	13.66	25	60.22	30.35	50	−1.11	0.892	0.983
glyA	12.45	3.26	26	17.05	6.12	36	−1.37	0.578	0.640
hdeA	19.18	3.65	19	242.60	371.09	153	−12.65	0.578	0.615
hdeB	11.27	4.25	38	12.44	7.34	59	−1.10	0.892	0.983
hfq	78.93	11.17	14	163.23	121.93	75	−2.07	0.578	0.640
mlrA	17.22	7.35	43	8.12	1.91	24	2.12	0.464	0.374
rpoS	62.93	23.74	38	19.11	8.62	45	3.29	0.416	0.129

Data include mean $2^{-\Delta C_T}$, standard deviation (SD), and coefficient of variation (CV). The fold-change values were normalized by tus gene expression using the $\Delta\Delta C_T$ method for comparison of B201 in M9GT vs. M9GT+glycine.

intermediate acid resistance-flocculation phenotype (**Table 1**). As expected, the addition of glycine to M9GT cultures of B241 had no effect (**Figure 1**). We examined the effects of exogenous glycine on the glycine cleavage system genes, curli expression, and acid induced chaperone proteins (**Table 4**). Expression of *csgA, csgD, gcvA, mlrA,* and *rpoS* was up-regulated in B201 compared to B201 supplemented with glycine (+1.07, +1.41, +1.66, +2.12, and +3.29 fold change, respectively). As expected, expression of *cycA, gcvB, gcvR, glyA, hdeAB, and hfq* was down-regulated (−1.32, −9.66, −1.11, −1.37, −12.65, −1.10, and −2.07 fold-change, respectively) in B201. Unexpectedly, *csgB* was also down-regulated (−1.39 fold-change). However, acid challenge of B201 in M9GT showed similar log reduction values with and without glycine supplementation (1.52 log reduction in M9GT and 1.45 log reduction in M9GT supplemented with glycine, data not shown).

Regulation of Curli Expression in Strain B250

We measured expression of selected curli and chaperone target genes in strain B250, which was determined to be acid resistant, but exhibited the flocculation phenotype (**Table 1**). RT-qPCR data revealed that expression of *csgA*, *cycA*, *gcvR*, *glyA*, *hdeAB*, *hfq*, and *rpoS* was significantly different ($p < 0.05$) from B241, where *csgA* exhibited a +3.69 fold change in B250 in comparison to B241, yet no significant difference ($p > 0.05$) was seen in *csgB* or *csgD* expression (+9.15 and −1.07 fold change, respectively; **Table 5**). Expression of *cycA* and *glyA* was down-regulated (−3.50 and −6.75 fold change, respectively) in B250 when compared to B241, which was consistent with the data for strains B201 and B241. Additionally, *gcvR* and *hfq* expression was down-regulated (−1.86 and −14.77 fold change, respectively) in B250, although no statistically significant difference ($p > 0.05$) was seen in *csgD, gcvA,* or *gcvB* expression between treatments. Expression of *hdeAB* and *rpoS* was down-regulated (−87.80, −5.11, −12.55 fold change, respectively) in B250 in comparison to B241 which may account for the differences in acid sensitivity.

DISCUSSION

All strains grew normally in LBG (a complex medium), however flocculation and curli production was found to correlate with acid sensitivity in a minimal medium, M9GT. However, two of the more acid resistant strains, B250 and B266, flocculated when grown in M9GT (minimal medium). Our findings agree with previous research that determined that *E. coli* does not express curli in static broth (LBG) or agar plates at 37°C (Barnhart and Chapman, 2006). The flocculation phenotype described in our work has been seen previously under environmental stress, including LB supplemented with NaCl, for the Shiga-toxin producing *E. coli* O111 serogroup (Diodati et al., 2015). In that study, environmental and outbreak strains displayed the aggregative curli phenotype, which was proposed to be related to biofilm formation, while sporadic-case strains did not. Furthermore, our curli gene deletion mutant, B201Δ*csgD*, was unable to express curli and did not flocculate when grown in M9GT. This observation proves that curli production is responsible for the flocculation phenotype observed in acid sensitive *E. coli* O157:H7 strains. In a study with *E. coli* O157:H7, curli production by strains grown in LB supplemented with NaCl was found to correlate with acid resistance (Carter et al., 2011). The data showed a 5-fold increase in the survival rate of non-curli variants after 6 h incubation in acidified broth when compared to curli variants challenged under the same conditions, although no regulatory link was proposed for curli and acid resistance. Carter et al. (2011) suggested that non-curli variants may be better fit for host colonization due to its acid resistance properties, whereas curliated *E. coli* O157:H7 may

TABLE 5 | Transcriptomics data for selected genes from strains B241 and B250.

Gene	B250 ($2^{-\Delta C_T}$)			B241 ($2^{-\Delta C_T}$)			Fold-change	*p*-values ($2^{-\Delta C_T}$)	*p*-values (ΔC_T)
	Mean	***SD***	**CV (%)**	**Mean**	***SD***	**CV (%)**			
csgA	53.49	23.93	45	14.48	0.78	5	3.69	0.104	**0.015***
csgB	85.93	108.23	126	9.39	1.90	20	9.15	0.340	0.120
csgD	6.32	0.86	14	6.79	1.91	28	−1.07	0.780	0.874
cycA	9.94	2.51	25	34.79	19.43	56	−3.50	0.173	**0.046***
gcvA	7.19	0.55	8	15.16	7.67	51	−2.11	0.190	0.161
gcvB	2.87	0.38	13	7.00	3.77	54	−2.44	0.190	0.079
gcvR	17.04	3.21	19	31.77	4.55	14	−1.86	**0.026***	**0.017***
glyA	9.50	2.16	23	64.15	8.95	14	−6.75	**0.003***	**0.002***
hdeA	9.31	1.93	21	817.16	123.86	15	−87.80	**0.003***	**<0.0001***
hdeB	8.55	2.21	26	43.69	11.16	26	−5.11	**0.019***	**0.005***
hfq	18.26	4.91	27	269.78	222.82	83	−14.77	0.190	**0.015***
mlrA	8.97	1.55	17	9.02	1.03	11	−1.01	0.977	0.933
rpoS	11.01	3.10	28	138.10	33.29	24	−12.55	**0.012***	**0.002***

Data include mean $2^{-\Delta C_T}$, standard deviation (SD), and coefficient of variation (CV). The fold-change was calculated with normalization expression data by tus gene expression using the $\Delta\Delta C_T$ method for comparison of B241 vs. B250. The bold values and asterisks denote statistically significant differences ($p < 0.05$) between strains for both $2^{-\Delta C_T}$-and ΔC_T-values.

mediate attachment better to environmental surfaces such as, soils and plant tissues. Curli production may compensate for the absence of other fimbriae, such as, long polar fimbriae (Lpf), involved in colonization of human intestinal epithelial cells. For example, enhanced curli production and adhesion to cultured intestinal epithelial cells was seen in *E. coli* O157:H7 *lpfA1* and *lpfA2* deletion mutants after exposure to T84 cells (Lloyd et al., 2012a,b). Although the absence of Lpf did not activate curli production itself, alternative structures may be required under various environmental pressures. This transition suggests a regulatory connection between curli and Lpf, but specific mechanisms are currently unknown (Lloyd et al., 2012a,b). Using a proteomic approach (Gunther et al., 2014), Gunther and colleagues proposed a regulatory connection between the *gad* genes and the curli phenotype. Our transcriptomic data showed that glutamate decarboxylase genes were among those showing the largest increase in expression (Supplementary Table 1) for strain B241 compared to strain B201, which could contribute to the PAR phenotype we observed in M9GT, although further research would be needed to confirm this observation.

The carbon storage network of *E. coli* has been found to promote glycolysis and flagella production during growth in a complex medium, but gluconeogenesis and biofilm formation were stimulated by low pH and acetic acid (Suzuki et al., 2002; Mondragón et al., 2006; Jonas and Melefors, 2009; Chavez et al., 2010). Due to the glucose consumption during growth in M9GT, organic acid (primarily D-lactic acid) is produced by *E. coli,* which lowers the pH. These conditions led us to investigate *csr* genes in relation to the flocculation phenotype. We hypothesized that there would be increased expression of the sRNAs (CsrB and CsrC) that would affect other *csr* genes in FAS strain B201 compared to PAR strain B241. We found that the relative expression of *csrA*, *flhDC*, *fliC,* and *pgaAB* (for flagella and biofilm formation), was significantly different between FAS and PAR strains. Expression of *csrBC,* sRNA regulators, was not significantly different between B201 and B241 (**Table 3**). These data agree with other studies showing that decreased *csrA* expression correlated with increased biofilm through poly-N-acetylglucosamine production. Interestingly, we report decreased *csrA* expression with increased expression of flagella genes, *flhDC*. This appears to contradict reported findings from other studies on flagella formation regulation (Wei et al., 2001; Wang et al., 2005). We found that the carbon storage network affects biofilm formation and probably contributes to the flocculation phenotype, although we did not see regulation through *csrC* and *csrB*, small changes in sRNAs may account for large changes in target gene expression. It is likely that other changes in carbon metabolism are affecting this system that we did not evaluate.

For quantitative PCR data, the cycle threshold, or ΔC_T, value is an exponential number based on a log-linear plot of the PCR signal vs. the cycle number. While some authors suggest statistical analysis on normalized untransformed ΔC_T-values (Yuan et al., 2006), Livak and Schmittgen (2001) suggest performing statistical analysis with normalized exponential values, because the untransformed ΔC_T-values may introduce more sample-to-sample variation. We found that the statistical significance for fold change for gene expression varied depending on method of calculation (transformed vs. untransformed), including *csgD* for comparisons between B201 and B241, as well as *csgA*, *csgD*, and *hfq* in comparison between B241 and B250. Changes in gene expression for these genes were statistically significant when analyzed with ΔC_T-values but were not when analyzed with the $2^{-\Delta C_T}$-values. Because *csgD* appears to be a central regulatory molecule in acid resistance and curli production (influencing g*adAB* and *csgBA* expression, respectively), we therefore included both analyses in our data (**Tables 3–5**). Further work may be necessary to determine

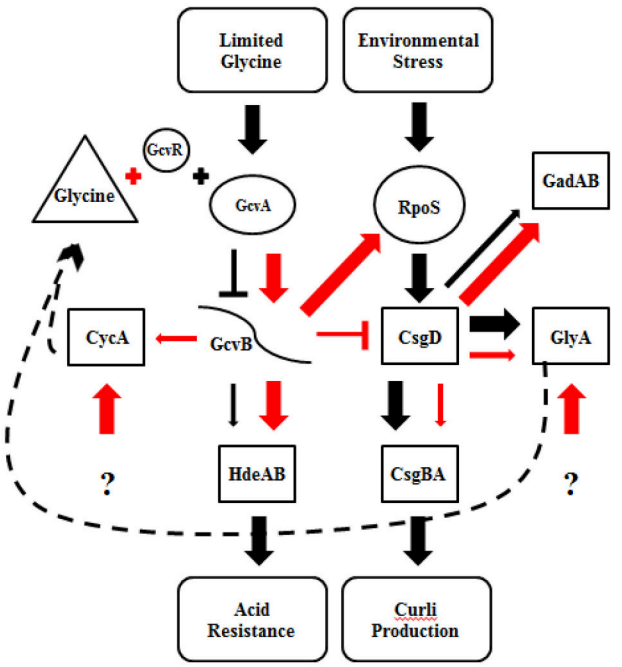

FIGURE 3 | Regulatory model for curli expression and acid resistance phenotype of STEC strains B201 and B241. Proposed gene regulation of FAS (black) and PAR (red) STEC strains. The thickness of the arrows represents the amount of transcription, where thinner arrows indicate less transcription than thicker arrows. Barred lines represent transcription repression, the arrows increased transcription. The dashed line represents a proposed glycine feedback loop. Gene target categories are grouped by shape; regulatory genes (boxes), glycine cleavage system and RpoS (circles), sRNA (curved line), environmental conditions (oval), and glycine (triangle). The question mark indicates unknown effectors.

the significance of these observations, perhaps with increased replication of measurements.

Curli production by *E. coli* may aid biofilm formation under various environmental stresses (Olsén et al., 1989; Vidal et al., 1998; Uhlich et al., 2006; Ogasawara et al., 2007). The results of the CRI assay provide evidence for curli production by acid sensitive strains (**Table 1**). Although the K-12 strain we tested was considered acid sensitive, the CRI assay did not provide evidence for curli production. This is likely because previous research has found that *E. coli* K-12 produces curli at 37°C, but not on agar plates at this temperature (Barnhart and Chapman, 2006). Interestingly, *mlrA* expression, a MerR-like receptor controlled by RpoS that activates CsgD expression (Chen et al., 2013; Uhlich et al., 2013), was only significantly different between B201 and B241 despite the differences seen in *rpoS* expression between B241 and B250 (**Tables 3**, **5**). This finding was surprising because PAR strain B241 had significantly higher expression of *rpoS* than B201 and B250, yet curli expression was higher in B201 and B250. This is in contrast to other studies showing that the Crl protein, required for curli production in most strains, up-regulates *rpoS* expression in response to temperature (Bougdour et al., 2004). RpoS then stimulates *mlrA* and thus *csgD* for curli production (Robbe-Saule et al., 2006; Ogasawara et al., 2009). Some strains of *E. coli* O157:H7 have been shown to differ in *rpoS* expression, with phage insertions in *mlrA* limiting curli production (Uhlich et al., 2013). Similar to our results, other research has shown that a sRNA, GcvB, helps activate RpoS as well as repress CsgD. Although we did not see statistical significance in *gcvB* expression between strains, we hypothesize that small change in sRNAs may cause large changes in target gene expression. This hypothesis is supported in both the RT-qPCR and global transcriptomics data via RNA-Seq, where the genes targeted by *gcvB*, *csgD,* and *hdeAB*, are affected according to previously reported gene regulation (Jin et al., 2009). These data support the regulatory pattern (i.e. lack of curli expression) we observed for PAR strain B241. While some PAR strains (B204, B244, B247, B264, and B269) had evidence of curli expression by the Congo red assay, the level of expression was evidently insufficient to result in flocculation.

Curli production has also been linked to the presence of glycine (Urbanowski et al., 2000; Pulvermacher et al., 2008; Jin et al., 2009). When levels of glycine are high in the environment, GcvA activates GcvB, which represses CsgD and curli production. GcvB also directly represses *cycA*, which acts as a transporter of extracellular glycine into the cell, under nutrient rich conditions (Stauffer and Stauffer, 2012b). Acid induced chaperone genes, *hdeAB* and *rpoS,* are positively regulated by the *gcv* system (Stauffer and Stauffer, 2012a). When levels of glycine are low in the environment, GcvA and GcvR repress GcvB, which relieves repression of CsgD and curli production (Urbanowski et al., 2000; Jorgensen et al., 2012). CsgD in turn regulates GlyA, which converts serine to glycine. We found that supplementing M9GT with glycine suppressed the flocculation phenotype in B201 (**Figure 1**), indicating that curli expression was prevented. Unexpectedly, no differences in gene expression were seen between strains B201 and B201 supplemented with glycine even though the flocculation phenotype was relieved when grown in the presence of exogenous glycine. Fold-change observations are consistent with a model of gene regulation depicting the relationship between curli production in minimal medium and acid sensitivity (**Figure 3**). It is possible that a glycine-based feedback loop mediated by GcvB in B201 modulates curli expression, although other factors (indicated by question marks in **Figure 3**) may affect GcvB control of CycA and GlyA under nutrient limitation.

Previous findings for strains grown in complex medium show that GcvB represses GlyA due to CsgD repression (Stauffer and Stauffer, 2012b). The phenotypes observed may be due to selective pressure, where survival of acid sensitive strains under nutrient starvation may depend on biofilm formation (presaged by curli), whereas more acid resistant strains do not require this survival strategy. Previously, Oh et al. (2009) compared resistant and sensitive strains and found that strains of animal origin may (in general) be more acid resistant than food or human isolates, although acid resistance mechanisms were not addressed. Further research will focus on determining the effects of gene knockouts and complementation of target genes, *gcvB* and *hdeAB*, on FAS and PAR phenotypes, as well as the generation of metabolomic, proteomic, and additional transcriptomic data for these strains. These experiments may help to confirm our proposed model and possibly identify

additional regulatory factors promoting curli formation in FAS and PAR STEC strains.

AUTHOR CONTRIBUTIONS

KK, GK, and DO performed laboratory analysis of acid resistance of STEC strains. KK conducted the quantitative reverse transcriptase PCR. PF, GB, and GK conducted transcriptomic analysis and KK and GB contributed to statistical analysis of the data. FB, AG, PF, and DO contributed to the original conception and design of the work. All authors contributed to the drafting the manuscript and have agreed to support submission for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01404/full#supplementary-material

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Simulating Intestinal Growth Conditions Enhances Toxin Production of Enteropathogenic *Bacillus cereus*

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Bacillus cereus is a ubiquitous bacterial pathogen increasingly reported to be the causative agent of foodborne infections and intoxications. Since the enterotoxins linked to the diarrheal form of food poising are foremost produced in the human intestine, the toxic potential of enteropathogenic *B. cereus* strains is difficult to predict from studies carried out under routine cultivation procedures. In this study, toxigenic properties of a panel of strains ($n = 19$) of diverse origin were compared using cell culture medium pre-incubated with CaCo-2 cells to mimic intestinal growth conditions. Shortly after contact of the bacteria with the simulated host environment, enterotoxin gene expression was activated and total protein secretion of all strains was accelerated. Although the signal stimulating enterotoxin production still needs to be elucidated, it could be shown that it originated from the CaCo-2 cells. Overall, our study demonstrates that the currently used methods in *B. cereus* diagnostics, based on standard culture medium, are not allowing a conclusive prediction of the potential health risk related to a certain strain. Thus, these methods should be complemented by cultivation procedures that are simulating intestinal host conditions.

Keywords: ***Bacillus cereus,*** **enterotoxins, simulated intestinal conditions, toxic potential, CaCo-2 cells**

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INTRODUCTION

Due to the secretion of various toxins linked to gastrointestinal as well as non-gastrointestinal diseases, *Bacillus cereus* presents a serious public health hazard (Stenfors Arnesen et al., 2008; Bottone, 2010). Since it is ubiquitous in nature and due to its capability to form stress resistant spores, it is not totally avoidable in certain food production and processing chains. Two different types of food poisoning are known, the diarrheal and the emetic syndrome. The emetic type, which manifests in nausea and vomiting, is caused by the heat-stable cyclic dodecadepsipeptide cereulide, which is produced in foods even before consumption (Agata et al., 1995; Ehling-Schulz et al., 2004; Messelhäusser et al., 2014). The onset of symptoms linked to the emetic toxin cereulide occurs rapidly within 0.5–6 h while the diarrheal type has a long incubation time lasting 5–16 h (Ehling-Schulz and Messelhäusser, 2012). Cause of the latter type of illness, which is associated

with a wide range of foods such as milk and milk products, salad, or meat, are enteropathogenic strains that produce heat-labile enterotoxins after outgrowth of the ingested *B. cereus* spores in the human intestine (Clavel et al., 2004). Most important are the two three-component toxin complexes hemolysin BL (Hbl) (Beecher et al., 1995) and the non-hemolytic enterotoxin Nhe (Lund and Granum, 1996; Fagerlund et al., 2010; Didier et al., 2012, 2016). The third known enterotoxin is the single protein CytotoxinK (Cyt K) (Lund et al., 2000). So far, the gene encoding the highly toxic variant CytK1 has been identified in very few strains, which were suggested as the separate species *Bacillus cytotoxicus* (Guinebretière et al., 2013).

Enterotoxin gene expression in *B. cereus* is a highly complex and multifactorial process integrating a vast number of environmental, nutritional, and intrinsic signals leading to an elaborate adaptation of gene transcription to the metabolic status of the cell. The global virulence regulator PlcR activates transcription of the enterotoxin genes during transition state in response to increasing cell density (Gohar et al., 2008). Maximal toxicity during the exponential growth phase has also been observed (Duport et al., 2004; Zigha et al., 2006). The PlcR regulon further comprises genes encoding cereolysin O, hemolysin III, as well as the three phospholipases C (PC-PLC, PI-PLC, SMase) and the immune inhibitor A2 protease (InhA2) (Gohar et al., 2002). *B. cereus* virulence is further closely associated with flagella and motility, aeration, the oxidation–reduction potential (ORP), and nutrients such as the carbon source or iron (Ceuppens et al., 2011; Mazzantini et al., 2016). This involves the environment-sensing regulator proteins Fnr, ResDE, CcpA, and CodY (Duport et al., 2006; Zigha et al., 2007; van der Voort et al., 2008; Esbelin et al., 2009, 2012; Messaoudi et al., 2010) as well as other, so far less characterized two component regulatory systems (Ceuppens et al., 2011).

Consistent with international standards, routine diagnostics detect and quantify *B. cereus sensu lato* colonies on selective culture media (Ehling-Schulz and Messelhäusser, 2013). With these methods, only *presumptive B. cereus* can be detected [ISO (International Organization for Standardization) 7932:2005-03], as the members of the *B. cereus* group cannot be differentiated and the potential health risk related to a new isolate cannot be assessed. Thus, *B. cereus* isolates are additionally grown in full media such as casein glucose yeast (CGY) or brain heart infusion (BHI) supplemented with 1% glucose to determine their enterotoxin producing ability as well as cytotoxic activity by immunochemical and cell culture methods (Ehling-Schulz et al., 2011; Jeßberger et al., 2015). The data generated under these established laboratory procedures, however, do not necessarily reflect the natural conditions leading to food poisoning in humans.

Different attempts have therefore been made to investigate the behavior of *B. cereus* under simulated gastrointestinal conditions. For instance, aiming to mimic the gastric passage, media were produced consisting of gastric electrolyte solution and J broth (JB) combined with different foods (Clavel et al., 2004, 2007). Gastric and intestinal fluids were simulated by including salts, bovine serum albumin, mucin, bile salts, and digestion enzymes such as lipases, pepsin, and pancreatin (Wijnands et al., 2006, 2009). Batch culture systems were used to study anaerobiosis, low oxido-reduction potential, and carbohydrate limitation, which the bacteria are confronted with in the small intestine (Clair et al., 2010). Other *in vitro* batch cultures were developed to simulate the gastrointestinal passage in different phases, from mouth to ileum, while even competing intestinal microbiota was considered (Ceuppens et al., 2012a,b). However, hitherto only few studies focused on the interaction of *B. cereus* with human gastrointestinal cells, such as adhesion, invasion, or germination (Wijnands et al., 2007; Minnaard et al., 2013).

In the present study growth, enterotoxin production, protein secretion and cytotoxicity of a set of 19 enteropathogenic and non-pathogenic *B. cereus* strains were investigated under intestinal conditions, simulated by RPMI 1640 medium pre-incubated with the human colon epithelial cell line CaCo-2, at 37°C and 7% CO_2. Key questions addressed are (I) the effects of the host intestine on growth, toxin gene transcription, protein secretion and enterotoxin production of the *B. cereus* strain set, (II) re-evaluation of the classification of strains as high or low toxic according to intestinal growth conditions, and (III) the distinctive characteristics of enteropathogenic and non-pathogenic strains in the intestine.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Sample Preparation

A set of 19 *B. cereus* strains was used for the comparative analyses carried out in frame of this study (**Table 1**; Jeßberger et al., 2015). Only enteropathogenic *B. cereus sensu stricto* were considered, i.e., strains negative for the emetic gene cluster *ces* and affiliated with clade I or II (**Table 1**; Didelot et al., 2009). The strains were assigned to different enterotoxin profiles depending on the presence of the toxin genes *hbl, nhe,* and *cytK2*. Within each toxin profile strains of different enterotoxigenic potential were chosen, including highly pathogenic (SDA KA 96, INRA C3, F837/76, NVH 0075-95, and others) and low or non-pathogenic (RIVM BC 934, F528/94, MHI 86, MHI 226, RIVM BC 90) strains (**Table 1**).

All strains were pre-cultured for 17 h in casein glucose yeast (CGY) medium with 1% glucose at 37°C and 125 rpm. For simulating intestinal growth conditions, RPMI 1640 medium (with stable glutamine; Biochrom GmbH, Berlin, Germany) was used, 2% casein hydrolysate was added and total glucose content was set to 1%. Fetal calf serum (FCS) was not added due to subsequent protein analyses. Differentiated CaCo-2 cells were washed twice with PBS (PBS Dulbecco, w/o Ca^{2+}, w/o Mg^{2+}, low endotoxin; Biochrom GmbH, Berlin, Germany) and incubated with this RPMI 1640 medium for 22 h. Thereafter, this "CaCo-2 treated," designated cRPMI, medium was subjected to sterile filtration using a 0.2 μm filter. The *B. cereus* strains were grown in 45 ml of cRPMI in 80 cm^2 cell culture flasks at 37°C under 7% CO_2 atmosphere in a cell culture incubator. Medium was inoculated to an OD_{600} of 0.05 and OD_{600} was recorded every 30 min. All strains were grown in triplicates. Eight milliliters (2 h) and six milliliters (4, 6, 8, and 10 h) samples were taken

TABLE 1 | Nineteen *B. cereus* strains used in this study.

B. cereus strain	Origin	Genotype clade (group)	Toxin gene profiling					CGY NheB titer	CGY NheB class.	CGY Vero tox.	CGY tox. class.	cRPMI NheB titer	cRPMI NheB class.	cRPMI CaCo-2 tox.	cRPMI tox. class.
			ces	*hbl*	*nhe*	*cytK2*	profile								
14294-3 (M6)	Icecream	I (III)	–	+	+	+	A	2062	m	332	m	3074	hi	109	hi
SDA KA96	Raw milk	I (III)	–	+	+	+	A	6481	hi	1228	hi	2375	hi	158	hi
INRA A3	Starch	II (IV)	–	+	+	+	A	1299	lo	256	lo	1859	m	66	m
INRA C3	Past. carrot	II (IV)	–	+	+	+	A	4460	hi	754	hi	3276	hi	340	hi
6/27/S	Human feces	II (IV)	–	+	+	+	A	1964	m	475	m	377	lo	110	hi
F3175/03 (D7)	Human feces	II (IV)	–	+	+	+	A	5157	hi	430	m	1312	m	87	m
RIVM BC 934	Lettuce	II (IV)	–	+	+	+	A	769	lo	118	lo	713	lo	75	m
F528/94	Beef and chow mein and rice, outbreak	I (II)	–	+	+	–	C	1759	lo	214	lo	776	lo	65	m
F837/76	Human, postoperative infection	I (III)	–	+	+	–	C	8598	hi	2106	hi	2449	hi	102	hi
RIVM BC 126	Human feces	I (II)	–	+	+	–	C	7757	hi	578	hi	1433	m	116	hi
MHI86	Infant food	I (III)	–	–	+	+	D	87	lo	0	lo	19	lo	19	lo
F4429/71	Vanilla pudding	I (III)	–	–	+	+	D	4907	hi	918	hi	2146	hi	73	m
RIVM BC 964	Kebab	II (IV)	–	–	+	+	D	10266	hi	858	hi	1837	m	145	hi
F3162/03 (D8)*	Human feces	I (III)	–	–	+	+	D	41	lo	858	hi	1246	m	69	m
MHI226**	Milk and milk products	I (III)	–	–	+	–	F	930	lo	91	lo	265	lo	37	lo
NVH 0075-95	Stew with vegetables, foodpoisoning	I (III)	–	–	+	–	F	7729	hi	674	hi	2751	hi	94	m
WSBC10035	Past. milk	I (III)	–	–	+	–	F	6205	hi	1100	hi	1357	m	86	m
RIVM BC 90	Human feces	I (III)	–	–	+	–	F	146	lo	10	lo	81	lo	6	lo
7/27/S	Human feces	I (III)	–	–	+	–	F	9011	hi	952	hi	1650	m	42	m

**Strain showed high toxicity but particularly low NheB titers in CGY due to binding failure of mAb 2B11 in sandwich EIA (Didier et al., 2015).*

***Sequence analysis revealed a truncated hbl operon; as strain is not able to produce Hbl L2 and Hbl B protein (negative in EIAs), it was allocated to profile F (Jeßberger et al., 2015).*

Genotyping of the strains had been conducted by sequence analyses of the genetic markers spoIIIAB and panC. PCR analyses with specific primers for ces and enterotoxin genes had been used for toxin profiling. A first classification of the strains as high or low toxic had been determined by sandwich EIAs against NheB and by WST-1-bioassays on Vero cells after growth of the strains under laboratory conditions (CGY medium). These preliminary data are part of and described in an earlier study (Jeßberger et al., 2015). Former classification (CGY) according to cytotoxicity was hi: >500; m: 250–500; lo: <250 and according to NheB titers hi: >4,000; m: 2,000–4,000; lo: <2,000. After growth under simulated intestinal conditions (cRPMI) in this study, NheB titers were again determined by sandwich EIAs and cytotoxicity titers were investigated in WST-1-bioassays on CaCo-2 cells. According to that, classification of the strains was adapted: cytotoxicity (hi: >100; m: 50–100; lo: <50) and NheB titers (hi: >2,000; m: 1,000–2,000; lo: <1,000).

and centrifuged for 15 min at room temperature and 3,500 rpm. Cell pellets were immediately frozen at −80°C and used for determination of toxin gene transcription and intracellular protein content. Supernatant was filtered through a 0.2 μm filter, split for protein quantification as well as for the determination of enterotoxins and frozen at −20°C. For enterotoxin analyses, 1 mM EDTA was added to the supernatant.

Cell Lines and Culture Conditions

CaCo-2 cells were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Cells were cultivated as recommended by the supplier. RPMI 1640 medium (with stable glutamine) was supplemented with 10% fetal bovine serum (Biochrom GmbH, Berlin, Germany). The CaCo-2 cells were cultivated in 80 cm^2 culture flasks in a humidified incubator at 37°C and 7% CO_2 and splitted 1:6 every 3–4 days. For differentiation, 2.15 million CaCo-2 cells per flask were cultivated for 14 days gaining a cell layer mimicking the intestinal epithelium. Medium was changed every 3–4 days. To obtain cRPMI medium, cells were treated as described above.

RNA Isolation, cDNA Synthesis, and Quantitative Real-Time PCR (qRT-PCR)

RNA isolation and DNAse I digestion were performed according to Dommel et al. (2010). To compare transcript levels of all 19 *B. cereus* strains at different time points the relative quantification method according to Livak and Schmittgen (2001) was chosen. cDNA synthesis and qRT-PCR (Real-Time Quantitative Reverse Transcription PCR) were carried out as described before (Jeßberger et al., 2015). In brief, random primers (qScript cDNA Supermix, Quanta Biosciences) were used for first strand synthesis of 1 mg of total RNA. Relative gene expression was determined by qPCR and calculation via the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen, 2001; Pfaffl et al., 2002; Lücking et al., 2009; Dommel et al., 2010). The primers used are published (Jeßberger et al., 2015). Transcription levels of the widely used house-keeping gene *rrn* (16S rRNA) served as reference for normalization applying the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen, 2001). This method is based on the following formula: amount of target transcript $= 2^{-\Delta\Delta C}$T with $-\Delta\Delta C_T = -(\Delta C_{T(sample)} - \Delta C_{T(calibrator)}) = -((C_{T(referencegene)} - C_{T(targetgene)})_{sample} - (C_{T(referencegene)} - C_{T(targetgene)})_{calibrator})$. C_T shows the cycle number of the amplification reaction that exceeds the quantification threshold. Relative transcription of a target gene in % was obtained by setting the *rrn*-normalized transcription level relative to the transcript level of an external calibrator and by multiplying with 100. Target gene transcript levels of all samples tested in this study were compared with the $2^{-\Delta\Delta C}$T method to the expression level of *hblD* of strain F837/76 at 6 h growth in CGY medium (Jeßberger et al., 2015), which served as external calibrator and was set to 100% (log-2 = 0).

Enzyme Immunoassays (EIAs)

All monoclonal antibodies (mAbs) used for detection of *B. cereus* enterotoxin components have been generated earlier at the Department of Veterinary Sciences, Faculty of Veterinary Medicine, Ludwig-Maximilians-Universität München by immunization of mice. Production and functionality of the specific mAbs against Hbl (Dietrich et al., 1999) and Nhe (Dietrich et al., 2005) have been described in detail.

Sandwich EIAs were carried out for detection of Hbl L2 and NheB. For that, microtiter plates were coated with 100 μl/well mAb 1A12 (Hbl L2; 10 μg/ml) in bicarbonate buffer or mAb 2B11 (NheB; 5 μg/ml) in PBS, respectively. After overnight incubation at room temperature, 30 min blocking with 150 μl/well 3% sodium-caseinate-PBS and 3x washing (wash buffer: 146 mM NaCl, 0.025% Tween 20), *B. cereus* culture supernatants were applied to the microtiter plates as serial dilutions in PBS with 0.5% Tween 20. The plates were incubated for 1 h at room temperature on a tumble shaker. To avoid cross-contaminations, samples were subsequently siphoned. After four washing steps the detection antibodies were applied (100 μl/well, anti Hbl L2 8B12-HRP 1:2,000 and anti NheB 1E11-HRP 1:4,000 in 1% sodium-caseinate-PBS). After 1 additional h at room temperature on a tumble shaker and 5 further washing steps, 100 μl/well 5% TMB (tetramethylbenzidin)-solution in citrate buffer were applied. The reaction was stopped after 20 min by addition of 1 M sulfuric acid and absorbance at 450 nm was measured immediately in a Tecan photometer using Ridawin software. Titers are defined as the reciprocal of the highest dilutions resulting in an absorbance value of $\geq$1.0.

Indirect EIAs were performed for detection of enterotoxin components Hbl L1 and Hbl B. The microtiter plates were coated with serial dilutions of the *B. cereus* culture supernatants in bicarbonate buffer. The mAbs 1E9 (Hbl L1; 1 μg/ml in PBS) and 1B8 (Hbl B; 2 μg/ml in PBS) served as primary antibodies. For detection, a polyclonal rabbit-anti-mouse-HRP conjugate was applied (1:2,000 in 1% sodium-caseinate-PBS). Washing and incubation steps were carried out analogously to the sandwich EIAs.

As an exception, for detection of NheB of strain F3162/04 (D8) the indirect assay solely based on mAb 1E11 was applied (Jeßberger et al., 2015).

Quantification of Total Protein Amount

The extracellular protein contents were determined from *B. cereus* culture supernatants. Protein amounts were quantified by Roti-Nanoquant Kit (Roth, Karlsruhe, Germany) in microtiter plates according to the manufacturer's instructions. Colorimetric reactions were measured with Infinite F200 reader (Tecan) at wavelengths of 610/450 nm. Protein concentrations were determined by quotient of optical densities OD 610/450 referred to an internal standard generated by Quick Start Bovine Serum Albumin Standard (Biorad).

Cytotoxicity Assays

WST-1 bioassays on CaCo-2 cells were performed as previously described (Didier et al., 2012; Jeßberger et al., 2014, 2015). Briefly, serial dilutions of the *B. cereus* culture supernatants in RPMI 1640 medium were applied to 96 well plates (100 μl/well). One hundred microliters/well CaCo-2 cell suspensions (2×10^4 cells/well) were added immediately. After 24 h incubation at 37°C and 7% CO_2, cell viability was determined by addition of WST-1 (Roche diagnostics). Optical density was recorded in a Tecan

photometer at 450 nm. Dose-response curves and thus 50% lethal concentrations were calculated with Ridawin software and are shown as reciprocal titers.

Propidium iodide influx tests (Jeßberger et al., 2014, 2015) were used to assess pore formation in the membranes of CaCo-2 cells. For that, 4×10^4 CaCo-2 cells were seeded in 200 µl RPMI 1640 medium/well in 96 well plates and incubated for 24 h at 37°C and 7% CO_2. After that, 100 µl medium were removed and 100 µl fresh RPMI 1640 medium were added containing 10 µg/ml PI (Sigma-Aldrich) and 1:20 dilutions of the *B. cereus* culture supernatants. Subsequently, fluorescence was measured in a Victor 1420 multilabel counter (Perkin Elmer) for 4 h every 2.5 min (excitation: 530 nm; emission: 616 nm; excitation time: 1 s; excitation strength: 20,000). Fluorescence curves were calculated using Microsoft Excel and samples were compared according to the highest linear slope of these curves.

RESULTS

Similar Growth Behavior under Simulated Intestinal Conditions

The 19 *B. cereus* strains were compared regarding their growth behavior under simulated intestinal conditions (in CaCo-2 treated cRPMI medium at 37°C and 7% CO_2) (**Figure 1**). All strains of toxin profile A grew quite similar. Only SDA KA96 reached a higher OD_{600} (>5) than the remaining strains (OD_{600} 3–4) (**Figure 1A**). In toxin profile C RIVM BC 126 showed delayed growth but a similar OD_{600} (3.7–4.3) than the remaining strains (**Figure 1B**). This strain was isolated from human feces, as was F3162/04 (D8), which grew to significantly higher OD_{600} (>7 compared to 2.7–3.6) than all other strains in toxin profile D (**Figure 1C**). For toxin profile F, similar growth of all strains was observed. NVH 0075-95 and the non-pathogenic strain MHI 226 showed slightly reduced OD_{600} (>3 compared to 4.5–5) than the remaining strains (**Figure 1D**).

Early Toxin Gene Transcription under Simulated Intestinal Conditions

qRT-PCR was used to determine transcription of the enterotoxin genes *nheB* (NheB) and *hblD* (Hbl L1) of all 19 *B. cereus* strains grown under simulated intestinal conditions. Transcription was analyzed after 2, 4, 6, 8, and 10 h of growth.

Relative *nheB* transcription was particularly high in strains 14294-3 (M6) and RIVM BC 126 compared to all other strains (**Figure 2A**). With the exception of RIVM BC 126, *nheB* transcription efficiency (relative transcript level/OD_{600}) of all strains was highest after 2 h growth. At later time points, transcription efficiency decreased significantly (**Figure 2B**). For 8 out of 10 strains, relative *hblD* transcription was higher than relative *nheB* transcription (**Figure 2C**). Strain INRA C3 showed the maximum relative *hblD* transcription at 2 h growth. For 9 out of 10 strains, *hblD* transcription efficiency was also highest after 2 h growth (**Figure 2D**).

It has been shown before that the toxic activity of a *B. cereus* isolate grown in CGY medium correlates with its ability to produce NheB and Hbl L1/B protein (Moravek et al., 2006;

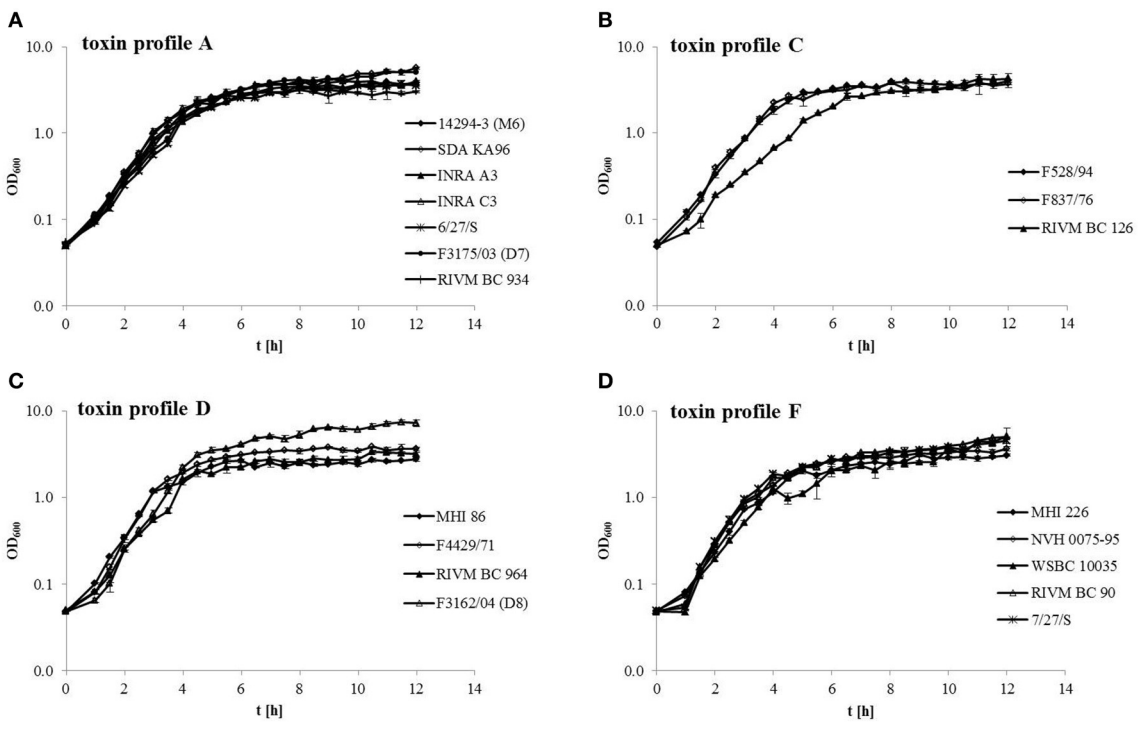

FIGURE 1 | Growth of 19 *B. cereus* strains under simulated intestinal conditions (RPMI medium treated with CaCo-2 cells, 37°C, 7% CO_2). Strains are grouped consistent with their toxin profile. **(A)** Toxin profile A (*nhe, hbl, cytK2*). **(B)** Toxin profile C (*nhe, hbl*). **(C)** Toxin profile D (*nhe, cytK2*). **(D)** Toxin profile F (*nhe*).

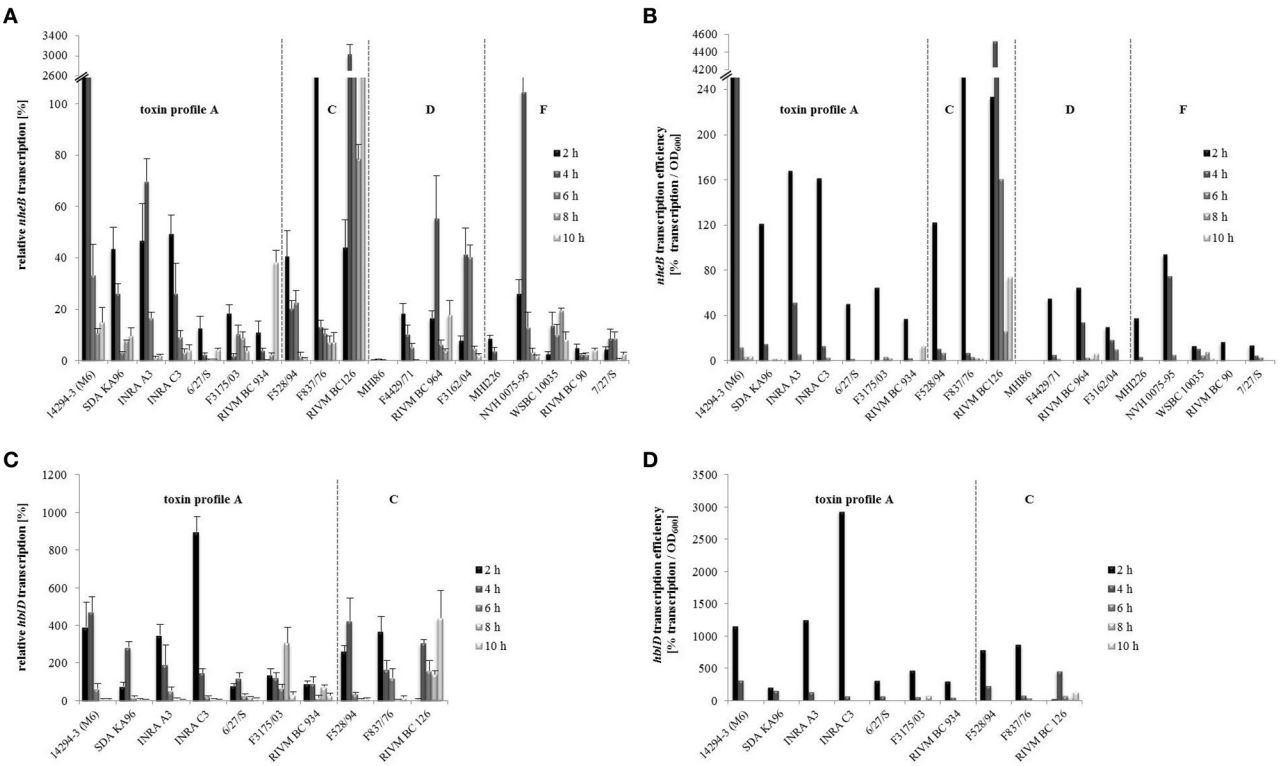

FIGURE 2 | Enterotoxin gene transcription of the *B. cereus* strains. *nheB* **(A)** and *hblD* **(C)** transcription was determined by qRT-PCR, normalized to 16S *rrn* levels of the same sample and relative to the transcript level of an external calibrator. *hblD* expression of reference strain F837/76 at 6 h growth in CGY medium (Jeßberger et al., 2015) was used as calibrator and set to 100% (log-2 = 0). With the $2^{-\Delta\Delta C}$T method, transcription of all other samples was compared to this calibrator. Transcription efficiencies of *nheB* **(B)** and *hblD* **(D)** were determined as relative transcript level/OD_{600}. Strains are grouped according to their enterotoxin gene profiles, which are separated by dotted lines.

Jeßberger et al., 2014). Generally, enterotoxin gene transcription under simulated intestinal conditions only partially correlated with the former classification of the strains as high or low NheB producing and high or low toxic (**Figure 2** and **Table 1**). Setting a random threshold of 20% relative *nheB* transcription, the following strains can be considered to transcribe high levels of *nheB*: the formerly classified as high toxic strains SDA KA 96, INRA C3, F837/76, RIVM BC 126, RIVM BC 964, F3162/04 (D8), and NVH 0075-95, but also the formerly low or medium toxic strains 14294-3 (M6), INRA A3, and F528/94. The formerly classified as low or non-pathogenic strains MHI 86, MHI 226, and RIVM BC 90 transcribed comparatively low levels of *nheB* under simulated intestinal conditions.

When a random threshold of 150% for *hblD* transcription was set, 8 out of 10 strains can be considered to transcribe high levels of *hblD*, among them 5 formerly high and 3 formerly low toxic strains.

Strain-Specific Enterotoxin Production Starts Early under Simulated Intestinal Conditions

Growth of the *B. cereus* strains under simulated intestinal conditions resulted in strain specific enterotoxin production, measured with specific EIAs (Dietrich et al., 1999, 2005; Jeßberger et al., 2015). Enterotoxin titers as well as productivity (titer/OD_{600}) are shown in **Figure 3**. Generally all titers were significantly lowered compared to growth of the *B. cereus* strains in CGY medium (Jeßberger et al., 2015), due to reduced growth under the defined intestinal conditions. Interestingly, NheB was detectable already after 2 h of growth (**Figure 3A**). After 4 h, comparably high NheB titers were measured; for 8 out of 19 strains these titers did not increase after prolonged growth. The formerly classified as non-pathogenic strains MHI 86 and RIVM BC 90 showed the lowest NheB titers, followed by MHI 226. Calculating enterotoxin productivity (titer/OD_{600}) demonstrated that NheB production/cell was most efficient at 4 h. On the other hand, with the exception of F4429/71, titers did also not significantly decrease over the time, indicating that only little proteolytic degradation takes place under simulated intestinal conditions.

The Hbl components L2, L1, and B were not detectable after 2 h. Except RIVM BC 126, all *hbl* positive strains produced comparably high amounts of Hbl L2 after 4 h, which even increased over time (**Figure 3B**). The highest titers were measured after incubation for 10 h. Nevertheless, Hbl L2 productivity, i.e., titers determined per OD_{600}, was generally highest after 4 h. Hbl L1 titers of 6 out of 10 strains increased significantly over time, but productivity was again highest at 4 h (**Figure 3C**). Five out of ten strains showed significantly reduced

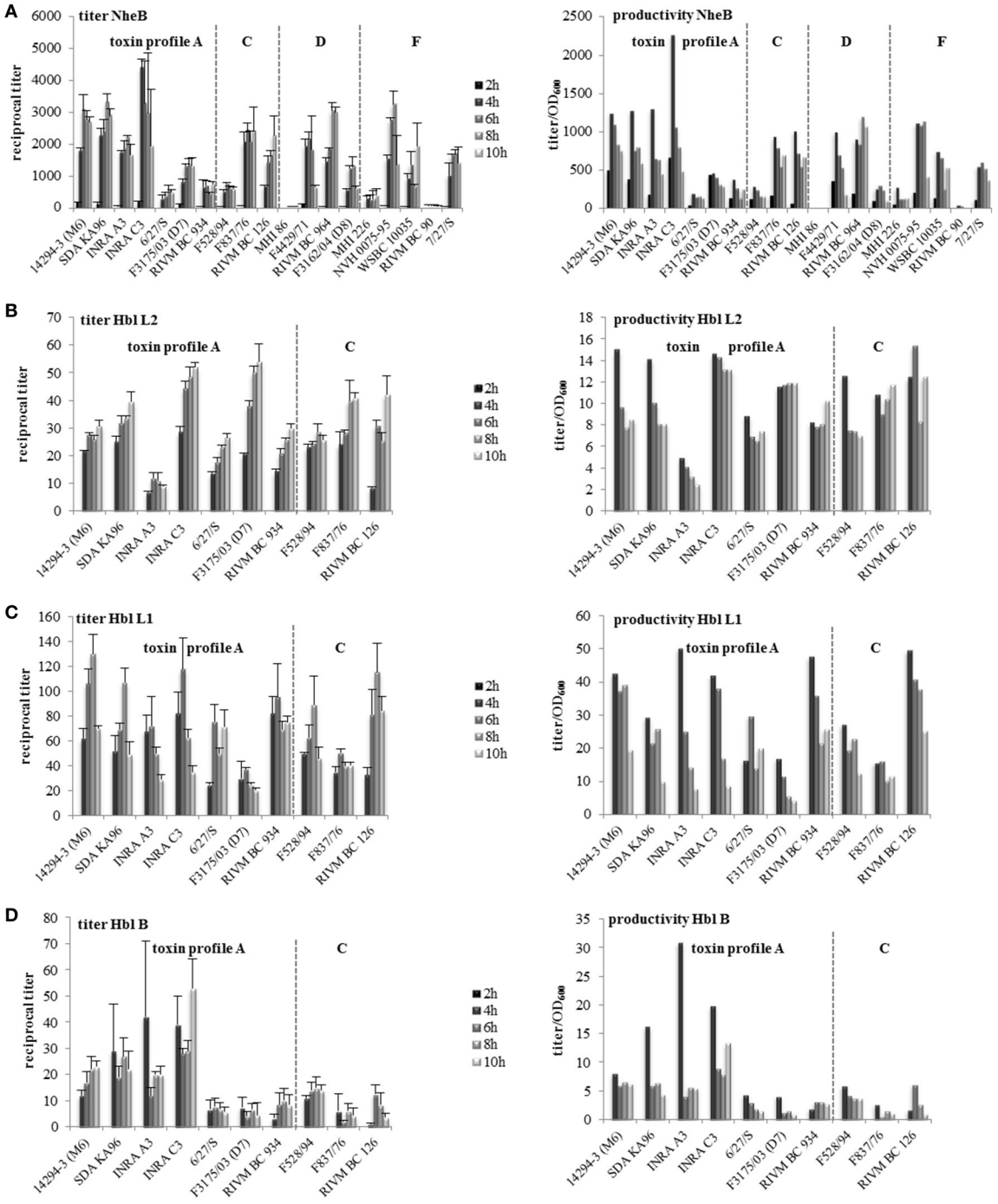

FIGURE 3 | Enterotoxin production of 19 *B. cereus* strains grown under simulated intestinal conditions. Reciprocal titers as well as the productivity (titer/OD_{600}) are shown. **(A)** NheB. **(B)** Hbl L2. **(C)** Hbl L1. **(D)** Hbl B. Strains are grouped according to their enterotoxin gene profiles, which are separated by dotted lines.

titers after 10 h, suggesting that the latter enterotoxin component might be more susceptible to proteolytic degradation. For Hbl B comparably high titers were detected after 4 h, which weren't significantly increased over time. Only strain RIVM BC 126 showed a significant increase of the Hbl B titer from 4 to 6 h.

Protein Secretion Starts Early under Simulated Intestinal Conditions

Total protein concentrations of all strains were determined as described previously (Jeßberger et al., 2015). After only 2 h growth, extracellular proteins were already detectable in all

strains at average concentrations of 20–25 ng/μl (**Figure 4A**). The highest extracellular protein concentrations were found in the supernatant of strain 6/27/S. To assess the efficiency of protein secretion, extracellular protein concentration was normalized to the optical density (OD_{600}). All strains showed highest secretion efficiency after 2 h growth, with RIVM BC 126 being the most efficiently protein secreting strain (**Figure 4B**). Intracellular protein concentrations were comparable among all tested strains (data not shown).

Despite Reduced Growth, Toxin Gene Transcription, Enterotoxin Production, and Protein Secretion Are Enhanced under Simulated Intestinal Conditions Compared to "Standard" Laboratory Cultivation

Due to oxygen and nutrient limitation, growth of all 19 strains was decreased under simulated intestinal conditions compared to laboratory conditions (**Figure 1** and Jeßberger et al., 2015). While in CGY medium strains grew to maximum optical densities of 20 (Jeßberger et al., 2015), the average maximum OD_{600} in cRPMI was 4–5. Log phases were shortened (2–4 h in cRPMI compared to 2–6 h in CGY). While strain MHI 226 showed decreased growth in CGY, this was no longer observed in cRPMI. On the other hand, in cRPMI, strain RIVM BC 126 grew significantly slower than the other strains of toxin profile C, while strain F3162/04 (D8) grew to a final OD_{600} significantly higher than all other strains.

Figure 5 compares efficiency of toxin gene transcription (*nheB*), enterotoxin productivity (NheB), and protein secretion efficiency of the 19 *B. cereus* strains grown in CGY medium (Jeßberger et al., 2015) and under simulated intestinal conditions (this study). Enterotoxin gene transcription turned out to be strain specifically activated after incubation for 2 h under simulated intestinal conditions (**Figure 5A**). Up to 40-fold increased amount of NheB toxin was found in the supernatant of all strains grown for 2 h in cRPMI compared to CGY (**Figure 5B**). This might partially result from enhanced *nheB* transcription, but also from generally increased protein secretion (**Figure 5C**). Likewise to standard laboratory conditions (Jeßberger et al., 2015), our current work showed that even under simulated intestinal conditions enterotoxin gene transcription provides no reliable information about the toxic potential of a *B. cereus* isolate, which points toward

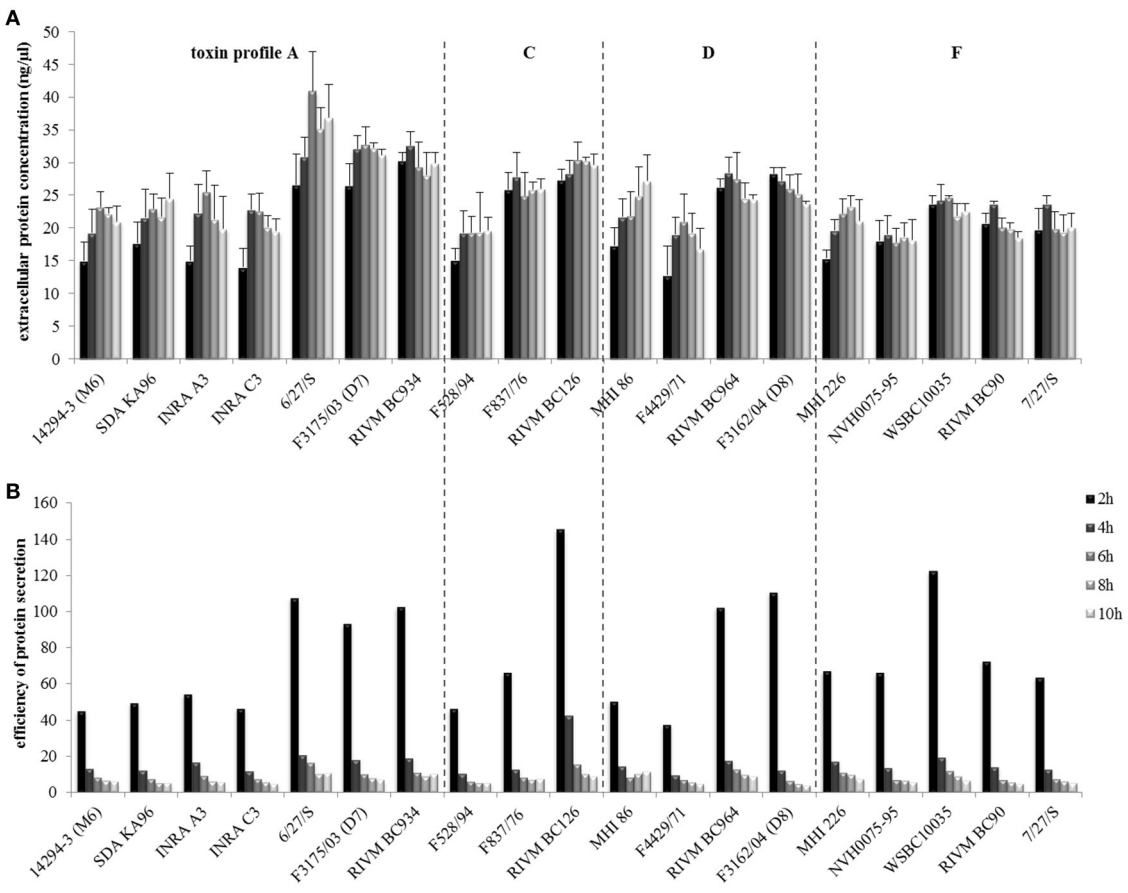

FIGURE 4 | Total protein secretion of the *B. cereus* strains grown under simulated intestinal conditions. (A) Quantification of extracellular protein after 2, 4, 6, 8, and 10 h growth. **(B)** Efficiency of protein secretion determined by normalization of extracellular protein concentrations to the OD_{600}. Strains are grouped according to their enterotoxin gene profiles, which are separated by dotted lines.

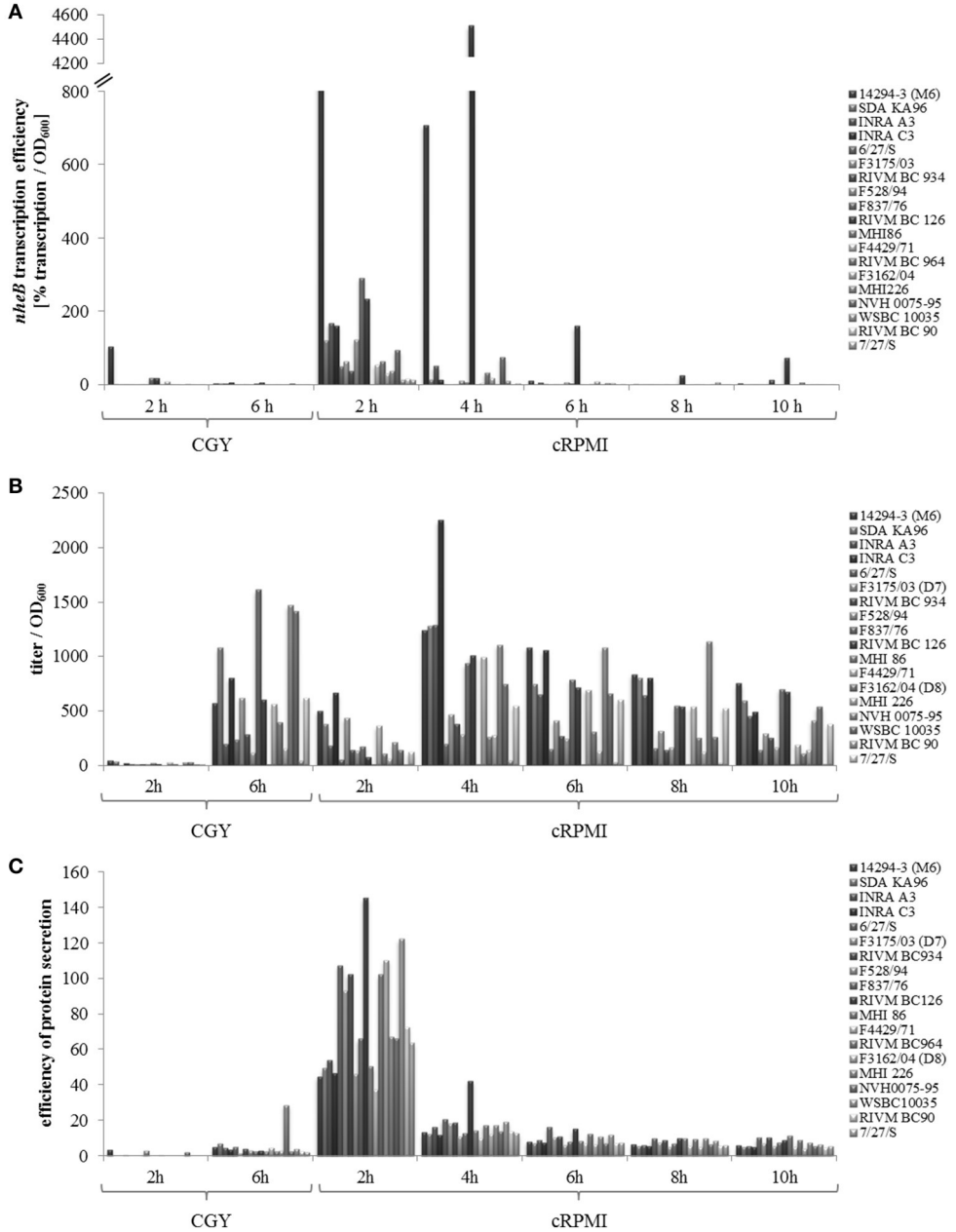

FIGURE 5 | Comparison of the *B. cereus* strain set grown in CGY (Jeßberger et al., 2015) and cRPMI (this study). (A) Transcription efficiency of *nheB* determined as % transcription per OD_{600}. **(B)** NheB productivity determined as reciprocal titer per OD_{600}. **(C)** Efficiency of protein secretion determined as extracellular protein concentration per OD_{600}.

additional posttranscriptional and posttranslational regulatory mechanisms.

Enhanced Enterotoxin Production Results from Pre-incubation with the CaCo-2 Cells

Prior experiments showed an increase of enterotoxin production and protein secretion under the chosen simulated intestinal growth conditions compared to standard laboratory conditions (**Figure 5**). Thus, the question arose whether this was due to the change of growth conditions (increased temperature, 7% CO_2 atmosphere, no agitation, nutrient limitation) or due to the pre-incubation of the medium with CaCo-2 cells. To examine this, seven selected *B. cereus* strains were compared in CaCo-2 treated cRPMI medium and in untreated RPMI 1640 medium under otherwise identical conditions in the cell culture incubator. While three strains showed no growth differences in the two compared media, four strains had a slightly accelerated log phase in the

CaCo-2 treated cRPMI medium (**Figure 6**). All of the tested strains showed enhanced enterotoxin production (represented by reciprocal titers of the toxin component NheB as well as by calculation of the productivity [titer/OD_{600}]) in the CaCo-2 treated cRPMI medium at early time points (2, 4, and partially 6 h after inoculation). After longer incubation times, NheB titers of six out of seven strains grown in the untreated RPMI 1640 medium caught up (**Figure 6**). This experiment shows that under both conditions enterotoxin production takes place, but that it is enhanced in the CaCo-2 treated cRPMI medium at early time points. Thus, it can be concluded that the signal triggering early enterotoxin production is present in the medium and indeed originates from the CaCo-2 cells.

Strain Specific Cytotoxicity

For cytotoxicity assays, the human colon carcinoma cell line CaCo-2 was used. Due to reduced growth under simulated intestinal conditions and thus, reduced amounts of enterotoxins (**Figures 1**, **3**), reciprocal titers obtained in WST-1 bioassays (**Figure 7A**) decreased compared to earlier tests (Jeßberger et al., 2015), and differences between strains formerly classified as high and low toxic seemed to be less distinct. Also pace of pore formation by the enterotoxins, determined by propidium iodide influx tests, was slightly decreased compared to earlier experiments (see **Figure 7B** and Jeßberger et al., 2015). Nevertheless, it became obvious that *hbl* positive strains caused much more rapid pore formation than solely Nhe producing strains, a fact that has been observed before (Jeßberger et al., 2014, 2015), even when growth and as a result enterotoxin (Hbl) production was limited. After 2 h no toxic effect was seen, and for 5 out of 10 *hbl* positive strains pace of PI influx was increased from 4 h to later time points.

DISCUSSION

To determine the toxic potential of a *B. cereus* isolate, bacteria are usually cultivated under laboratory conditions, which stimulate maximal growth and thus enhance the amount of secreted toxins (Jeßberger et al., 2015). These procedures, however, do not match the situation in the human gastrointestinal tract where the bacteria are enfacing nutrient limitation and anaerobic or microaerobic conditions (Clair et al., 2010). Hitherto, no suitable animal system is established to simulate growth behavior and enterotoxin production of *B. cereus* in the human intestine. In search of a relatively simple and easy to handle *in vitro* system simulating the human intestine, we applied "CaCo-2 treated" cRPMI medium, which was pre-incubated with the human epithelial colorectal adenocarcinoma cell line CaCo-2.

Routinely, the cytotoxic potential of a new *B. cereus* isolate is determined in WST-1 bioassays on Vero cells (Dietrich et al., 1999; Moravek et al., 2006; Jeßberger et al., 2015). Titers are determined as reciprocal value of the supernatant dilution that results in 50% loss of mitochondrial activity. High titers represent high toxic potential. The comparison of a variety of *B. cereus* isolates enables their classification as high, medium or low toxic (see **Table 1**). Vero, an epithelial cell line from the kidney of the African green monkey, is often used because of its high susceptibility toward bacterial toxins (Miyamura et al., 1974; Yutsudo et al., 1987). A recent study showed that *B. cereus* enterotoxins are harmful to a variety of different cell lines. Of these, CaCo-2 cells seem to be best suited to simulate the human intestine, although they are generally less susceptible and respond rather to Hbl than to Nhe in comparison to Vero cells (Jeßberger et al., 2014). When the *B. cereus* isolates were cultivated under simulated intestinal conditions and CaCo-2 were used as target cells, the criteria for the classification as high, medium or low toxic (see **Table 1** and Jeßberger et al., 2015) had to be adjusted, as otherwise most of the strains were underestimated. Thus, in cRPMI, more strains were now classified as medium toxic (**Table 1**), which should still be considered potentially dangerous. The general tendency of high, medium, or low toxicity remained, whether the strains were cultivated in CGY or cRPMI (**Table 1**), but under simulated intestinal conditions the differences between high and low toxic strains were decreased (**Figure 7**).

This study has clearly shown that enterotoxin gene expression, enterotoxin production, and total protein secretion are enhanced and start extremely early under the chosen simulated intestinal conditions. This might be due to time limitation, as the bacilli pass the human intestine to a large extent (Camilleri et al., 1989).

Our data further revealed a discrepancy between enterotoxin gene transcription and enterotoxin titers in the culture supernatants (compare **Figures 2**, **3**). This had also been observed when the analyses were performed after growth of the strains under laboratory conditions (CGY medium). At that time it was concluded that further posttranscriptional and posttranslational processes might be involved, such as alternative regulation by non-coding RNA (riboswitches) (Jeßberger et al., 2015). Unusually long 5′ untranslated regions (UTR) upstream of the start codons of both the *nhe* and the *hbl* operon have been identified (Böhm et al., 2016) suggesting posttranscriptional regulation via formation of regulatory mRNA structures. An involvement of riboswitches in gene expression has already been demonstrated for *B. subtilis* and *B. anthracis* (Welz and Breaker, 2007; Wilson-Mitchell et al., 2012). Furthermore, posttranslational regulation may also add to the complex regulatory network of enterotoxin gene expression, as it was recently shown for the regulation of cereulide toxin production in emetic *B. cereus* (Ehling-Schulz et al., 2015; Lücking et al., 2015; Kranzler et al., 2016). Overall, these multiple levels of regulation complicate the predictability of enterotoxin production.

Stimulation by intestinal conditions has also been observed for *Clostridium perfringens*, another Gram positive, toxin producing species. Compared to *in vitro* growth, rapid upregulation of toxin genes in the presence of CaCo-2 cells was observed, followed by enhanced protein secretion. This was detectable after only 1 h of infection and even enhanced after 2 and 3 h (Vidal et al., 2009). When growth and toxin production of selected *B. cereus* strains were compared in CaCo-2 treated cRPMI medium and in untreated RPMI 1640 medium under otherwise identical conditions, enhanced and early enterotoxin production was observed in the presence of the CaCo-2 cells or their supernatant (**Figure 6**). Thus, we concluded that a soluble factor present in the medium and originated from the CaCo-2 cells stimulates toxin production. Considering that enterotoxin titers of six out of seven

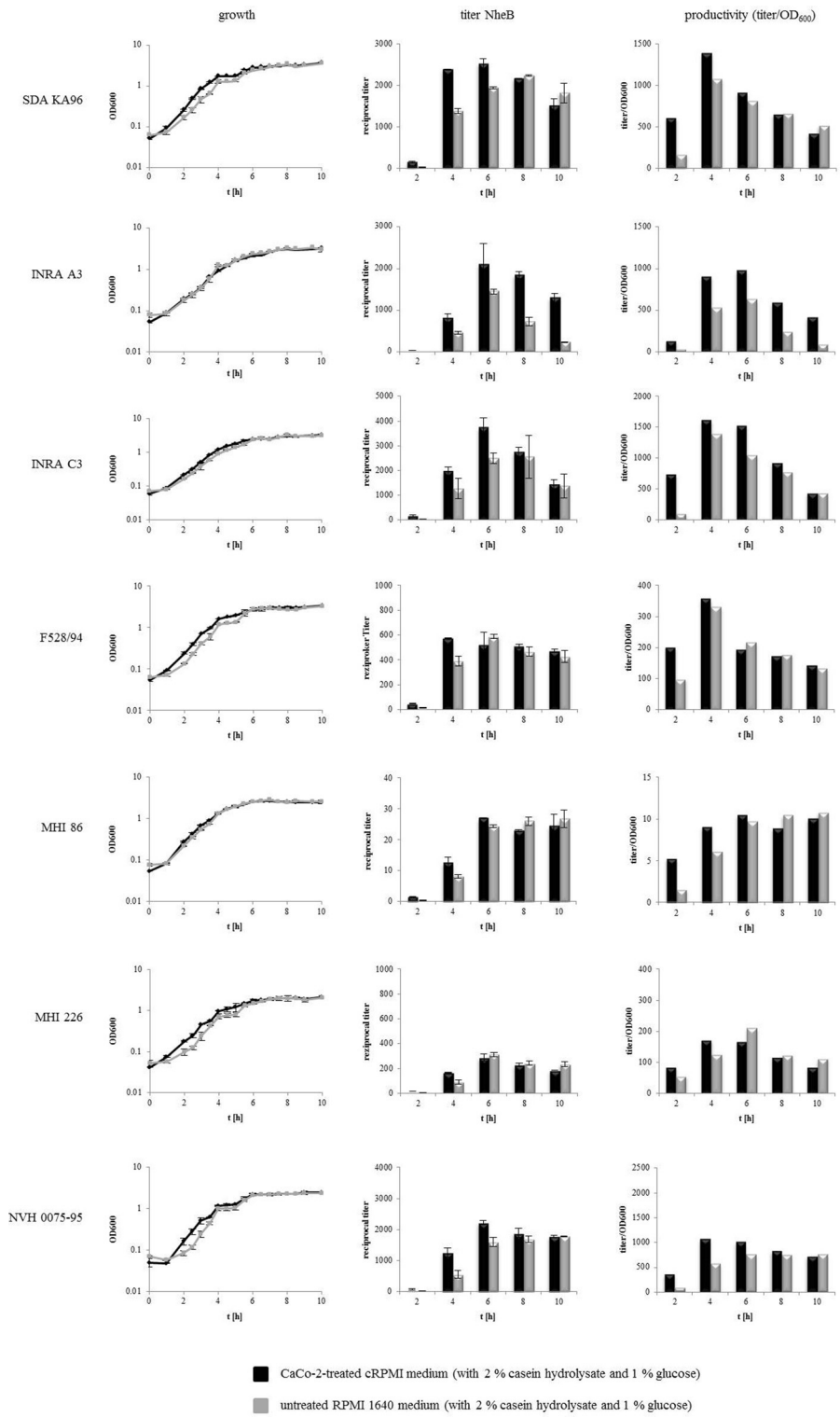

FIGURE 6 | Growth and enterotoxin production of 7 selected *B. cereus* strains comparing CaCo-2 treated cRPMI medium with untreated RPMI 1640 medium. Both media types were supplemented with 2% casein hydrolysate and 1% glucose. cRPMI medium had been pre-incubated for 22 h with differentiated CaCo-2 cells. Bacteria were grown in 80 cm^2 cell culture flasks at 37°C under 7% CO_2 atmosphere. OD_{600} was recorded for 10 h and every 2 h samples of the supernatant were taken. Enterotoxin production was determined via sandwich EIA specific for NheB.

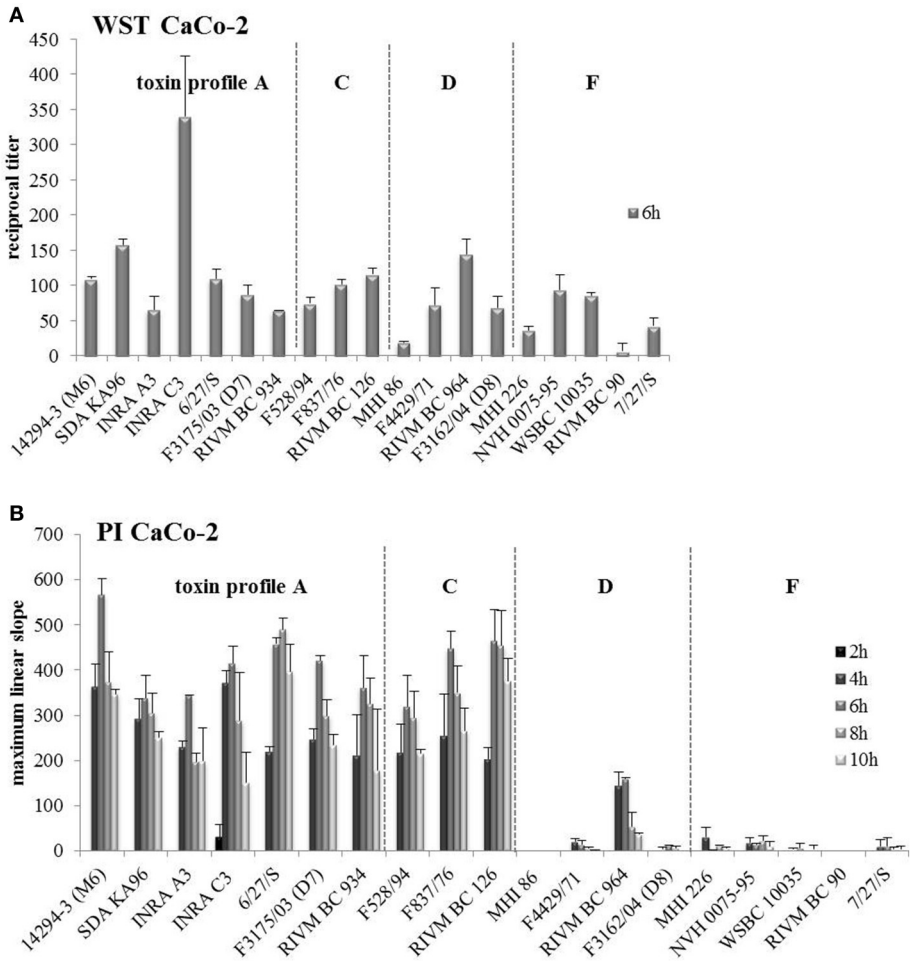

FIGURE 7 | Toxicity tests of the 19 *B. cereus* strains on CaCo-2 cells. (A) Reciprocal titers for 50% lethal doses obtained in WST-1 bioassays are shown. The assay was performed only after 6 h of growth under simulated intestinal conditions. **(B)** Propidium iodide influx tests were performed after 2, 4, 6, 8, and 10 h of growth. The maximum linear slope of the fluorescence curves of each strain is shown. Strains are grouped according to their enterotoxin gene profiles, which are separated by dotted lines.

strains in untreated RPMI 1640 medium converged with those in cRPMI after 6–8 h (**Figure 6**), one could also speculate that the factor accelerating toxin production is used up by the bacteria. On the contrary, only pre-infected CaCo-2 cells triggered toxin production of *C. perfringens* (Vidal et al., 2009). It was concluded that rapid host-cell stimulated secretion of most *C. perfringens* toxins is triggered by an unknown host factor present during infection and that close contact between the bacteria and the host cells is required, as the factor is Caco-2-surface-bound (Vidal et al., 2009). In both cases, the signal (factor) stimulating the bacteria could not be further localized. It has been suggested that a lack of glucose activates *B. cereus* enterotoxin gene transcription *via* CcpA-dependent catabolite repression (van der Voort et al., 2008). Hence, we measured glucose concentrations in cRPMI for 36 h. It appeared that glucose was not used up until the bacilli reached the end of the exponential growth phase (data not shown). So it is assumed that enterotoxin production of *B. cereus* is stimulated by a so far unidentified host factor. In an earlier study it has been observed that germination of 8 out of 11 *B. cereus* strains was induced by CaCo-2 cells and also by their (heated) supernatant. Thus, it was concluded that this heat stable germinant is released by the eukaryotic cells and thereupon bound or degraded by the *B. cereus* spores (Wijnands et al., 2007).

Activation of virulence factor gene expression and of protein secretion upon host contact has been primarily described for invasive pathogens. It has been reported that contact with epithelial cells induces transient assembly of appendages on the surface of *Salmonella typhimurium* (Ginocchio et al., 1994). After host cell contact and initial formation of A/E (attaching and effacing) lesions, enteropathogenic *Escherichia coli* (EPEC) increase transcription of genes involved in adherence and virulence (Leverton and Kaper, 2005). For example EspC (autotransporter protein) secretion is stimulated when bacteria are grown in cell culture medium and increased in the presence of epithelial cells (Vidal and Navarro-García, 2006). Transcription of virulence genes of *Shigella flexneri* has been found to be

transiently regulated by the type III secretion machinery upon entry into epithelial cells (Demers et al., 1998). After contact with host cells *Helicobacter pylori* produces parts of the Hp T4SS (*Helicobacter pylori* type IV secretion system) and activates two different invasion mechanisms (Rohde et al., 2003). Our study showed that contact with the CaCo-2 treated medium enhances enterotoxin gene transcription as well as total protein secretion of *B. cereus*. Up to now it is not quite clear how the main virulence factors, the enterotoxin complexes Nhe and Hbl, are secreted. The occurrence of Sec-type secretion signal peptides at the proteins' N-terminus points to secretion via the *sec* pathway (Fagerlund et al., 2010; Vörös et al., 2014), but evidence has also been found for involvement of flagellar export complexes similar to type III secretion systems of Gram negative bacteria (Senesi and Ghelardi, 2010). In contrast to invasive pathogens, according to our data *B. cereus* does not necessarily need close contact to the host cells. Sensing the intestinal environment seems to be sufficient for stimulating the production of enterotoxins, which subsequently attack the host cells via ingenious mechanisms of pore formation (Heilkenbrinker et al., 2013; Didier et al., 2016; Zhu et al., 2016).

CONCLUSION

As revealed by our study, cultivation of *B. cereus* under "standard" laboratory conditions does not allow conclusive predictions of the toxic potential of enteropathogenic *B. cereus* strains in the human intestine. Simulated intestinal growth conditions, such as the ones presented here, have to be included in the standard cultivation procedure. cRPMI medium, pre-incubated with host cells, was shown to accelerate and enhance enterotoxin production per cell. The cRPMI medium represents an interesting alternative for the determination of the enterotoxigentic potential of *B. cereus* strains in a more host simulating setting, as long as the signal originating from the CaCo-2 cells, which stimulates enterotoxin production, is unknown. Elucidating the latter one is subject of ongoing research.

AUTHOR CONTRIBUTIONS

NJ performed growth experiments and sample preparation and wrote the manuscript. VK and MB carried out transcription experiments. CR carried out the protein secretion studies. AM and NJ determined enterotoxin production and cytotoxicity. RD, SS, ME, and EM were involved in experimental setup and writing of the manuscript.

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Tetracycline Resistance Genes Identified from Distinct Soil Environments in China by Functional Metagenomics

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Soil microbiota represents one of the ancient evolutionary origins of antibiotic resistance and has been increasingly recognized as a potentially vast unstudied reservoir of resistance genes with possibilities to exchange with pathogens. Tetracycline resistance is one of the most abundant antibiotic resistances that may transfer among clinical and commensal microorganisms. To investigate tetracycline resistance genes from soil bacteria in different habitats, we performed functional analysis of three metagenomic libraries derived from soil samples collected from Yunnan, Sichuan, and Tibet, respectively, in China. We found efflux transporter genes form all the libraries, including 21 major facilitator superfamily efflux pump genes and one multidrug and toxic compound extrusion (MATE) transporter gene. Interestingly, we also identified two tetracycline destructase genes, belonging to a newly described family of tetracycline-inactivating enzymes that scarcely observed in clinical pathogens, from the Tibet library. The inactivation activity of the putative enzyme was confirmed *in vitro* by biochemical analysis. Our results indicated that efflux pumps distributed predominantly across habitats. Meanwhile, the mechanism of enzymatic inactivation for tetracycline resistance should not be neglected and merits further investigation.

Keywords: functional metagenomics, soil, tetracycline resistance, efflux transporter, tetracycline destructase

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INTRODUCTION

The widespread of antibiotic-resistant bacteria is an escalating global problem, underscoring the need for a larger arsenal of antibiotics and a further understanding of the ecology of the antibiotic "resistome", including their origins, reservoirs and the underlying resistance mechanisms (Wright, 2007). It is now well accepted that resistance genes originated in environmental organisms long before the anthropogenic use of antibiotics (Forsberg et al., 2014; Perry and Wright, 2014; Perry et al., 2014; Graham, 2015). Growing evidences implicated that the resistance genes were transmitted between environmental bacteria and pathogens via horizontal gene transfer (Forsberg et al., 2012; Rolain, 2013). Besides, numerous resistance genes have been detected in different kinds of food (Aquilanti et al., 2007; Osimani et al., 2017), and the diet is one of the main route for the entrance of antibiotic resistance genes and bacteria within the humans (Wang et al., 2012;

Rolain, 2013). Worse still, the exchange of antibiotic resistance genes has also been found from soil to food-producing animals (Wang et al., 2017). Thus, it emphasizes the important role of environmental bacteria as a vast reservoir of antibiotic resistance genes. It has also been suggested that these resistance genes in their original reservoirs may serve very different functions from the "weapon-shield" role they play in clinic. Therefore, the occurrence of these reserve genes that could express resistance function by "functional shift" may be greatly unexplored (Martinez, 2008). Vast majority of these unknown genes in environmental organisms are unable to be identified by traditional survey methods like PCR or microarray which generally based on known sequences. Functional metagenomics allows for the exploration of novel resistance genes, and is independent of culture and sequence bias (Allen et al., 2009; Sommer et al., 2009; Vercammen et al., 2013; Gibson et al., 2015).

The tetracyclines is one of the most widely used classes of broad spectrum antibiotics in clinic and agriculture due to their excellent therapeutic index, low cost, oral administration, and few side effects (Thaker et al., 2010). After over 60 years of extensive use of tetracyclines, the prevalence of tetracycline resistance has alarmingly increased, and become one of the most abundant antibiotic resistances among clinical and commensal microorganisms. To date, more than 50 different tetracycline resistance genes have been identified, conferring resistance primarily through three mechanisms: active efflux, ribosomal protection and enzymatic inactivation of the antibiotics. The first two mechanisms currently predominate in clinical settings (Roberts, 2005; Thaker et al., 2010). Due to the horizontal exchange of genes on mobile genetic elements such as plasmids and transposons, numerous tetracycline efflux, and ribosomal protection genes spread rapidly in a variety of species and ecosystems over time (Roberts, 2005; Knapp et al., 2010; Thaker et al., 2010). In contrast, enzyme inactivation, a common and preferred resistance mechanism of most natural-product antibiotics (Walsh, 2000), was far scarcely observed for tetracycline resistance. Only one enzyme, Tet(X), has been found in human pathogens with confirmed *in vitro* activity (Yang et al., 2004; Leski et al., 2013). However, a recent finding of a family of tetracycline destructases from soil metagenomic sources has brightened the importance of this long been neglected mechanism (Forsberg et al., 2015). The present study was directed toward investigating tetracycline resistances from distinct soil environments in China using functional metagenomics. The exploration of diverse resistance genes in environmental organisms and its mobilization to clinic would aid in our understanding of the evolution of the resistome as well as anticipation of the emergence of new resistance mechanisms in clinic.

MATERIALS AND METHODS

Metagenomic Library Construction

Three metagenomic libraries were constructed using soil samples collected from distinct area of China (**Table 1**). All of the sampling sites have no known exposure to antibiotics. The

TABLE 1 | Cosmid libraries constructed with soil samples collected from distinct area of China.

Library name	Sampling site	Altitude (meters)	Vector	Vector resistance mark [a]	Host	Average insert size (kb)	No. of clones	Gb of cloned DNA
Yunnan	Forest (N23°44′, E101°71′)	2100	pWEB-TNC	Chl, Amp	*E. coli* EPI100-T1^R	38	1.28×10^7	486
Tibet	Mount Qomolangma (N28°21′, E86°56′)	5500	pWEB-TNC	Chl, Amp	*E. coli* EPI100-T1^R	38	1.04×10^7	395
Sichuan	Forest (N29°34′, E103°20′)	900	pJTU2554	Apr	*E. coli* JTU007	30	4.80×10^4	1.44

[a]*Chl, chloramphenicol; Amp, ampicillin; Apr, apramycin.*

construction of the Yunnan and Tibet libraries were performed following the published methods (Brady, 2007; Gu et al., 2015). In brief, 200 g of meshed soil was used to extract the environmental DNA (eDNA). The eDNA was then purified and blunt-ended (Epicentre, Charlotte, United States). The resulting eDNA was ligated with the pWEB-TNC vector (Epicentre, Charlotte, United States), packaged (Epicentre, Charlotte, United States) and transfected into *Escherichia coli* (*E. coli*) EPI100-T1^R (Epicentre, Charlotte, United States). The Sichuan library was constructed with pJTU2554 (Li et al., 2008) vector in the *E. coli* JTU007 (Zhou et al., 2012) host using the same methods.

Screening of Tetracycline Resistant Clones

The tetracycline-resistant clones were selected as previously described (Allen et al., 2009). Five thousand individual clones of the metagenomic libraries were inoculated in 3 ml Luria–Bertani (LB) broth containing 50 μg/ml chloramphenicol and 100 μg/ml ampicillin (clones form Tibet and Yunnan libraries), or 50 μg/ml apramycin (clones from Sichuan library). After 2–4 h at 37°C with shaking, cultures were then plated at ∼5 × 10^5 CFU/plate on LB agar plates containing 10 μg/ml tetracycline together with 50 μg/ml chloramphenicol or 50 μg/ml apramycin, according to the selection marker on the vectors. Plates were incubated at 37°C for up to 2 days, and then resistant colonies were picked for further study.

Identification and Sequencing of Active Genes

The gene responsible for tetracycline resistance was identified by subcloning. The cosmid DNA from the resistant clone was partially digested with *Sau* 3AI (Takara Bio, Dalian, China), and DNA fragments of 2–3 kb were then recovered and ligated into *Bam* HI digested and alkaline phosphatase treated pTG19-T vector (Generay Biotech, Shanghai, China), electroporated into *E. coli* EPI100-T1^R, and plated onto LB screening agar plates for resistant subclones. All screened subclones were verified by restriction digestion and retransformation to confirm the phenotypes. The resulting positive recombinant clones were sequenced by using T7 promoter and M13 primers at first, then by primer-walking sequencing.

Sequence analysis was carried out with the BLASTx program[1]. Amino acid alignment was performed with the ClustalW2.0 program and phylogenetic analysis (Neighbor-joining tree) was done with MEGA6.0 using neighbor-joining (500 bootstrap replicates).

Tetracycline Susceptibility Testing

The susceptibilities of the positive subclones to tetracycline were tested using the standardized broth microdilution method. All tests were performed and results were evaluated according to the Clinical and Laboratory Standards Institute guidelines (CLSI) document CLSI M100-S24 (2014). Assays were performed in duplicate and experiments were conducted twice with *E. coli* ATCC 25922 and *E. coli* EPI100-T1^R carrying the empty pTG19-T vector as the reference strains.

Construction of Expression Recombinant Plasmids

The full-length ORF encoding putative tetracycline destructases was amplified by PCR with the following primer pair: 1435-F (5′-ggaattccatatgtctgctacaaataaaattctcgt-3′)/1435-R (5′-ccgctcgagcttttcataatctggcaaagaaatgg-3′). *Nde* I or *Xho* I sites added for cloning are underlined. *Nde* I/*Xho* I-digested PCR product was cloned into *Nde* I/*Xho* I-digested pET30a(+) vector. The construct was then transformed into *E. coli* BL21 (DE3) for overexpression.

Enzyme Expression and Purification

The putative tetracycline-inactivating enzyme was expressed and purified as previously described with some modification (Gu et al., 2015). Briefly, overnight culture was used to inoculate 500 ml kanamycin supplemented LB broth (50 μg/ml) (1:1000 dilution) and cultured at 37°C with shaking until the $OD_{600\ nm}$ value reached 0.6. The sample was then cooled to 18°C and 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression for an additional 18 h. Cells were harvested by centrifugation at 4°C, resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1% Triton X-100), and subsequently disrupted by sonication. The protein mixture was collected at 15,000 rpm for 15 min at 4°C. Proteins were purified via Ni-NTA resin (Bio-Rad, Hercules, United States). After washing with 10 column volumes of washing buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl), the bound proteins were recovered with elution buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 500 mM imidazole). The purified protein was analyzed by SDS-PAGE electrophoresis, flash frozen and stored at –80°C.

In Vitro Enzymatic Reactions

In vitro enzymatic reactions were performed following the method previously described with a little modification (Forsberg et al., 2015). A total volume of 564 μL reaction mixture contained 1.4 mM tetracycline and 350 μg purified enzyme was prepared with a NADPH regenerating system. The reaction started with the addition of thawed enzyme. Then, 2000 μL quencher solution (equal parts methanol and 0.25 M HCl) was added to each reaction mixture at indicated time points. The enzymatic degradation products were extracted by ethyl acetate, and redissolved with 400 μL methanol for reverse phase HPLC analysis.

Analysis of Tetracycline Degradation Products by HPLC

Products generated from enzymatic inactivation of tetracycline were analyzed by reverse phase HPLC (InertSustain® C18, 4 × 250 mm, 5 μm) using a linear gradient of H_2O:MeOH (20:80 to 80:20) over 20 min at 1 ml/min.

[1] http://blast.ncbi.nlm.nih.gov

Nucleotide Sequence Accession Numbers

All nucleotide sequences have been deposited in GenBank with the following accession numbers: KX161706–KX161713, KY697282–KY697295, and KY853665–KY853666.

RESULTS

Construction of the Metagenomic Libraries

We collected three soil samples from distinct area of southwest China (**Table 1**). The sampling sites have different altitude and climate. DNA isolated from soils collected in Yunnan and Tibet was used to construct cosmid libraries that contained 12.8 and 10.4 million unique members, respectively. The average insert size of these two libraries was approximately 38 kb. Besides, a cosmid library containing about 4.80×10^4 individual members was also constructed using eDNA extracted from Sichuan soil, with an average insert size of 30 kb.

Screening of Tetracycline Resistant Clones and Susceptibility Assays

To identify tetracycline resistant clones, we subjected the metagenomic libraries to a functional selection on LB agar amended with tetracycline. In total, we obtained thirty clones that conferred resistance on *E. coli*. We determined the MICs of those thirty clones that confer reproducible decrease in susceptibility to tetracycline (**Table 2**). All of these clones showed increased resistance to tetracycline compared to the vector-only control. The overall MIC values ranged from 16 to 256 mg/ml, and 50% of the clones presented high-level resistance to tetracycline (MIC $\geq 8 \times$ resistant breakpoint). The mean MIC values of the Yunnan and Tibet clones (58 and 65 mg/ml, respectively) were much higher than that of the Sichuan clones (32 mg/ml).

Identification of Tetracycline Resistance Genes and Phylogenetic Analysis

The genes responsible for tetracycline resistance were identified by sequencing and BLAST analyzing the resistant subclones, and 24 unique resistance genes of different types were obtained (**Table 2**). Of these functional genes, 22 showed homology to previously reported tetracycline transporters, including twenty one major facilitator superfamily (MFS) transporters and one multidrug and toxic compound extrusion (MATE) family efflux. MFS transporter genes were obtained in all of the three libraries screened in this study, suggesting a wide distribution of this gene family across the area and diverse soils. The deduced amino acid sequences of them showed a moderate similarity with one another (average pairwise amino acid identity 47.3% $\pm$ 7.3%). The two Sichuan-derived MFS transporters, SC6 and SC2451, shared the same amino acid sequences with YN2306 and YN1469 from Yunnan library, respectively, implying a horizontal transfer potential of this element. In contrast, the amino acid sequences of MFS transporters from Tibet were divergent from that of Yunnan-derived ones, and therefore clustered separately in the phylogenetic tree (**Figure 1A**). From Tibet library, we also identified a putative MATE efflux protein. BLAST analysis of the putative MQ1629 protein yielded the best match with a MATE efflux from Acidobacteria bacterium OLB17 (KXK01510.1, 59%). The MATE family efflux was reported to confer resistance to a wide spectrum of cationic toxic agents such as organic acids, plant hormones and secondary metabolites (Li and Nikaido, 2009). To evaluate the activity of this functional MATE transporter gene, we additionally determined the antimicrobial susceptibility of clone MQ1629 for ceftazidime, gentamycin, ciprofloxacin. However surprisingly, this putative efflux conferred resistance only to tetracycline (32 μg/ml) (data not shown).

In addition, we also identified two functional genes showed homology to a novel family of tetracycline destructase newly identified from soil metagenomes (Forsberg et al., 2015; **Figure 1B**). The two deduced proteins we reported here, sharing 73% amino acid identity with each other, harbored an average identity of 61.6% with those nine previously found tetracycline destructases. But they showed low identities of 20 and 21%, respectively, to Tet(X), the only one confirmed tetracycline inactivation enzyme from clinical bacterium.

In Vitro Enzymatic Inactivation

The two putative tetracycline destructases we found shared high amino acid identity with each other and the same MIC to tetracycline (64 μg/ml). We deduced that the inactivation mechanism of these two enzymes may be the same, so MQ1435 clone was chosen for further study. The relative molecular mass of the putative MQ1435 enzyme was estimated to be 43 kDa by SDS-PAGE analysis (**Figure 2**). Reverse-phase HPLC was used to monitor the progression of each inactivation reaction. The result indicated that the tetracycline substrate eliminated with time (**Figure 3B**), however, no obvious new stable product was observed during the *in vitro* inactivation reaction. For control reaction that performed without enzyme, no obvious change in tetracycline substrate was generated (**Figure 3A**).

DISCUSSION

Using a functional metagenomics approach, we identified 21 MFS transporter genes from all three soil libraries. The Tibet-derived MFS transporters showed higher sequence similarity from each other, and clustered separately from those derived from the other two libraries. In addition, sequence and phylogenetic analysis showed that they were more homologous to the deduced MFS transporters obtained from sequenced metagenomes than those derived from cultured bacterium. The MATE transporter and inactivation enzyme genes were only found from Tibet library. These distinctions may be due to the specificity of the sampling site of this library. The soil was sampled at 5500 m altitude of Mount Qomolangma and there was no known exposure to antibiotics at the sampling point. Soil resistome composition has been demonstrated to be closely associated with different soil types and microbial taxonomic structures (Forsberg et al., 2014). Our results verified this correlation to some extent, and implied that the microbe

TABLE 2 | Clones from three soil metagenomic libraries that confer tetracycline resistance on *Escherichia coli*.

Clone	Tetracycline MIC (μg/ml)	Mode of action	Similarity (organism)	Accession no. of closet match (% identity)
MQ203	32	Efflux pump	Tetracycline efflux MFS transporter (*Microbacterium sp*. Root280D1)	WP_056279670.1 (92)
MQ730	16	Efflux pump	Tetracycline efflux MFS transporter (*Microbacterium* sp. *CGR1*)	WP_053097549.1 (88)
MQ1411	64	Efflux pump	Tetracycline efflux MFS transporter (*Micrococcales bacterium* 72–143)	OJX66894.1 (78)
MQ1715	128	Efflux pump	Tetracycline efflux MFS transporter Tet(42) (*Micrococcus sp*. SMCC G887)	WP_063855882.1 (88)
MQ1987	64	Efflux pump	Tetracycline transporter (*Microbacterium sp*. Leaf320)	WP_056514290.1 (91)
MQ1629	32	Efflux pump	MATE efflux family protein (*Acidobacteria bacterium* OLB17)	KXK01510.1 (59)
MQ776	64	Enzyme inactivation	Tetracycline destructase Tet(47) (Uncultured bacterium)	AKQ05891.1 (73)
MQ1435	64	Enzyme inactivation	Tetracycline destructase Tet(47) (Uncultured bacterium)	AKQ05891.1 (80)
YN141	256	Efflux pump	MFS transporter (*Streptomyces griseus*)	WP_044369073.1 (78)
YN623	32	Efflux pump	MFS transporter (*Labrys* sp. WJW)	WP_068282769.1 (80)
YN629	32	Efflux pump	MFS transporter, EmrB/QacA subfamily (*Nocardioides terrae*)	SFC16744.1 (86)
YN652	32	Efflux pump	MFS transporter (*Labrys* sp. WJW)	WP_068282769.1 (86)
YN1039	64	Efflux pump	Tetracycline resistance MFS efflux pump [*Devosia sp*. 66-22]	OJX53909.1 (80)
YN1046	64	Efflux pump	Tetracycline resistance MFS efflux pump [*Streptomyces griseus*]	WP_044369073.1 (78)
YN1332	64	Efflux pump	MFS transporter, EmrB/QacA subfamily (*Nocardioides lianchengensis*)	SDD59586.1 (88)
YN1338	128	Efflux pump	MFS transporter (*Aeromicrobium* sp. Root344)	WP_056210997.1 (85)
YN1469	32	Efflux pump	MFS transporter Tet (G) (*Burkholderia* sp. BT03)	WP_007744101.1 (98)
YN1865	32	Efflux pump	MFS efflux pump (*Streptomyces* sp. LUP30)	WP_069766755.1 (90)
YN2297	64	Efflux pump	MFS efflux pump Tet (A) family (*Pelomonas* sp. Root1237)	WP_056192001.1 (92)
YN2306	64	Efflux pump	MFS efflux pump Tet (A) family (*Pelomonas* sp. Root1237)	WP_056192001.1 (89)
YN2386	16	Efflux pump	MFS efflux pump (*Gordonia soli*)	WP_007619561.1 (81)
YN2560	32	Efflux pump	MFS transporter, DHA1 family, bicyclomycin/chloramphenicol resistance protein (*Dyella* sp. OK004)	SFS19133.1 (82)
SC6	32	Efflux pump	MFS efflux pump Tet (A) family (*Pelomonas* sp. Root1237)	WP_056192001.1 (89)
SC2451	32	Efflux pump	MFS transporter Tet(G) (*Burkholderia* sp. BT03)	WP_007744101.1 (99)

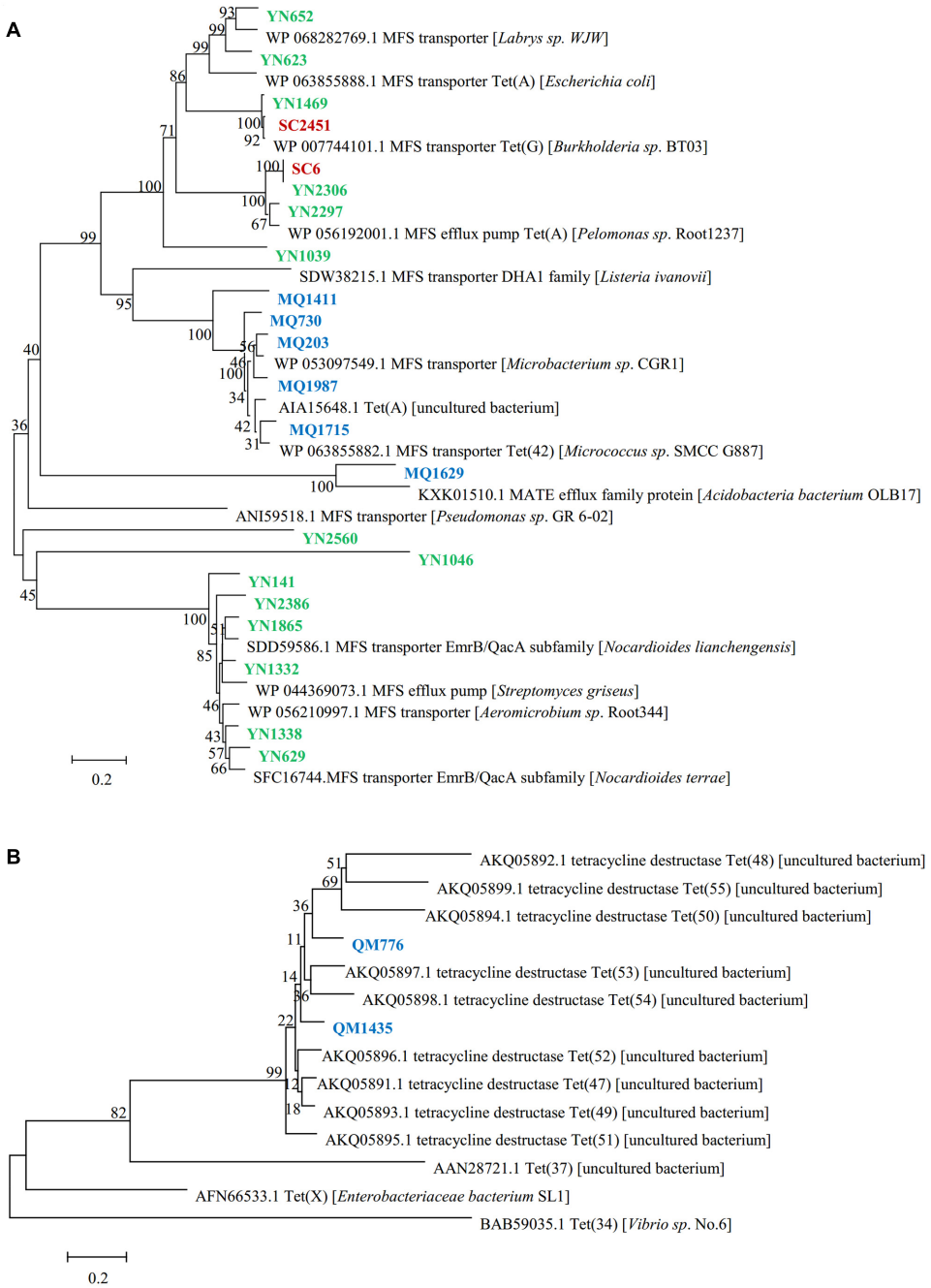

FIGURE 1 | Phylogenetic tree inferred with neighbor-joining for the predicted amino acid sequences of **(A)** tetracycline resistance transporter, **(B)** tetracycline destructase. Tetracycline resistant clones from Mount. Qomolangma soil metagenomic library are shown in blue, clones from Yunnan library are colored in green and clones from Sichuan library are colored in red.

derived from pristine environments could be a potential reservoir for discovering novel resistance genes, considering that the clinical resistome driven by antibiotics induced selection pressure is typically encoded by a much smaller pool of circulating resistance genotypes compared to the vast diverse environmental resistome (Davies and Davies, 2010; Martinez et al., 2015).

Drug inactivation, a common mechanism of pathogens to resist most naturally derived antibiotics such as the aminoglycosides and β-lactams (Walsh, 2000), was far scarcely observed for tetracycline. *tet*(X) was the first tetracycline inactivation gene identified early in 1988 from *Bacteroides fragilis* (Park and Levy, 1988). It has been demonstrated that Tet(X) is a flavin-dependent monooxygenase that inactivates

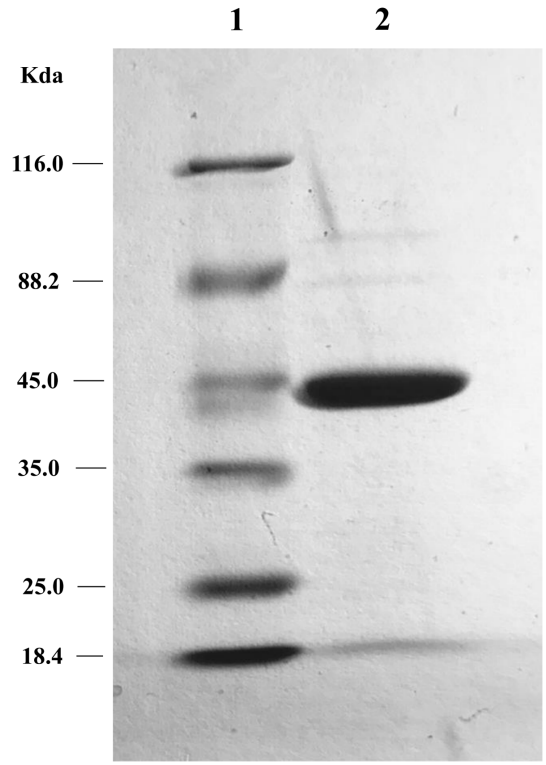

FIGURE 2 | SDS-PAGE gel electrophoresis of the tetracycline inactivation enzyme. Lane 1: protein molecular weight marker; lane 2: purified protein of MQ1435.

tetracycline by monohydroxylation followed by non-enzymatic decomposition of unstable product. Later, another two predicted tetracycline inactivation genes, *tet*(34) and *tet*(37), were identified from *Vibrio sp*. no. 6 isolated from intestinal contents and an oral metagenome, respectively (Nonaka and Suzuki, 2002; Diaz-Torres et al., 2003). Strikingly, *tet*(X) was the only tetracycline inactivation gene that have been reported in human pathogens to date (Leski et al., 2013). Therefore, tetracycline inactivation has long been considered as a rare mechanism for tetracycline resistance. However, Forsberg et al. (2015) recently discovered nine tetracycline destructase genes from agricultural and grassland soil metagenomes, although three of them showed no *in vitro* activity. Here, we further identified two putative inactivation enzyme genes from a remote soil collected from Mount Qomolangma. Previously study with Tet(X) indicated that enzymatic degradation products of tetracyclines are stable at low pH (Yang et al., 2004; Moore et al., 2005). Similar with the results observed for the five of six functional destructases described by Forsberg et al. (2015), no obvious signatures of new stable product was detected in our study, indicating that these enzymes might inactivate tetracycline via diverse oxidative mechanisms different from Tet(X). Although the exact degradation mechanism of the enzyme we found remains unclear, our findings confirm that tetracycline inactivation genes may be much more widely distributed in natural soil habitats than previously recognized, and obviously brighten the importance of this long been neglected mechanism. Nevertheless, further work is needed to figure out how pH condition affects the stability of the degradation product of our enzyme, as well as to gain clear understanding of the degradation mechanism.

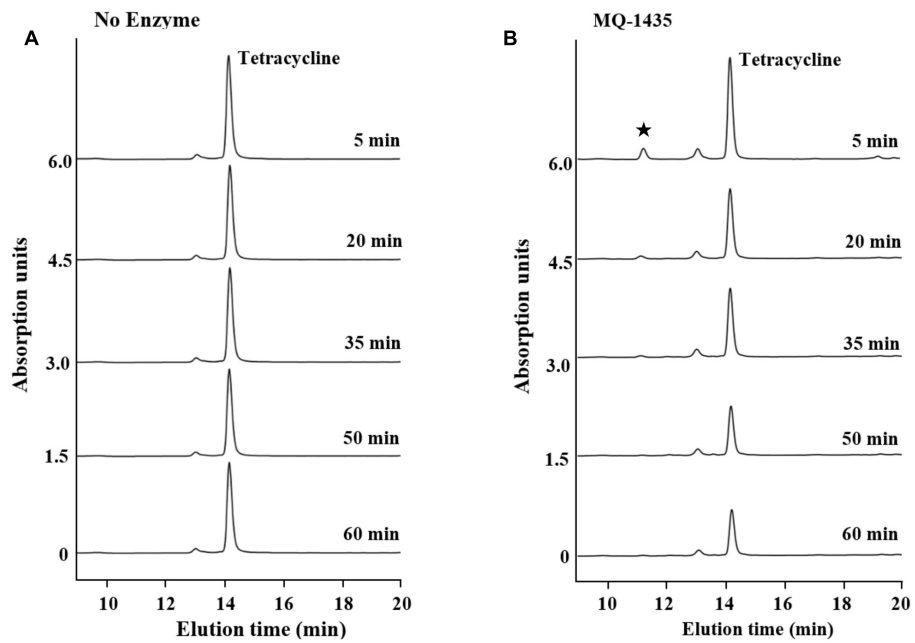

FIGURE 3 | Reverse-phase PHLC separation of tetracycline and enzymatically catalyzed degradation product at 260 nm. **(A)** Reaction with no enzyme; **(B)** Reaction with the purified enzyme of MQ-1435. ★indicates the unstable degradation product.

Functional metagenomic approach is able to reveal novel resistance genes that are unrecognizable as resistance genes on the basis of sequence from the vast environmental resistome (Allen et al., 2009; Gibson et al., 2015). Further, given that all the genes yielded by this approach are functional in *E. coli*, they clearly have the potential to be transferred and functional in pathogens. If there is a barrier to gene transfer between environmental microbes and pathogens, it is not due to functional compatibility. Several studies have revealed that the exchange of antibiotic resistance genes between bacteria from soils/animals and clinical pathogens occurred via horizontal gene transfer, and the frequency of gene exchange might be greatly underestimated (Smillie et al., 2011; Forsberg et al., 2012; Liu et al., 2016; Ma et al., 2016). Worse still, resistance genes residing in the abundant environmental reservoir were thought to have a larger threat for entering the circulating pathogenic resistome (Davies and Davies, 2010; Martinez et al., 2015), emphasizing the clinical importance of the environmental resistome. Consequently, further exploration of the diverse resistome in extend environments is critical and essential for a deeper understanding of the antibiotic resistance from an ecological perspective.

In this study, through functional metagenomic selection, we discovered several tetracycline resistance genes of different classes from soil metagenomes. Efflux pump genes were found to be predominant distributed across soil types and habitats, while two enzymatic inactivation genes were also identified. The tetracycline degradation activity of the expressed enzyme was confirmed *in vitro*. The results demonstrate that the enzymatic inactivation mechanism for tetracycline resistance should not be neglected and merits further investigation to figure out the diverse degradation mechanisms. Besides, it is necessary and urgent to monitor their spread in clinical settings.

AUTHOR CONTRIBUTIONS

SW, XG, and ZF designed research; SW, XG, YG, YL, MC, ZX, and LZ performed research; SW, XG, and ZF analyzed data; WS and ZF wrote the paper.

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REVIEW
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A Review on the Applications of Next Generation Sequencing Technologies as Applied to Food-Related Microbiome Studies

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The development of next generation sequencing (NGS) techniques has enabled researchers to study and understand the world of microorganisms from broader and deeper perspectives. The contemporary advances in DNA sequencing technologies have not only enabled finer characterization of bacterial genomes but also provided deeper taxonomic identification of complex microbiomes which in its genomic essence is the combined genetic material of the microorganisms inhabiting an environment, whether the environment be a particular body econiche (e.g., human intestinal contents) or a food manufacturing facility econiche (e.g., floor drain). To date, 16S rDNA sequencing, metagenomics and metatranscriptomics are the three basic sequencing strategies used in the taxonomic identification and characterization of food-related microbiomes. These sequencing strategies have used different NGS platforms for DNA and RNA sequence identification. Traditionally, 16S rDNA sequencing has played a key role in understanding the taxonomic composition of a food-related microbiome. Recently, metagenomic approaches have resulted in improved understanding of a microbiome by providing a species-level/strain-level characterization. Further, metatranscriptomic approaches have contributed to the functional characterization of the complex interactions between different microbial communities within a single microbiome. Many studies have highlighted the use of NGS techniques in investigating the microbiome of fermented foods. However, the utilization of NGS techniques in studying the microbiome of non-fermented foods are limited. This review provides a brief overview of the advances in DNA sequencing chemistries as the technology progressed from first, next and third generations and highlights how NGS provided a deeper understanding of food-related microbiomes with special focus on non-fermented foods.

Keywords: next generation sequencing, food microbiome, 16S rDNA, metagenomics, metatranscriptomics

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INTRODUCTION

It is well known that foodborne diseases cause considerable morbidity and mortality in humans particularly in immunocompromised individuals and in young children (Stein et al., 2007; Tauxe et al., 2010). Many foodborne diseases are caused by bacteria, viruses, and parasites (Scallan et al., 2011). Consequently, sporadic infections or outbreaks are reported regularly in many countries.

In addition, increased globalization has resulted in the transmission of foodborne pathogens across international borders, severely impacting trade and food security (Frank et al., 2011b; Bernard et al., 2014). This has led national governments and international bodies to establish elaborate controls to improve food safety. Therefore, there is a burden on different food production systems to provide food safe for consumption.

Food, an indispensable part of everyday life, undergoes many processing steps before reaching the consumer. The total population of all microorganisms (microbiome), play important roles in any food matrix ranging from fermentation, contamination and spoilage. Deep taxonomic understanding of the microorganisms and their communities is required either to enhance desired food processes like fermentation or to mitigate detrimental occurrences like contamination and spoilage. Historically, conventional techniques including the classical Gram stain along with individual biochemical characteristics are used for the isolation, identification and characterization of bacteria from clinical, food or environmental origins. Even though considered as the "gold standard," culture dependent techniques can only detect 0.1% of a complex community, such as that found in the human intestinal microbiota. Therefore, to extend the understanding of an ecological niche, such as food, techniques are needed to identify or characterize microorganisms and predict the functional interactions of different microbiological communities present in the sample. To this end, contemporary advances in multi-omic technologies have enabled microbial community profiling, monitoring population fluctuations in different microbial ecosystems and characterization of different microbial species in food matrices.

The rapid development of nucleic acid sequencing technologies over the past four decades has improved the capacity to characterize the microbiomes of complex matrices associated with food or environmental samples. The ubiquitous nature and specificity of nucleic acids make the molecule an ideal target for bacterial or microbiome characterization. Utilizing significant advancements in sequencing chemistries, DNA sequencing gradually evolved from low throughput DNA fragment sequencing to high throughput next generation (NGS) and third generation sequencing techniques (Loman and Pallen, 2015).

Traditionally, most NGS related food microbiome studies have focussed on fermented foods, such as cheese, kimchi and sausages (Patra et al., 2016; De Filippis et al., 2017). Different studies have not only enabled the characterization of the microbial composition of fermented foods but also identified the changes in microbial structure overtime, along with changes in the gene expression patterns related to different fermentation stages (Bokulich et al., 2012; Jung et al., 2013; Ahn et al., 2014; Lessard et al., 2014; Połka et al., 2014). Compared to fermented foods, studies to identify and characterize the microbiome of non-fermented foods are scarce. This review will present the basic principles of the currently available DNA sequencing techniques and how some of these strategies played a key role in understanding microbiomes associated with food, with special emphasis on non-fermented foods.

WHOLE GENOME SEQUENCING

Scientific advances in whole genome sequencing proceeded through three major technological revolutions: first *generation sequencing* (whole genome shotgun sequencing), *next generation sequencing* (NGS high throughput sequencing) and the *third generation of sequencing* (single molecule long read sequencing) (Loman and Pallen, 2015).

First Generation DNA Sequencing (Whole Genome Shotgun Sequencing)

The first DNA sequencing strategy was the *Sanger Chain Termination Method*. Whole genome shotgun DNA sequencing is a capillary based, semi-automated version of the original Sanger strategy. Here, DNA is randomly fragmented, cloned into high copy number plasmid and transformed into *Escherichia coli*. The cloned region is amplified using flanking PCR primers. Each PCR cycle is stochastically terminated by the incorporation of a fluorescently labeled dideoxyribonucleotide (ddNTP), corresponding to the nucleotide identity at the terminal position. DNA fragments are then separated in a high resolution electrophoretic capillary containing polymer gel, and upon exit from the gel, the fluorescent label is excited by an argon laser and the emission spectrum is captured. Read lengths of approximately 1,000 base pairs were obtained with an accuracy of 99.99%. However, low throughput results together with high operational costs, restricted the application of this method.

NGS High Throughput Sequencing

The advantages of NGS over Sanger sequencing can be summarized as follows (1) *in vitro* construction of the sequencing library; (2) *in vitro* clonal amplification of DNA fragments; (3) array based sequencing enabling DNA fragments to be multiplexed (4) solid phase immobilization of DNA. Based on the different methods used to immobilize DNA on a solid substrate, three technologies were commercialized; (a) high throughput pyrosequencing on beads, (b) sequencing by ligation on beads and **(c)** sequencing by synthesis on a glass substrate.

(a) High throughput pyrosequencing on beads. The first next-generation high throughput sequencer to be made commercially available was the 454 GS20 pyrosequencing platform (Roche) (Margulies, 2006). A DNA molecule is first sheared with enzymatic based digestion or sonication and ligated with oligonucleotide adapters. Each ligated fragment is then attached to a 28-μm bead, PCR amplified in an oil-water emulsion and pyro sequenced (Ronaghi et al., 1996). Amplicon bearing beads are then captured in a picolitre sized well and the immobilized DNA fragments are pyrosequenced. In each pyrosequencing cycle, the addition of an unlabelled nucleotide will result in the enzyme-mediated release of an inorganic pyrophosphate (PPi) molecule that is detected computationally. These iterative pyrogenic cycles generate a DNA sequence with a mean read length of 400 nucleotides. The main disadvantage of this technique is reading through homopolymeric sequences, where on occasion n nucleotides are read as n-1 nucleotides, making this technology prone to

high error rates. *Myxococcus xanthus*, a soil inhabitant, was the first bacterium to be sequenced using this technology (Vos and Velicer, 2006). Subsequently this method was used in a survey of microbial populations from different environments *viz.* underground mine water, marine, fresh water, fish, corals terrestrial animals and mosquitoes (Dinsdale et al., 2008).

(b) Sequencing by ligation on beads. SOLiD technology was based on the Multiplex Polony Sequencing technology (Shendure et al., 2005). Adaptor flanking template DNA fragments were initially attached to 1-μm paramagnetic beads and PCR amplified in an oil-water emulsion. Beads with attached PCR amplicons were immobilized on a solid planar substrate and hybridized with a universal PCR primer complementary to the adaptor. Each sequencing cycle proceeds through the ligation of a fluorescently labeled DNA octamer to the universal primer revealing the positional identity of the nucleotide. Subsequent chemical cleavage leaves a pentamer on the DNA template. Progressive iteration of this process reveals the DNA sequence. Since this platform utilizes a two-base coding system, miscalls are more readily identified resulting in 99.94% accuracy.

(c) Sequencing by synthesis on a glass solid phase surface. The Illumina Genome Analyser (SOLEXA) was described in 2006 and 2008 (Fedurco et al., 2006; Turcatti et al., 2008). The DNA library preparation involves random fragmentation of template DNA and the ligation of oligonucleotide adaptors. The DNA amplification strategy involved is referred to as Bridge PCR (Adessi et al., 2000; Fedurco et al., 2006). Both forward and reverse primers, with complementarity to the adaptor, are attached to a glass surface by a flexible linker. The adaptor flanked DNA fragments are hybridized on to the forward and reverse primers attached to the glass surface. Bridge PCR then amplifies the DNA fragment using formamide based denaturation and *Bst* DNA polymerase, resulting in a "*cluster*" of clonal amplicons. Amplicons produced from a single DNA fragment will cluster in a single physical location on the array. Following cluster generation, the sequencing primer hybridizes to the universal sequence flanking the region of interest. Sequencing then proceeds in cycles with a modified DNA polymerase and four nucleotides. Nucleotides are labeled with a chemically cleavable fluorescent reporter group at the 3′-OH end thereby allowing only a single base incorporation in each cycle. Each cycle extends a single base followed by the chemical cleavage of the fluorescent reporter that will identify the incorporated nucleotide. Advanced chemistry designs have allowed paired end reads of 2 × 300 bp from each DNA fragment. The basic challenges of Illumina technology are signal decay and dephasing caused by incomplete fluorescent label cleavage or terminating moieties. Average raw error rates are of the order 1–1.5%.

Third Generation Sequencing (Single Molecule Long Read Sequencing-SMRT)

A recognized limitation of NGS technologies is the requirement for a PCR amplification step, which introduces a bias in read distribution ultimately affecting the coverage. Third generation sequencing technology was designed to address this limitation. Here single DNA molecules are directly sequenced thereby reducing low error rates by avoiding amplification associated bias, intensity averaging, phasing or synchronization problems.

The first commercially released long read methodology was single-molecule-real-time (SMRT®) technology (Pacific Biosciences) (Eid et al., 2009). In this case the library preparation step constructs a closed circular DNA molecule by ligating an adaptor molecule to both ends of the target DNA molecule to be sequenced. The circular DNA molecule is then loaded into a SMRT® cell containing 150,000 zeptolitre wells. Each well has a single immobilized DNA polymerase at its base. DNA polymerase then binds to the hairpin adaptors on the circular target DNA molecule and initiates replication. Four fluorescently labeled nucleotides are then introduced into the reaction wells. As each base is enzymatically incorporated, a light pulse is produced that identifies the base and analyzed iteratively to generate the DNA sequence (Rhoads and Au, 2015). The main advantage of the SMRT® sequencing is the read length obtained. The original C1 generation sequencer produced a read length of about 1,500 bp. More recent C4 chemistry protocols provided for an average read length of 10-kbp. The typical throughput of a PacBio RS II system is 0.5–1 billion bases per SMRT® cell. However, the platform has significantly higher error rates (approximately 11- to 15%).

HeliScope (Braslavsky et al., 2003) is another example of single DNA molecule sequencing. In that case single DNA molecules are sequenced by synthesis using a highly sensitive fluorescence detection system. A DNA library is prepared by random DNA fragmentation followed by poly A tailing. The poly A tail is then hybridized to surface tethered poly T oligomers. This yields an array of primer annealed single molecule DNA templates. To this primer, DNA polymerase adds a single nucleotide resulting in a template-dependent extension. Each nucleotide has a fluorophore attached and these are introduced one nucleotide at a time. The recorded image is analyzed to identify the nucleotide being incorporated into the growing strand. The cycle is then repeated with a new species of nucleotide.

MinION (Oxford Nanopore Technology) was released in 2014 through the MinION Access Programme (MAP). Here, electrophoresis is used to move the DNA/RNA molecule through a nanopore. This system involves the use of electrolytic solutions and the application of a constant electric field. As the nucleic acid passes through the nanopore, the change in the current pattern and magnitude is measured. During the library preparation step, double stranded DNA is sheared using a Covaris g-TUBE and fragmented DNA is repaired using a PreCR step. Blunt ended DNA molecules are then created using an end repair step before a poly A tail is added to the 3′-OH end. Two adaptors are then added to the DNA, a Y adapter (so called, due to its shape) and a hair pin adaptor. A motor protein unzips the double stranded DNA at the Y adapter and feeds the DNA as a single strand through the nanopore. Base calling is then performed and a read length of a few hundred thousand base pairs is achieved with an accuracy ranging from 65 to 88%. If information from only one strand is used, base calling is 1-dimensional (1D), otherwise

it is 2-dimensional (2D) system (Lu et al., 2016). Due to the small size of the instrument, low cost and the real-time nature of this platform, the MinION platform is attracting interest in the genomics community particularly for pathogen surveillance and diagnostics (Judge et al., 2015; Quick et al., 2015).

SEQUENCING TECHNIQUES APPLIED TO CHARACTERIZE FOOD-RELATED MICROBIOMES

16S rDNA Sequencing

This is one of the most important culture independent methods used for conventional microbiome analysis. Most bacteria contain 16S rDNA gene which is made up of nine hypervariable regions flanked by conserved sequences (Neefs et al., 1993). This offers a unique opportunity to design generic PCR primers to amplify and sequence these hypervariable loci to identify the corresponding bacterial taxonomy of the species associated with the food matrix. Similarly, the 18S rDNA gene can be used to identify fungi. Based on the nucleotide sequence similarity, these sequences are clustered into Operational Taxonomic Units (OTU). OTUs are then compared against databases to identify the microorganisms present in the microbiome. The first attempt to characterize a microbiome utilizing this approach was the identification of microbial population from Sargasso sea picoplankton (Giovannoni et al., 1990).

Traditional Sanger sequencing allows only a smaller proportion of amplicons to be sequenced. This results in less abundant members of the microbiome population being missed, thus compromising the comprehensive description of the microbial community. The subsequent inclusion of NGS platforms in 16S rDNA sequencing increased the capacity for a more thorough identification of the bacterial members of the community by several orders of magnitude and at a much lower cost. Since only a short amplicon was sequenced, much higher coverage per sample was obtained (Claesson et al., 2009). In addition, pyrosequencing allowed individual samples to be indexed and facilitated multiplexing during each instrument run (Hamady et al., 2008). This latter step provided a breakthrough in the way environmental prokaryotes could be analyzed. Since then 16S rDNA sequencing has become one of the most popular techniques to identify the microbiome members associated with food matrices. One of the main advantages of using the 16S rDNA sequencing approach is the availability of many bioinformatic tools designed for sequencing data analysis which are free and easy to operate. Commonly used software to analyze 16S rDNA data from food/environmental samples include QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010), mothur (Schloss et al., 2009), and USEARCH (ultra-fast sequence analysis) (Edgar, 2010).

Due to shorter reads obtained from NGS protocols, especially from Illumina platforms, bacterial classification using 16S rDNA sequencing often cannot be identified beyond the genus level (Claesson et al., 2010). Furthermore, 16S rDNA sequencing was found to underestimate the contribution of Gram negative bacteria when compared to bacterial counts observed using transmission electron microscopy and Gram staining (Hugon et al., 2013). A further challenge to this approach is related to the choice of nine hypervariable regions (V1–V9) contained within the 16S rDNA gene. The selection of the hypervariable region for 16S rDNA sequencing has not been generally dependent on the sample environment but rather on published or in-house designed protocols. Many authors have favored different hypervariable regions, such as V1/V2/V4 (Sundquist et al., 2007), V2/V3/V4 (Liu et al., 2008), V2/V4 (Wang et al., 2007), and V2/V3 (Chakravorty et al., 2007). A systematic survey of the efficiency of different hypervariable regions identified that PCR primer pairs targeting V4/V5 was best to identify the microbiome with reduced amplification bias compared to the standard V3/V4 (Claesson et al., 2010). PCR amplicon fragments as short as 82 bp targeting the 16S rDNA V5 variable region have proved to be of sufficient length for bacterial classification at the phylum level (Lazarevic et al., 2009), and a longer amplicon fragment of 100 bp combined with proper primer design and downstream analysis was capable of displaying the same clustering information as longer 16S rDNA sequence (Liu et al., 2007). Even with longer variable regions, pyrosequencing and higher coverage, the amplification of different polymorphic regions resulted in a bias in assessing the microbiome. Moreover, the focus on one marker gene, neglecting other genomic biomarkers makes this technique unsuitable for isolate-specific identification. Nevertheless, considering the low cost per sample and the requirement of low input template DNA concentrations, 16S rDNA sequencing remains one of the most popular high-throughput sequencing methods.

Metagenomic and Metatranscriptomic Sequencing

Metagenomics refers to the application of high throughput techniques to sequence the entire DNA (or RNA) content found in a sample, independent of its origin. Template DNA contained in a sample of interest is subject to sequencing directly, without any prior marker gene amplification step. Metagenomic data not only provides an in-depth taxonomic identification of the microbiome but can also simultaneously compare the relative abundance of all organisms present in the microbiome. Substantial amounts of sequencing data generated using a metagenomic approach is then queried against databases, such as k-mer (Compeau et al., 2011) and SILVA (Quast et al., 2013) to determine the taxonomic composition of microorganisms within the sample. The main advantage of metagenomic approaches over 16S rDNA sequencing is the ability to characterize bacteria present in the microbiome to their species/strain level. In addition, metagenomics also provides comprehensive information on the entire repertoire of genes, structure and organization of the genomes, microbial community structure and evolutionary relationships present in the sample. Consequently, this approach has significant advantages over the 16S rDNA marker gene approach.

The main challenge of the metagenomic approach is the amount of sequence data generated. This procedure is expensive compared to 16S rDNA-based strategies. Moreover, data analysis

requires high-end bioinformatics requiring a long term financial investment, which is possible only in specialized laboratories. The lack of specially designed reference databases also makes the use of this technology challenging when attempting to extract biological information on a routine basis.

Another concern is that metagenomics approaches cannot distinguish viable microbial populations within a microbiome (Ercolini, 2013). Treatment of such samples with propidium monoazide (PMA) has been demonstrated to be a useful approach to distinguish the viable members of a microbiome (Wagner et al., 2008; Erkus et al., 2016). PMA selectively binds to free DNA present in the matrix and as this compound cannot penetrate a cell with an intact cell membrane, it targets the substrate from dead bacteria and other cells. Treating a food matrix sample with PMA prior to DNA extraction will enable the binding of PMA to the free DNA present in the sample and this step will inhibit subsequent sequencing. DNA from viable cells are unaffected and are thus available to be subsequently sequenced.

Alternatively, RNA can also be used as a template to distinguish the viable population within the microbiome. Sequencing total RNA purified from a sample is the basic principle underpinning metatranscriptomic analysis. Apart from distinguishing the viable members within the population contained in a microbiome, this technique is invaluable for providing a functional characterization of the different bacterial members of the microbiome. In a complex microbiological sample, such as food, different microbiological communities interact with each other, either to degrade, spoil or ferment the organic constituents of the matrix. Sequencing RNA purified from these samples would provide a basic description of how the communities interact with each other.

APPLICATION OF NGS TECHNIQUES IN SELECTED MICROBIOME STUDIES

Determining the optimal sequencing approach useful for the study of different food matrices depends upon the complexity of the sample to be analyzed and the depth of bacterial taxonomic information required. An initial 16S rDNA sequencing based profile would provide a broad overview of the microbial composition within a food sample. Nonetheless, this technique lacks the necessary resolution required to provide species-level/strain-level identification. Further, it will not provide an assessment of the functional capability of these organisms, contained within the sample. Therefore, for in-depth species-level or for strain level identification or detailed functional characterization of the different members in the microbiome, metagenomics and metatranscriptomics would be useful.

Examples of Different Sequencing Approaches Used to Characterize Food Microbiomes

For the purpose of this review, a literature search was performed on the current NCBI PubMed database (https://www.ncbi.nlm.nih.gov/pubmed) using a group of seven phrases as follows: (1) 16S rDNA sequencing food microbiome, (2) 16S rRNA sequencing food microbiome (3) 16S metagenetics, (4) metagenomic food sequencing, (5) metagenomic food, (6) metatranscriptomic food and (7) metatranscriptomic food sequencing.

Between 2011 and June 2017, a total of 126 papers were published describing the characterization of various food microbiomes (**Figure 1A**). Of all the publications using different NGS techniques in the analysis of food microbiome more than half (63%) used 16S rDNA sequencing, suggesting that this strategy was widely applied for the analysis of food/food production related studies (**Figure 1B**). NGS-based methods were more often applied to characterize fermented (63%) type food matrices compared to non-fermented foods (**Figures 1C,D**). Since 2012, metagenomic and metatranscriptomic approaches were more often used for food microbiome characterizations and the numbers of studies using these latter approaches gradually increased (**Figure 1A**). The utility of high throughput sequencing approaches in fermented foods was extensively reviewed previously (Patra et al., 2016; De Filippis et al., 2017). To provide a contrast to these data, this review focuses on the compositional analysis of microbiomes associated with non-fermented foods.

Application of NGS-based Strategies to Raw Materials and Production Environment Surveillance

Hygiene is an essential step in the *farm-to-fork* continuum and all production/processing lines are designed to maintain sanitary standards at all stages of food manufacture. Improvements in the maintenance of sanitary standards have enhanced food safety by reducing the number of pathogenic bacteria colonizing these environments and ultimately cross contaminating the final food product. The quality of the final food product not only depends on the hygiene of the processing environment but also on the quality of the raw materials used. For example, a 16S rDNA sequencing-based approach reported that milk with higher somatic cell counts was associated with a higher abundance of certain bacterial taxa including *Acinetobacter*, *Enterobacteriaceae*, *Corynebacterium*, and *Strepococcus* species (Rodrigues et al., 2017), along with *Thermoanaerobacterium*, a genus identified for the first time in the core microbiome of a milk sample. The presence of spoilage organisms (such as *Acinetobacter*, *Thermoanaerobacterium* among others) along with pathogenic bacteria (including *Enterobacteriaceae*, *Corynebacterium* species, and *Strepococcus*) in the raw material can potentially contaminate the resident microbiome of bulk milk tank in which the raw material is stored representing a threat to the entire downstream production line. The influence of the ecology of processing environments on food quality was shown in a more elaborate study involving bulk milk tankers (Kable et al., 2016). Using a 16S rDNA sequencing-based approach, it was shown that the microbiome of milk containing silos was influenced by that of the bulk milk tankers feeding them, and that distinct bacterial communities were evolved

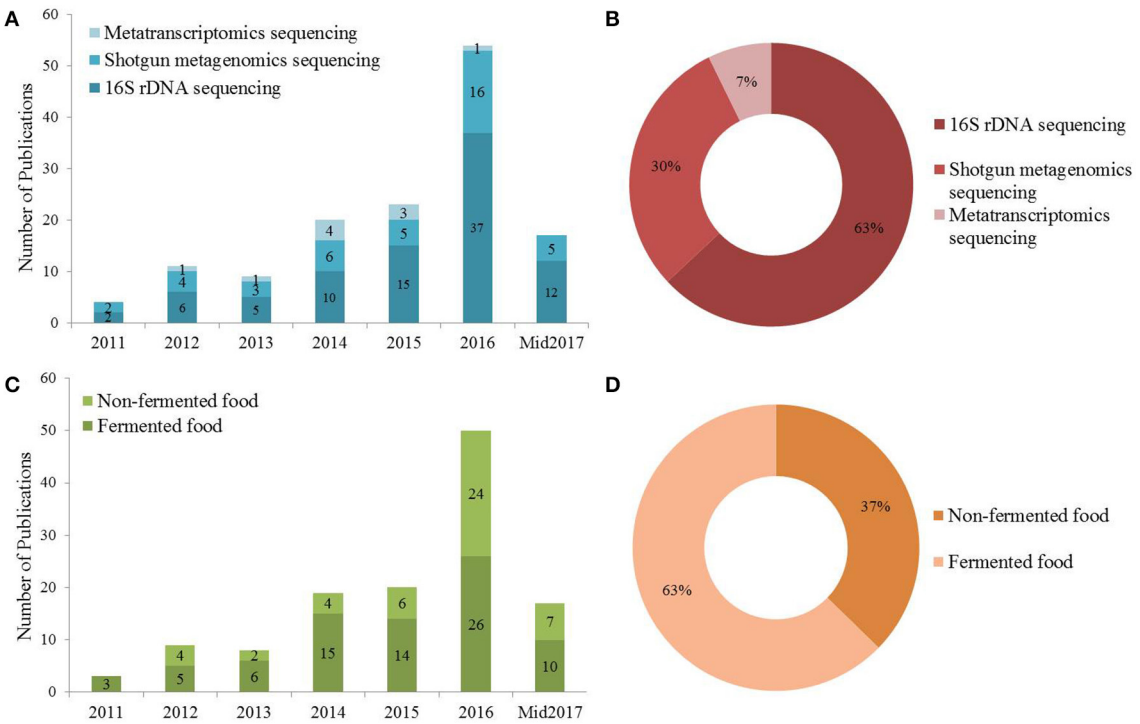

FIGURE 1 | (A) The total number of publications utilizing NGS technology investigating the microbiome associated with fermented and non-fermented food between 2011 and June 2017. **(B)** The proportion of different sequencing strategies used in the publications mentioned in **(A)**. **(C)** The number of publications utilizing NGS strategies to investigate fermented and non-fermented food. **(D)** The percentage of publications utilizing NGS approaches in investigating fermented and non-fermented foods.

within different milk silos. Furthermore, the study showed that the composition of the milk microbiome varied seasonally, as higher bacterial diversity was associated with the spring season. These observations, however, were not limited to dairy facilities. In cheese production sites, the *in house* environmental flora dominated the cheese microbiome (Bokulich and Mills, 2013). This demonstrated that the presence of spoilage microorganisms in the immediate environment of the processing line increase the chances of subsequent spoilage of the final product. Identification of spoilage bacteria, such as *Pseudomonas, Acinetobacter* and *Psychrobacter* species were found along with the core microbiota, indicating the potential of these bacteria to contaminate the processing line (Stellato et al., 2015). Clearly, the hygienic status of the processing facility is a crucial factor that can influence the microbiome of the food matrix. A comprehensive list of publications utilizing NGS approaches in the identification of microbiomes associated with food production chains is given in **Table 1**. In an effort to gain a better understanding of the microbiome of a powder infant formula (PIF) processing facility, 16S rDNA sequencing approach was used to identify the microbiome of the designated low, medium and high care areas (Anvarian et al., 2016). The medium care area had the highest bacterial diversity, possibly due to the moisture associated with these locations. These studies provide a novel insight into the dynamics of the microbiome and demonstrate how storage, processing or temporal fluctuations in the production environment may influence the microbiological quality and safety of food products.

Monitoring the Surface Microbiome of Ready-To-Eat Food Product(s)

Vegetables are known to be vehicles of pathogenic microorganisms and in several cases and have led to outbreaks of foodborne illness (Buchholz et al., 2011; Frank et al., 2011a). Farm waste water used for irrigation contains high numbers of coliform bacteria and is one of the factors responsible for the surface contamination of vegetables (Van Dyk et al., 2016). Phyllosphere, the part of plant surface located above ground, is colonized by several microorganisms (Lindow and Brandl, 2003). Recent studies using NGS-based methods have identified how the phyllosphere microbiome can be affected by season, irrigation, soil type and other parameters. The most direct correlation between the composition of a plant microbiome with temporal fluctuations was shown in the case of the Romaine lettuce phyllosphere microbiome (Williams et al., 2013). Firmicutes dominated the microbiome in June whilst proteobacteria dominated in the period August-October. In addition to temporal fluctuations, different plant irrigation methods also influence the compositions of plant microbiome. For example, in a study conducted on tomato plants, when irrigated with ground water, the microbiome contained mainly Proteobacteria

TABLE 1 | A comprehensive list of publications using next generations sequencing approaches to study the environmental microbiome along the food production chain.

Research target	Country	Methods	Sequencing platform	Conclusions	References
Artisan cheese factory and cheese samples	United States	16S rDNA sequencing (V4); qPCR	Illumina MiSeq	Facility-specific "house" microbiota play an important role in shaping site-specific characteristics in products	Bokulich and Mills, 2013
Wine factory equipment surface	United States	16S rDNA sequencing (V4)	Illumina MiSeq	Winery surface microbiomes have no obvious link with spoilage microbes in wine under normal operating conditions	Bokulich et al., 2013
Carcass, processing environment and beefsteaks	Italy	16S rDNA sequencing (V1-V3)	Roche 454 GS Junior	4°C aerobic storage led to dramatic decrease in beef microbial complexity; spoilage-associated bacteria originated from carcasses and carried through the production chain to the products	De Filippis et al., 2013
Brewery plant environment and beer product	United States	16S rDNA sequencing (V4, for bacteria); Fungal internal transcribed spacer (1 loci, for fungi); T-RFLP; Droplet digital PCR)	Illumina MiSeq	Most microbes found in the brewery environment originated from raw ingredients; beer-spoilage and hop-resistance genes were found throughout the brewery, but little beer spoilage occurred	Bokulich et al., 2015
Sausage processing environment and product	Finland	16S rDNA sequencing (V1-V3)	Roche 454 Titanium FLX	Abundant mesophilic psychrotrophs were prevalent throughout sausage production chain microbiomes, and with different characteristic patterns of contamination for different genera	Hultman et al., 2015
Ready-to-eat meal plant environment and product	Not mentioned	16S rDNA sequencing (V1-V3)	Roche 454 GS Junior	*L. gelidum* was identified to be dominant in ready-to-eat meal samples at the end of shelf-life, its spoilage characteristic and ability of growing under cold storage should raise industries' concern	Pothakos et al., 2015
Cheese factory environment and cheese product	Italy	16S rDNA sequencing (V1-V3, for bacteria); 26S rDNA sequencing (D1-D2, for fungi)	Roche 454 GS Junior	Coexistence of lactic acid bacteria and possible spoilage-associated bacteria was found in core microbiota of cheese factory environment and cheese samples	Stellato et al., 2015
Powdered Infant Formula plant environment	Ireland	16S rDNA sequencing (V3-V4); Flow cytometry	Illumina MiSeq	Bacteria present in low, medium and high care area of a powdered infant formula plant environment were mostly associated with soil, water, and humans, respectively	Anvarian et al., 2016
Environment samples alone beef production chain	United States	Shotgun metagenomics sequencing	Illumina HiSeq 2000	No antimicrobial resistant determinants (ARD) were identified in final beef products, indicating slaughter interventions may reduce ARD transmission risk	Noyes et al., 2016
Dairy farm agroecosystems	United States	Shotgun metagenomics sequencing	Ion Torrent Personal Genome Machine	The most abundant antimicrobial resistant genes in dairy agroecosystems were grouped under multidrug transporters	Pitta et al., 2016
Butchery meat and environment samples	Italy	16S rDNA sequencing (V1-V3)	Roche 454 GS Junior platform	The type of retail (large- or small-scale distribution) had no apparent effect on initial fresh meat contamination	Stellato et al., 2016
Environment samples along beef production chain	United States	Shotgun metagenomics sequencing	Illumina HiSeq 2000	Usage of standard antimicrobial interventions in beef processing system significantly reduced the diversity of remaining microbiomes	Yang et al., 2016

while surface water gave rise to the plant microbiome containing Firmicutes, Actinobacteria, and Verrocomicrobia (Telias et al., 2011).

Soil is another factor that influences vegetable and fruit microbiomes. A study using 16S rDNA sequencing reported that the microbiomes of leaves, flowers and fruits shared a greater proportion of taxa with the soil microbiome in which the plants were grown (Zarraonaindia et al., 2015). Therefore, irrigation water containing pathogenic microorganisms may contaminate the soil resulting in cross-contaminating vegetables or fruits (Van Dyk et al., 2016), which led to outbreaks (Buchholz et al., 2011). Each plant product may also harbor bacterial communities that are unique (Leff and Fierer, 2013). Sprouts, spinach, lettuce, tomato, pepper and strawberries exhibited a high abundance of Enterobacteriaceae, whilst other fruits like apples, peaches, grapes and mushrooms were found to be dominated by Actinobacteria, Bacteriodetes, Firmicutes and Proteobacteria. Since all these food product types are consumed with minimal processing, individuals can be exposed to these bacterial phyla. A comprehensive list of publications utilizing NGS approaches in the identification of microbiomes associated with raw and ready to eat food products is given in **Tables 2**, **3**, respectively.

All the above studies provide insights into the role that NGS-based strategies played in uncovering the dynamic changes in the microbiome. Careful analysis of NGS data can be used to facilitate

TABLE 2 | A comprehensive list of publications using next generations sequencing approaches used in characterizing the microbiome of raw food products.

Research target	Country	Methods	Sequencing platform	Conclusions	References
Broiler filet strips	Finland	16S rDNA sequencing (V1-V3); T-RFLP	Roche 454 GS FLX	Marination process led to increased lactic acid bacteria in broiler meat microbiome, resulting in enhanced CO_2 production and acidification	Nieminen et al., 2012b
Broiler filet strips	Finland	16S rDNA sequencing (V1-V3); Shotgun metagenomics sequencing	Roche 454 GS FLX; Roche 454 GS FLX;	Marination altered broiler fillet strips' microbial community by favoring the spoilage associated bacteria *L. gasicomitatum*	Nieminen et al., 2012a
Spoiled retail foodstuffs	Belgium	16S rDNA sequencing (V1-V3)	Roche 454 GS Junior	Characterization of psychrotrophic lactic acid bacteria that cause unexpected food spoilage cases in Belgian retail food	Pothakos et al., 2014
Store bought meat	United States	Shotgun metagenomics sequencing	Illumina Miseq	Primary characterization of viruses commonly found in US store-bought meats	Zhang et al., 2014
Beef burger	Italy	16S rRNA sequencing (V1-V3); PCR-DGGE	Roche 454 GS Junior	Nisin-based antimicrobial packaging reduced the abundance of microbes that produce compounds of specific metabolic pathways related to spoilage	Ferrocino et al., 2016
Raw pork sausage	France	16S rDNA sequencing (V1-V3); qPCR	Roche 454 GS FLX++ Titanium	Salt reduction, particularly when combined with CO_2-enriched packaging, resulted in faster spoilage of raw sausages by lowering the overall bacterial diversity	Fougy et al., 2016
Raw milk	Finland	16S rDNA sequencing (V1-V2);	Illumina MiSeq	Bacterial diversity is better preserved in bovine raw milk by additional flushing with N_2 gas compared to cold storage at 6°C alone	Gschwendtner et al., 2016
Raw milk	United States	16S rDNA sequencing (V4); qPCR	Illumina MiSeq	raw milk microbial community structure can be influenced during low-temperature, short-term storage	Kable et al., 2016
porcine musculature	Austria	16S rDNA sequencing (V1-V2); qPCR	Roche 454 GS-FLX Titanium	Pork sample microbiota was dominated by psychrophilic spoilers; *E. coli* was present in all pork samples and can be used as marker species in pork contamination cases	Mann et al., 2016
Raw milk	Australia	16S rDNA sequencing (V5-V8)	Roche 454	Spoilage bacteria growth was delayed by at least 7 days in CO_2 treated raw milk sample	Lo et al., 2016
Bulk tank milk	United States	16S rDNA sequencing (V4); qPCR; Flow cytometry	Illumina MiSeq	Spoilage and spore-forming bacteria were ubiquitous in all dairy farms	Rodrigues et al., 2017
Common carp filets	China	16S rDNA sequencing (V3-V4)	Illumina HiSeq 2500	Use of cinnamon essential oil extended vacuum-packaged common carp fillets shelf-life by approximately 2 days based on sensory and other analysis, but showed no significant differences in dominant microbiota composition compared with non-treated samples at the end of shelf-life	Zhang et al., 2016

the development of safer production processes thereby reducing risk for the consumer.

Monitoring Microbiomes Associated with Food Storage Conditions

Control measures like refrigeration, modified atmospheric packaging (MAP), nisin treatment and others are often used to extend the shelf life of many perishable food products. The microbiome undergoes considerable compositional change when food products are stored under defined conditions. It is possible that these fluctuations in the microbiome may finally affect the quality of the food product. NGS techniques have been increasingly applied to study how these variations contribute to improved shelf life. The most common response noted in refrigerated food products was a reduction in bacterial diversity associated with the microbiome. A 16S rDNA sequencing approach noted this reduction in bacterial diversity in refrigerated spinach (Lopez-Velasco et al., 2011). Long-term storage of spinach at low temperatures selected for *Pseudomonas* species and *Enterobacteriaceae* family members. Similarly in beef steaks, reduction in bacterial diversity correlated with the increase in the abundance of spoilage agents like *Pseudomonas* species and *Brochothrix thermosphacta* (De Filippis et al., 2013). These bacterial species contaminated the raw meat during animal slaughter and proliferated during refrigerated storage. Since bacterial diversity is reduced during refrigeration, the competition for nutrients is similarly reduced. This development provides an opportunity for spoilage organisms to overgrow resulting in deterioration of the food product following long-term refrigerated storage.

Marination is another traditional treatment method frequently used during food production process. It is a process of soaking foods in a seasoned, often acidic, liquid before cooking. The derivation of the word refers to the use of brine or a water solution containing a significant amount of salt. The most common examples are used for curing, preserving, and developing flavor in foods, such as that used in the pickling process. NGS-based approaches have been used to investigate the microbiome diversity and structure

TABLE 3 | A comprehensive list of publications using next generations sequencing approaches in characterizing the microbiome of ready to eat food.

Research target	Country	Methods	Sequencing platform	Conclusions	References
Bagged leaf vegetables	United States	16s rDNA sequencing;	Roche 454 GS-FLX Titanium	No significant differences found on microbial compositions between organic and conventionally grown, surface-sterilized and non-sterilized leaf vegetables	Jackson et al., 2013
Store-bought fruits and vegetables	United States	16S rDNA sequencing	Roche 454	Microbial communities of certain type product are more similar than different types, but Significant difference identified between conventional and organic product within the same type	Leff and Fierer, 2013
Field grown lettuce	United States	16S rDNA sequencing (V5-V9); qPCR	Roche 454 GS-FLX Titanium	Lettuce phyllosphere microbiome are affected by seasonal, irrigation, and biological factors	Williams et al., 2013
Carrots	United Kingdom	Metatranscriptomics qPCR	Illumina MiSeq	Carrot yellow leaf virus are strongly associated with carrot internal necrosis	Adams et al., 2014
Basil leaves	Belgium	16S rRNA sequencing (V1-V3); PCR-DGGE	Roche 454 GS-FLX Titanium	Spoilage of commercially grown basil leaves was caused by tissue injuries and visual defects rather than by specific bacterial growth	Ceuppens et al., 2015
Cilantro	United States	16S rRNA sequencing (V1-V3); Shotgun metagenomics sequencing (with pre-enrichment)	Illumina MiSeq; Illumina MiSeq	A 24 h non-selective enrichment identified *Salmonella* spiked in cilantro	Jarvis et al., 2015
Bagged spinach	United States	Shotgun metagenomics sequencing (with pre-enrichment)	Illumina MiSeq	Eight h pre-enrichment and sequencing depth identified' spiked Shiga toxin-producing *E. coli* as low as 100 CFU/100 g in bagged spinach	Leonard et al., 2015
field-grown and retail lettuce	United States	Shotgun metagenomics sequencing; Metatranscriptomics	Illumina HiSeq 2500; Illumina HiSeq 2500	Virome of iceberg lettuce from fields and produce distribution center were dominated by plant pathogenic viruses but human and animal viruses were also identified	Aw et al., 2016
Oregano	United States	Shotgun metagenomics sequencing (with pre-enrichment)	Illumina MiSeq	Addition of corn oil during pre-enrichment of oregano samples led to increased overall abundance of Gram negative microorganism and a ≥50% recovery rate of *Salmonella*	Beaubrun et al., 2016
Bagged spinach	United States	Shotgun metagenomics sequencing (with pre-enrichment)	Illumina MiSeq	Shotgun metagenomics sequencing identified Shiga toxin-producing *Escherichia coli* (STEC) spiked into fresh bagged spinach	Leonard et al., 2016
Cheese	Ireland	16S rDNA sequencing (V4-V5); Shotgun metagenomics sequencing; qPCR	Roche 454 GS-FLX; Illumina HiSeq 2000	Carotenoid-producing bacteria, genus *Thermus*, is linked with pink discoloration defect in cheese	Quigley et al., 2016

alterations during marination, and to determine whether, such process can extend product shelf life. Sometimes contrary to its intended use, marination increased the speed of spoilage. In a typical example, the poultry product was rapidly spoiled when marinated with acetic acid and subsequently packaged in a modified atmospheric environment. Investigation on the bacterial composition contributing to spoilage using 16S rDNA sequencing indicated a heterofermentative lactic acid bacteria *Leuconostoc gasicomitatum* being detected as the spoilage organism. It was also noted that the process of marinating meat diminished the proportion of *Brochothrix thermosphacta, Clostridium* species, and *Enterobacteriaceae* in the sample, while increasing the proportion of spoilage associated *Leuconostoc gasicomitatum* (Nieminen et al., 2012a) which contributed to faster spoilage. The same study used metagenomics to identify *Vagococcus* species and *Vibrio* species both of which are predominating in un-marinated meat, a hitherto unobserved phenomenon.

Extended shelf life can also be achieved through the addition of the bacteriocin nisin, a polycyclic antibacterial peptide secreted by *Lactococcus lactis* which is applied to suppress the growth of spoilage and pathogenic microorganisms. 16S rDNA analysis confirmed that nisin treatment showed remarkable reduction in nisin susceptible taxa including *Kocuria rhizophila, Staphylococcus xylosus, Leuconostoc carnosum*, and *Carnobaterium divergens* (Ferrocino et al., 2016). The extension in shelf life could also be associated with the suppression of bacteria including *C. divergens*, a spoilage bacterium associated with refrigerated fish and meat products (Borch et al., 1996).

Addition of NaCl to meat is known to improve texture, flavor and taste whilst also improving shelf life by reducing water activity. In one study using high salt concentration along with low temperature and CO_2 enriched packing to improve the shelf life of sausage meat, the reduction of salt concentration was not surprisingly associated with faster spoilage in products. The 16S rDNA sequencing-based investigation revealed that

reduction in salt concentration led to an overall reduction in bacterial diversity which caused faster spoilage. Improvements in sausage meat processing with higher salt concentrations combined with vacuum packaging increased the abundance of a subpopulation consisting of *Enterobacteriaceae*, *Enterococcaceae* and *Leuconostocaceae* families, which also helped with delayed spoilage (Fougy et al., 2016).

The selected studies cited above demonstrate the potential of NGS approaches and facilitated a better understanding of bacterial food spoilage. These sequencing based investigations not only identified the spoilage agent in many cases but also showed how different bacterial communities interacted with each other to counteract spoilage.

The Status of Metagenomic and Metatranscriptomic Approaches in Understanding Food Microbiome

Metagenomics/metatranscriptomics is also a valuable tool to understand how bacterial communities interact with each other in fermented foods. The first metagenomic study used a 454 GS FLX titanium platform to describe the microbiome of a ferment food, kimchi, with the predominant microbial population identified as *Leuconostoc mesenteriodes* and *Lactobacillus sakei* (Jung et al., 2011). Interestingly, a substantial proportion of bacteriophage DNA was also detected. Bacteriophage DNA were further identified in large proportions during the fermentation of shrimp, kimchi, sourdough and sauerkraut (Park et al., 2011), suggesting an important role for phages in the fermentative process. Even though the analysis of the *Lactobacillus sanfranciscensis* genome suggested phages could affect fermentation (Vogel et al., 2011), no role could be assigned for them during the fermentation of sourdough (Foschino et al., 2005). This simultaneous detection of phage- and bacterial-DNA would not have been possible by conventional 16S rDNA sequencing approaches, and was one of the main advantages of metagenomics over the former.

Metatranscriptomics also played a pivotal role in understanding the process of fermentation, especially the ripening of cheese. Camembert-type cheese ripening is driven by fungal microflora including *Geotrichum candidum* and *Penicillium camemberti*. Functional gene expression studies using metatranscriptomics identified that genes associated with metabolic pathways/cell growth/stress responses were differentially expressed during cheese ripening and that changes in expression patterns occurred over the first 2 weeks of the ripening period (Lessard et al., 2014). Metagenomics identified that genes responsible for amino acid catabolism were involved in flavor production. A combined effort utilizing metagenomics, metatranscriptomics and biochemical analysis aided the understanding of the process involved in surface ripened cheese (Dugat-Bony et al., 2015). In that case, metatranscriptomic data facilitated the characterization of the interactions between the dominant microbial species like *Lactococcus lactis*, *Debaryomyces hansenii*, *Geotrichum candidum*, *Kluyveromyces lactis*, and *Corynebacterium casei* during degradation of the dairy matrix. *Lactococcus lactis* produced excessive amounts of lactate from lactose and *Debaryomyces hansenii*/*Geotrichum candidum* up-regulated their lactate dehydrogenase genes, showing that lactate was metabolized by the latter. Genes associated with amino acid catabolism were also involved in the flavor production studies of Reblochon-type cheese, identifying the differences in amino acid catabolism between two yeast species, *Debaryomyces hansenii* and *Geotrichum candidum*, during cheese fermentation. The former took over 30 days to add its flavor constituents while the latter organism, added its flavor constituents during the first phase of ripening. An increase in ripening temperature promoted the expression of many genes including those involved in proteolysis, lipolysis and amino acid catabolism, consistent with the metabolomics profile and volatile organic compounds detected in cheese (De Filippis et al., 2016).

All these insights above showed that metagenomic/metatranscriptomic analysis provided an in-depth analysis of how microbial communities interacted with each other during fermentation. However, reports on the usage of these techniques in non-fermentative foods are rare, and metagenomics/metatranscriptomics approaches have not been utilized to their full potential in investigating food microbiomes of non-fermentative foods. Considering the limitations of 16S rDNA sequencing approaches, metagenomics/metatranscriptomics could be increasingly used in future to extend understanding of the functional microbiome of non-fermented food.

The Utility of NGS Approaches in Identification and Characterization of Specific Bacteria from a Microbiome

Many bacteria are non-culturable, either because they are unknown or they are known but not recoverable in the laboratory conditions. Genomic approaches have played a major role in understanding such non-culturable bacteria, and in some cases, have led to development of new media that can be subsequently used for their cultivation. A classic example is the case of *Tropheryma whipplei*, an organism that can only be grown within a human fibroblast cell line. Genome data from this organism was used to identify specific metabolic deficiencies, providing support to design an axenic growth medium which was in turn used to culture this bacterium (Renesto et al., 2003). A similar example is pink discoloration of cheese. Pink discoloration is a defect affecting cheese leading to significant loss of revenue to the dairy industry. Despite decades of research, the cause of pink discoloration remained elusive. Combining 16S rDNA shot gun metagenomics and quantitative PCR (qPCR), the spoilage bacterium associated with pink discolored cheese was identified as *Thermus thermophilus* (Quigley et al., 2016). The carotenoids produced by the bacterium were responsible for pink discoloration. Subsequent, successful culture of the bacterium from pink colored cheese confirmed the diagnosis.

NGS approaches can be utilized in the identification and characterization of specific pathogenic or spoilage bacteria from food or food production microbiomes. Some examples include the identification of *Leuconostoc gasicomitatum* as the spoilage agent in marinated poultry (Nieminen et al.,

2012a), along with *Pseudomonas* species and *Brochothrix thermosphacta* in refrigerated beefsteaks (De Filippis et al., 2013). Unfortunately, 16S rDNA sequencing cannot provide any further characterization of the organism other than identification. Since whole genomes are sequenced, data from metagenomic approaches can be used to characterize any specific microorganism within a microbiome. This is particularly helpful when the organism is non-culturable. One of the earliest examples, using metagenomic approaches, was the construction of the complete genome of an non-culturable α-propteobacteria *Candidatus Liberibacter asiaticus*, the causative agent of Citrus huanglongbing (Duan et al., 2009). Genes associated with motility, transport and virulence were identified in this genome. Similar metagenomics approaches were used in another study to confirm that carrot yellow leaf virus was responsible for carrot internal necrosis (Adams et al., 2014). The most recent example is the determination of the draft genome of *Listeria monocytogenes* from an ice-cream microbiome using metagenomics (Ottesen et al., 2016).

Since the sequence data obtained from a metagenomics approach are detailed, these data could be used to characterize organisms other than bacteria in the microbiome. Metagenomics approaches were used to study the virome associated with store-derived beef, pork and chicken identified a novel bovine polyomavirus in beef and a novel gyrovirus species in chicken (Zhang et al., 2014). These reports highlight the utility of NGS approaches in the deep taxonomic characterization of the microbiome.

Monitoring Environmental Spread of Antimicrobial Resistance Genes

Dissemination of antibiotic resistance in human pathogens is a matter of global concern. Antibiotics are used in food-producing animals and aquaculture as therapeutic agents, for prophylactics and in some jurisdictions as growth promoters (Phillips, 2004). Although the growth promoting effects of antibiotics on livestock was known for years, there were no experimental data substantiating this claim. NGS played an important role in proving the growth promoting effect of penicillin in broiler chickens (Singh et al., 2013). 16S rDNA sequencing demonstrated an increased proportion of phylum Firmicutes and decreased proportion of phylum Bacteriodetes in the gut microbiota of chicken post penicillin treatment. A similar shift in gut microbiota was noted in obese- compared to lean-individuals, suggesting that penicillin treatment had an indirect growth promotion effect. Metagenomics based assays identified the origin and the distribution pattern of antibiotic resistance encoding genes in a wide variety of samples including animal feaces, manure and soil (Pitta et al., 2016). However, the overuse of antibiotics for growth promotion or prophylaxis has contributed to an increased dissemination of antibiotic resistance genes among bacteria, including major human pathogens leading to widespread antibiotic resistance (Casewell et al., 2003). Collections of antibiotic resistant genes, often referred to as the resistome, were uncovered in reservoirs, such as human, animals, food and the environment (Rolain, 2013). Genes in resistomes are mobilizable and can therefore be transferred to human pathogens, generating drug resistant strains. NGS-based techniques played an important role in demonstrating the transferable nature of resistome-related genes. Initially a study using high capacity qPCR observed a co-enrichment of antibiotic resistance genes and transposons demonstrating the mobility of antibiotic resistant genes (Zhu et al., 2013). A resistome comprising genes active against 18 antimicrobial classes was observed across all samples. Metagenomics was also used to study the effect of antibiotic treatment on swine microbiota and demonstrated that there was a shift in the bacterial phylotypes driven by an increase in *Escherichia coli* populations 14 days post treatment (Looft et al., 2012). Along with an increase in the abundance of antibiotic resistance-encoding genes, the up-regulation of genes encoding aminoglycoside O-phosphotransferases showed that antibiotic treatment of farm animals promoted cross-resistance.

CONSIDERATIONS TO IMPROVE THE CURRENT NGS BASED FOOD MICROBIOME STUDIES

NGS methods are highly efficient for microbiome related studies but there are still challenges and limitations to consider when applying these techniques to specific cases on food and food-related environments.

Quality and Quantity of Recovered Nucleic Acid

Natural environment samples, such as soil and water, along with stool and saliva, and fermented food samples, such as cheese and kimchi, contain high numbers of microorganisms. Consequently, these samples can offer sufficient template nucleic acid for subsequent analysis. In contrast, sanitary control and maintenance of strict food production environment standards have made isolation of total DNA from these environments challenging (Anvarian et al., 2016). Frequent exposure to detergents from routine cleaning can cause injury to microorganisms colonizing this niche, thereby compromising the ability to recover sufficient template nucleic acid for analysis. Moreover, metal ions (Bickley et al., 1996), lipids and proteins (Rossen et al., 1992), detergent residues (Schrader et al., 2012), are all likely to inhibit nucleic acid isolation (Ahn et al., 2014). Further these are likely to inhibit downstream experimental procedures, leading to the recovery of low quality nucleic acid or PCR reaction failure. These inhibitors can be difficult to remove and this problem is compounded especially when samples contain a low bacterial load. Since an amplification step is involved, 16S rDNA approach is well suited to analyze the microbiome from such samples (Anvarian et al., 2016).

Different enrichment methods can be carried out to increase nucleic acid concentrations in samples. Prior to nucleic acid purification, selective or non-selective cultivation based pre-enrichment or enrichment can be used to generate microbiomes with higher target bacterial counts (Duan et al., 2009; Leonard

et al., 2015). After nucleic acid purification and before downstream sequencing, whole genome amplification can be carried out to linearly amplify genomic material (Duan et al., 2009). However, these approaches may lead to amplification bias giving rise to changes in the microbiome composition (Kim and Bae, 2011). Nonetheless, it provides genomic DNA in sufficient quantity for subsequent sequencing. Total RNA is also able to be amplified using a similar whole transcriptome amplification strategy (Wu et al., 2011).

Utilization of Adequate Controls during Sequencing

Internal controls are essential in multi-omic strategies. Often these are never considered. A sequencing control should be designed to contain DNA sequences from known bacterial species, processed in parallel with other samples during sequencing, which could finally provide an estimation of the sequencing errors during downstream bioinformatics analysis. An ideal positive control could contain DNA isolated from mixture of multiple reference strains constituting a *mock community* either from NCBI (National Center for Biotechnology Information) and/or NCTC (National Collection of Type Cultures) collections (Kozich et al., 2013; Schloss et al., 2015). Strains closely related to the target of interest, such as lactic acid bacteria strains for fermented food microbiome studies (Humblot and Guyot, 2009), or a single strain as in *Staphylococcus aureus* DNA as in a gut microbiome study (Bull-Otterson et al., 2013) should be used. Ideally, a positive control should be applied in all 16S rDNA sequencing studies due to error associated with sequencing accuracy.

As with the positive, a negative control is also relevant in sequencing runs. Being high throughput, conventional amplicon based or whole genome sequencing experiments can analyze DNA at nanomolar concentrations. Trace levels of contamination present in the reagents used during DNA isolation or library preparation can introduce an undesirable bias in the analysis. This may interfere with final sequencing results giving rise to background contamination noise, especially when the target contains very few microorganisms (Salter et al., 2014). Water as template for PCR amplification is generally included as negative control in NGS experiments, while the usage of a no template control during 16S rDNA PCR is also common (Park et al., 2012). Currently few food related 16S rDNA sequencing studies take negative controls into consideration (Claesson et al., 2010; Wu et al., 2011; Park et al., 2012; Bull-Otterson et al., 2013; Burns et al., 2015; Burke and Darling, 2016), and most of these use negative controls applied only in during the PCR step (Salter et al., 2014).

CONCLUSIONS AND FUTURE PERSPECTIVES

The utility of culture dependent approaches in the study of bacterial diversity is inherently limited in sensitivity. Nucleic acid sequencing platforms are being increasingly used to characterize bacteria and to identify bacterial communities from complex environmental matrices. With respect to food, popular sequencing technologies used to identify microbial communities include 16S rDNA sequencing, metagenomics and metatranscriptomics. 16S rDNA sequencing is currently the most commonly applied technique, while metagenomics and metatranscriptomics approaches are still underutilized to date. The latter approaches provide deep insights into the compositional and functional characteristics of a fermented food microbiome. Compared to fermented food, these technologies are rarely used to characterize the non-fermented food microbiome. In addition to discussing different sequencing chemistries currently available, this review has provided an overview of non-fermented food-related microbiome studies based on these next generation approaches. Overall, the potential benefits of multi-omic approaches in improving food safety was emphasized.

Even though, most metagenomic approaches use NGS platforms to sequence DNA, third generation sequencing (TGS) strategies are not routinely used for microbiome studies. Recently, a third generation sequencing MinION platform was used to sequence full length 16S rDNA amplicons generated from a synthetic community of ten microbial species with varying relative abundance (Li et al., 2016). The MinION based whole 16S rDNA amplicon profiling identified species correctly and the abundance profiles correlated with defined abundances. The latter technique offers some improvements in terms of the depth of data obtained when compared to the current 16S rDNA strategies by allowing species level characterization of the microbes. Soon it can be expected that TGS platforms will gain wider applications related to food production. In time, these data can be carefully analyzed to design new techniques to enhance process efficiency and product quality.

AUTHOR CONTRIBUTIONS

YC, SF, and SS organized the draft and wrote the manuscript. YC, SF, SP, KJ, and SS participated in the critical revision of the manuscript. All authors read and approved the manuscript.

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The Role of Ribonucleases and sRNAs in the Virulence of Foodborne Pathogens

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Contaminated food is the source of many severe infections in humans. Recent advances in food science have discovered new foodborne pathogens and progressed in characterizing their biology, life cycle, and infection processes. All this knowledge has been contributing to prevent food contamination, and to develop new therapeutics to treat the infections caused by these pathogens. RNA metabolism is a crucial biological process and has an enormous potential to offer new strategies to fight foodborne pathogens. In this review, we will summarize what is known about the role of bacterial ribonucleases and sRNAs in the virulence of several foodborne pathogens and how can we use that knowledge to prevent infection.

Keywords: RNA metabolism, foodborne pathogens, control of gene expression, RNase E, RNase III, PNPase, RNase R, YbeY

INTRODUCTION

Globalization has important implications for food safety considering the exchanging of food around the world. Contaminated food represents a risk for human health and has an enormous negative impact in economy. Scientists are making great progresses in securing food by studying the biology, life cycles, and infection processes of foodborne pathogens.

RNA metabolism has recently been exploited for the development of new antimicrobials. Ribonucleases (RNases) are crucial enzymes for the cell, since they degrade RNA and are involved in the processing and quality control of transcripts (Arraiano et al., 2010). RNases can be divided into endoribonucleases, which cleave RNA internally, and exoribonucleases, which degrade the RNA by removing terminal nucleotides from the extremities. In the last years, the discovery of small non-coding RNAs (sRNAs) unraveled a new world of post-transcriptional regulatory networks, which cooperate with RNases in the control of gene expression. With the development of new technologies, many sRNA molecules were identified and shown to be important players in bacterial virulence.

The aim of this review is to provide a broad look to what is known about the role of bacterial ribonucleases and sRNAs in virulence of several foodborne pathogens and discuss their potential for the development of new therapeutic applications.

EXORIBONUCLEASES

Polynucleotide Phosphorylase (PNPase)

Polynucleotide phosphorylase (PNPase) is a phosphorolytic exoribonuclease that degrades RNA molecules from the 3′ end. However, when inorganic phosphate concentration is low, PNPase is also able to add heteropolymeric tails to the 3′ end of the RNAs. It is also involved in the exchange reaction between free phosphate and the β-phosphate of ribonucleoside diphosphates (Grunberg-Manago et al., 1955; Grunberg-Manago, 1963; Littauer and Soreq, 1982).

Polynucleotide phosphorylase is widely conserved and is not essential for survival at optimal temperatures (Donovan and Kushner, 1986). However, PNPase has been reported to be essential for adaptation and growth at low temperatures in *Escherichia coli*, *Yersinia*, *Salmonella, Staphylococcus aureus,* and *Campylobacter jejuni* (Goverde et al., 1998; Beran and Simons, 2001; Clements et al., 2002; Rosenzweig et al., 2005, 2007; Haddad et al., 2009; Bonnin and Bouloc, 2015). Refrigeration is typically used for the control of bacterial growth in food. The ability of foodborne pathogens to persist and grow at lower temperatures is a major problem for food safety and public health. The role of PNPase in adaptation to cold could be very important in the development of new strategies for food conservation.

Polynucleotide phosphorylase also seems to be directly or indirectly involved in the expression of different virulence factors in some pathogens. In *Salmonella*, PNPase activity decreases the expression of genes from the pathogenicity islands SPI-1 (containing genes for invasion) and SPI-2 (containing genes for intracellular growth) (Clements et al., 2002). In *Yersinia*, PNPase modulates the type three secretion system (T3SS) by affecting the steady-state levels of T3SS transcripts, controlling the secretion rate. This is probably the reason why PNPase deletion results in a less virulent strain in a mouse model (Rosenzweig et al., 2005, 2007). In *E. coli* O157:H7 the deletion of PNPase decreased its adhesion to epithelial cells and colonic epithelial tissues (Hu and Zhu, 2015). In *C. jejuni* it was shown that PNPase mutants have swimming limitations, colonization delay, decrease of cell adhesion/invasion ability, and reduced virulence (Haddad et al., 2012).

RNase R

RNase R is a hydrolytic enzyme that degrades RNA processively in the 3′–5′ direction. Its activity is modulated according to growth conditions and is induced under several stress conditions, namely in cold shock (Cairrão et al., 2003; Chen and Deutscher, 2005). It has also been shown to be required for virulence in several pathogens.

In *Aeromonas hydrophila*, an emerging foodborne, RNase R was shown to be crucial for cell survival at low temperatures, and it influences the motility and virulence in mice (Erova et al., 2008). In *Shigella flexneri*, the causative agent of dysentery, RNase R is extremely important for the expression of a variety of invasion factors, and decreases expression of virulence factors, epithelial cell invasion, and hemolytic activity (Tobe et al., 1992; Cheng et al., 1998). In *C. jejuni*, RNase R was shown to be active in a wide range of conditions, which could be crucial for the adaptation during the infection process. This exoribonuclease was also shown to be involved in adhesion and invasion of eukaryotic cells (Haddad et al., 2014). In *Helicobacter pylori* this enzyme was shown to promote apoptosis of host cells while enhancing bacterial chemotaxis and motility, features that are required for optimal virulence (Tsao et al., 2009).

ENDORIBONUCLEASES

RNase E

RNase E is essential for cell growth in all organisms where it is present. RNase E cleaves internally single-stranded RNA preferring AU-rich regions (Arraiano et al., 2010). In *Salmonella*, the major role of RNase E is to degrade mRNAs, including essential genes and genes implicated in virulence and intracellular growth (Chao et al., 2017). RNase E was shown to cleave the *mgtC* mRNA important not only for virulence in a murine model but also for survival within macrophages (Lee and Groisman, 2010). Moreover, studies using as infection model *Galleria mellonella* demonstrated that a *Salmonella* RNase E mutant is less virulent, the motility is impaired, and the proliferation inside the host is reduced (Viegas et al., 2013). Additionally, RNase E also contributes for biofilm development and antibiotic susceptibility in *Salmonella* (Saramago et al., 2014b). In *Y. pseudotuberculosis* RNase E was shown to be important for survival within macrophage-like cells and in the regulation of the T3SS (Schiano and Lathem, 2012).

RNase III

RNase III is a hydrolytic enzyme specific for double stranded RNAs (dsRNAs). It was shown to have an important role not only in rRNA and tRNA maturation but also in mRNA decay. Although not essential, in the absence of RNase III cells show defective growth when compared to the wild-type (Arraiano et al., 2010). In *Salmonella*, RNase III has been implicated in the control of important virulence factors (Lee and Groisman, 2010). Using *G. mellonella* as a host model, a mutation in RNase III reduced the *Salmonella* virulence capacity (Viegas et al., 2013). It was also shown that RNase III contributes for biofilm development and antibiotic susceptibility in *Salmonella* (Saramago et al., 2014b).

In *S. aureus* RNase III regulates the expression of factors implicated in cell adhesion and escape to immunity by degrading sRNA/mRNA duplexes (Bonnin and Bouloc, 2015). In *C. jejuni* RNase III was shown to be active in different conditions which can be important during infection. Moreover, it was able to complement the *E. coli* RNase III mutant restoring the 30S rRNA processing and PNPase regulation (Haddad et al., 2013). Considering the important functions that PNPase play in *C. jejuni* virulence (see above), this also confers to RNase III an important role in *C. jejuni* biology.

YbeY

YbeY is a metalloprotein important for rRNA processing, which is also involved in gene silencing (Jacob et al., 2013; Saramago et al., 2017). In *Vibrio cholerae* YbeY is essential and is involved in pathogenesis, as its depletion was shown to have a negative impact in cholera toxin production and a reduced colonization of mouse intestines (Vercruysse et al., 2014). In *Y. enterocolitica*, YbeY also has a high impact in the pathogenicity of this bacterium. In the absence of YbeY several virulence factors are differentially expressed, resulting in a drastic defect in adhesion and a decreased ability of *Y. enterocolitica* to infect eukaryotic cells (Leskinen et al., 2015).

Other Endoribonucleases

In *Listeria monocytogenes*, the inactivation of the putative RNase HII, called Imo1273, caused a strong attenuation of virulence in a mouse model. Interestingly, overexpression of this protein also attenuates virulence, which means that Imo1273 is expressed in an appropriate level to promote an efficient infection (Bigot et al., 2009). As such, Imo1273 seems a good target to be further studied to control *L. monocytogenes* virulence.

In *S. aureus*, it was discovered an RNase Y ortholog that regulates virulence genes (Bonnin and Bouloc, 2015). It was also shown that RNase Y and PNPase act in an opposing manner to regulate *S. aureus* virulence (Numata et al., 2014).

RNA REGULATORS

Hfq

The bacterial Hfq is an RNA-chaperone critical for the sRNA-mediated regulation and it is mainly known for facilitating the imperfect base-pairing between sRNA and their targets (Vogel and Luisi, 2011; Andrade et al., 2013). Hfq has been related to pathogenesis in many microbes. Inactivation of Hfq in *Cronobacter sakazakii* leads to an attenuated virulence phenotype. *In vivo* dissemination, invasion ability and survival in host cells are negatively affected in the absence of Hfq (Kim et al., 2015). The RNA-chaperone is also important for *Y. pseudotuberculosis, Salmonella, L. monocytogenes* and *S. flexneri* virulence processes (Christiansen et al., 2004; Sharma and Payne, 2006; Sittka et al., 2007; Schiano et al., 2010) and can therefore serve as a potential therapeutic target in foodborne-related diseases.

sRNAs

sRNAs have been extensively implicated in the adaptation of pathogenic bacteria to different environments upon entry in the host (for a thorough review see Silva et al., 2011; Ortega et al., 2014; Saramago et al., 2014a; Svensson and Sharma, 2016).

SgrS is a bifunctional sRNA expressed in several organisms. It is specifically associated with the glucose-phosphate stress preventing inhibition of cell growth (Gorke and Vogel, 2008). In *Salmonella* this sRNA also regulates the expression of the *Salmonella*-specific virulence effector SopD which is injected into the host upon infection (Brumell et al., 2003; Papenfort et al., 2012). SgrS binds directly to the early coding sequence of *sopD* mRNA leading to the translation inhibition with the concomitant degradation of the mRNA target (Papenfort et al., 2012). The deletion of this sRNA leads to an attenuation in *Salmonella* virulence in mice (Santiviago et al., 2009).

Contrarily to what happens in other pathogenic organisms, there are few sRNAs described so far in *S. flexneri*. Interestingly, all of these sRNAs are connected with virulence processes and are expressed in specific environmental conditions (for a review see, Fris and Murphy, 2016). A curious example is RnaG sRNA that is encoded in the *S. flexneri* virulence plasmid pINV. RnaG is known to downregulate the expression of the virulence gene *icsA* that encodes an outer membrane protein crucial for bacteria spreading inside the host (Bernardini et al., 1989; Giangrossi et al., 2010). RyfA1 is another sRNA that affects *S. flexneri* multiplication in the host cells. Specifically, its over-expression leads to the elimination of *ompC* mRNA that encodes the major outer membrane protein C. The dramatic disappearance of the *ompC* transcript results in an inhibition of cell-to-cell spread, a mechanism that is crucial for pathogenesis (Bernardini et al., 1993; Fris et al., 2017). Interestingly, the expression of RyfA1 was shown to be negatively regulated by other sRNA, RyfB1 (Fris et al., 2017).

The Csr system is a global regulator of bacterial central metabolism that regulates several important mechanisms including virulence (Heroven et al., 2012; Vakulskas et al., 2015). The main component of this system, CsrA, regulates the expression of several mRNAs and is regulated by two sRNAs, CsrB, and CsrC that sequester CsrA inhibiting its activity (Liu et al., 1997; Weilbacher et al., 2003). In the absence of both RNAs, *S. flexneri* was able to invade eukaryotic cells more efficiently (Gore and Payne, 2010), due to the consequent increasing of free CsrA. In *Salmonella*, both the loss and overexpression of CsrA were shown to decrease invasion (Altier et al., 2000), highlighting that the level of CsrA and its sRNAs regulators must be controlled to allow a correct invasion. In *Y. pseudotuberculosis*, these sRNAs were also associated with virulence through their role in the regulation of RovA, a global transcriptional factor (Heroven et al., 2008). There were several other riboregulators described and associated with *Yersinia* virulence processes (for a review see, Nuss et al., 2017).

Besides its role on iron metabolism, RyhB was shown to be important for *Shigella dysenteriae* virulence by inhibiting the transcription of *virB* (Murphy and Payne, 2007), a transcription factor that promotes the expression of several virulence genes and is encoded in the *S. dysenteriae* large virulence plasmid (Adler et al., 1989). The repression of *virB* using a strain overexpressing RyhB affects the invasion efficiency of eukaryotic cells, plaque formation and effector protein secretion (Murphy and Payne, 2007). The expression of the two RyhB *Salmonella* homologes, RfrA and RfrB, was shown to be increased inside fibroblasts, and in murine macrophages (Padalon-Brauch et al., 2008; Ortega et al., 2012; Leclerc et al., 2013) emphasizing their importance in *Salmonella* intracellular replication.

In *Vibrio parahaemolyticus* it was shown that the sRNA Spot 42 downregulates VP1682 – a chaperone protein of

the T3SS1 – in an Hfq-dependent fashion. Deletion of Spot 42 led to an increase in cytotoxicity in Caco-2 cell cultures (Tanabe et al., 2015). In *V. cholerae*, the sRNA VrrA has major implications to bacterial fitness and virulence. VrrA regulates the expression of outer membrane porins, outer membrane vesicles (OMVs) and biofilm formation. This sRNA represses OmpA by base-pairing with the Shine-Dalgarno and coding region of the target mRNA. Downregulation of OmpA correlates with an increase of OMV, which has been associated with new pathways of toxin delivery and evasion of the host immune system (Song and Wai, 2009). Deletion of VrrA sRNA resulted in an attenuation of virulence of *V. cholerae* in a mouse model, linking this sRNA to pathogenesis (Song et al., 2014).

The pathogenicity island locus of enterocyte effacement (LEE) present in the enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) encodes the T3SS responsible for the delivery of effector molecules to the infected host cells. Overexpression of the Hfq-dependent sRNA350 was shown to activate expression of LEE encoded operons by regulating the master regulator *ler*. Interestingly, sRNA350 is located within LEE and originates from 3′-UTR region of the *cesF* mRNA, a chaperone necessary for EPEC virulence (Gruber and Sperandio, 2015). This sRNA does not appear to be processed and it carries out its function as part of the *cesF* transcript (Bhatt et al., 2016). Consequently, sRNA350 constitutes an example for the diverse nature of sRNA biogenesis and action.

Others

CRISPR RNAs are long repeat sequences interspaced by non-repetitive spacer sequences that provide bacteria with an adaptive immunity against foreign genetic elements. *L. monocytogenes* possesses a cas-less CRISPR system (RliB-CRISPR) with an unusual base-pairing between the repeat sequences and the adjacent spacers (Sesto et al., 2014). RliB sRNA is important for *L. monocytogenes* virulence as it was found to be upregulated in bacteria grown in mouse intestine. Moreover, upon RliB inactivation bacterial cells accumulated much faster than the parental strain in mouse liver (Toledo-Arana et al., 2009). Bacteriophages can often impact the expression of bacterial virulence factors. Therefore, the RliB-CRISPR system can pose as an important mechanism for regulation of *L. monocytogenes* virulence by modulating the crosstalk between bacteriophages and bacteria during infection.

In the 5′ UTR of *shuA*, a virulence gene that encodes an outer-membrane heme receptor, it was discovered the first RNA thermometer described in *S. dysenteriae*. This RNA structure senses the temperature of the human body upon invasion (Mills and Payne, 1995, 1997; Kouse et al., 2013). ShuA enables the utilization of heme as a source of iron and is only expressed when iron is depleted and at temperatures corresponding to the human body (Kouse et al., 2013). In *L. monocytogenes* it was shown that the virulence regulator PrfA was controlled by a RNA thermometer located at the 5′ UTR of the *prfA* transcript. At lower temperatures, the 5′ UTR of *prfA* forms a secondary structure which results in translation inhibition. At 37°C the secondary structure is not formed, and translation occurs (Johansson et al., 2002). These are good examples of sophisticated mechanisms that efficiently activate virulence genes at 37°C.

CONCLUSION

Growth of foodborne pathogens in food is mainly controlled by using refrigerated temperatures. Foodborne pathogens are able to survive at low temperatures by adapting their gene expression, which represents a challenge to developing new strategies for making food safer. The ribonucleases PNPase and RNase R are very important for the adaptation to lower temperatures by regulating cold survival transcripts and increasing their levels upon a cold shock. As such, these proteins could be very interesting in the developing of new strategies to prevent food contamination. In the last years, there was a great development in the production of polymers that can be used for food packaging, namely nanofibers that can be designed to soak certain compounds that confer antimicrobial protection (Torres-Giner, 2011). Hence, RNases offer a new perspective for developing antimicrobials that could be used in combination with such polymers. Although the discovery of inhibiting compounds is still a challenge, the use of small molecules that target RNase E in *E. coli* was already described (Kime et al., 2015). Moreover, in *S. aureus*, it was successfully identified and validated an inhibitor of RnpA, the protein component of RNase P (Olson et al., 2011; Eidem et al., 2015).

Ribonucleases often cooperate with sRNAs in the regulation of key cellular processes, namely, in the adaptation to different extracellular environments and in the regulation of virulence factors of foodborne pathogens. By providing knowledge on the sRNA-mediated virulence and adaptation networks we are widening the scope in the look for new antimicrobial targets. Another hypothesis is the use of antisense RNAs as they were shown to inhibit growth of *E. coli, S. enterica*, and *S aureus* by targeting essential genes (revised in Thomason and Storz, 2010). Antisense RNAs detain the advantage of being very specific and can affect multiple targets. However, there are many variables that still need to be tested before its use.

The knowledge that we have on RNA regulators and their involvement in pathogenesis is still the tip of the iceberg. In this review, we covered only a few examples of what is known in the field. In addition, with the constant development of new tools many important players in virulence will be certainly identified. These findings will certainly be exploited in the near future to pursue good antimicrobial candidates, opening a new world of possibilities.

AUTHOR CONTRIBUTIONS

RGM, JC, CB, RFdS, IJS, and CMA wrote the manuscript, RGM and CMA revised and coordinated the work.

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REVIEW
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Current Perspectives on Viable but Non-culturable State in Foodborne Pathogens

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The viable but non-culturable (VBNC) state, a unique state in which a number of bacteria respond to adverse circumstances, was first discovered in 1982. Unfortunately, it has been reported that many foodborne pathogens can be induced to enter the VBNC state by the limiting environmental conditions during food processing and preservation, such as extreme temperatures, drying, irradiation, pulsed electric field, and high pressure stress, as well as the addition of preservatives and disinfectants. After entering the VBNC state, foodborne pathogens will introduce a serious crisis to food safety and public health because they cannot be detected using conventional plate counting techniques. This review provides an overview of the various features of the VBNC state, including the biological characteristics, induction and resuscitation factors, formation and resuscitation mechanisms, detection methods, and relationship to food safety.

Keywords: VBNC, foodborne pathogens, induction, detection method, resuscitation

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INTRODUCTION

The viable but non-culturable (VBNC) state, a special physiological state, was first discovered and presented by Xu et al. (1982). As bacteria are subjected to some environmental stress, they cannot grow on conventional culture medium and maintain their activity. Another bacterial non-culturable state that is similar to the VBNC state is dormancy, which is defined operationally as a reversible state of metabolic shutdown (Kell et al., 1998). The VBNC state is presented slightly differently compared with dormancy because VBNC cells exhibit measurable metabolic activity, which is not detected in dormant cells (Mukamolova et al., 2003). However, many authors consider the VBNC state and dormancy as different terms that are used for the same physiological state (Oliver, 2005; Ayrapetyan and Oliver, 2016).

The concept of VBNC has attracted great attention in the fields of microbiology, because it has upset the traditional concept of microorganismal growth. Unlike normal cells, VBNC cells cannot be grown in conventional culture medium, and thus, conventional methods of detection cannot be used to detect bacterial pathogens in the VBNC state. Thus, challenges are encountered in the detection of pathogens. To date, researchers have identified 85 species of bacteria that can enter the VBNC state, including 18 non-pathogenic species and 67 pathogenic species. Some foodborne pathogens retain virulence after entering the VBNC state, which may be due to their rapid resuscitation into culturable cells under certain conditions (Li et al., 2014).

Many people believe that VBNC pathogens are simply in a stage preceding cell death or adaptation to stress (Sachidanandham and Gin, 2009), which cannot induce disease despite the

retention of virulent properties. VBNC pathogens are generally considered to be unable to initiate disease, but the virulence of VBNC pathogens can be recovered or maintained after resuscitation, leading to disease/infection (Du et al., 2007b; Nicolò and Guglielmino, 2012). For example, the VBNC cells of *Listeria monocytogenes* resuscitated by incubation with embryonated egg regained virulence identical to that of culturable cells (Cappelier et al., 2007). More seriously, large amounts of evidence have shown that VBNC pathogens may be involved in foodborne outbreaks. For example, a foodborne outbreak caused by salted salmon roe contaminated with Enterohemorrhagic *Escherichia coli* O157 was reported in Japan. Makino et al. (2000) performed numerous experiments and proposed that *E. coli* O157 might enter the VBNC state in salted salmon roe. In another outbreak in Japan, Asakura et al. (2002) suggested that *Salmonella Oranienburg* might become VBNC cells in response to NaCl stress in the outbreak caused by dried processed squids, and this hypothesis was confirmed by resuscitation experiments. Although there is no evidence to confirm that these outbreaks were directly caused by VBNC pathogens, the above studies adequately demonstrate that the potential presence of VBNC pathogens can pose a serious risk to food safety and public health. Foodborne pathogenic bacteria that use food as a carrier are some of the most important human pathogens, causing foodborne disease in human beings (Zhao et al., 2014, 2016). The foodborne pathogens may enter the VBNC state during food processing techniques, such as high temperature, high pressure, disinfectant, preservation, and low temperature storage, and they have become a potential risk for food safety. The VBNC cells of foodborne pathogenic bacteria are easily missed using the conventional plate counting technique and can be recovered with pathogenicity under certain conditions, resulting in a serious threat to human health. Therefore, research investigating VBNC foodborne pathogens is very important, and the establishment of a rapid and effective detection method for bacteria in the VBNC state has become a key to resolving the current crisis, as well as guaranteeing food safety and human health. To provide references for the safety control of foodborne pathogens, the biological characteristics, induction and resuscitation factors, detection methods and formation mechanism of VBNC foodborne pathogens are reviewed in this article.

FOODBORNE PATHOGENS IN THE VBNC STATE

After the discovery and presentation of VBNC cells in 1982, researchers found different species of bacteria that can exist in a VBNC state in recent years. For example, Oliver reviewed 52 species of VBNC bacteria in 2005 (Oliver, 2005), and added four new species in his review of pathogenic bacteria published 5 years later (Oliver, 2010). Moreover, 51 species of human pathogens have been reported to exist in the VBNC state (Li et al., 2014). Recently, Pinto et al. (2015) presented a list of 68 species of pathogenic bacteria in the VBNC state which was described. An increasing number of VBNC cells have been found in various environments, which cannot be ignored because of their negative impact on public health. Food is frequently exposed to a limited environment during processing, transportation and storage, which can provide more opportunities for the induction of VBNC cells. Unfortunately, it has been widely documented that foodborne pathogens are induced to enter the VBNC state in various foods, such as grapefruit juice (Nicolò et al., 2010), milk products (Gunasekera et al., 2002; Barron and Forsythe, 2007), and vegetables (Dinu and Bach, 2011, 2013). Additionally, most foodborne pathogens can be induced to enter the VBNC state in response to environmental stress. For example, *E. coli* O157:H7 VBNC cells were induced by low temperature on the surface of lettuce and spinach plants (Dinu and Bach, 2013) and by UV disinfection (Zhang et al., 2015). *Campylobacter jejuni* VBNC cells were observed under oxygen-rich (Oh et al., 2015) and low temperature conditions (Chaisowwong et al., 2012). The cells of *L. monocytogenes* entered a VBNC state within 24 h in the presence of potassium sorbate at pH 4.0 (Cunningham et al., 2009). *L. monocytogenes* and *Bacillus cereus* were also changed into VBNC cells by treatment with a pulsed electric field (Rowan, 2004). The above examples fully illustrate that food samples cannot be considered free from pathogens if the plate counting result is negative. Therefore, an understanding of the VBNC state is essential to comprehend the challenges associated with and how to avoid the risk of VBNC pathogens.

In this review, we specifically focus on foodborne pathogenic bacteria that can enter VBNC state. An overview of 35 foodborne pathogens with a confirmed VBNC state is provided in **Table 1**, with their main survival environments and VBNC cells induction and resuscitation conditions. The list includes many pathogens, such as *E. coli* O157:H7, *Staphylococcus aureus* and *C. jejuni*, which cause foodborne disease in healthy individuals, as observed in outbreaks of epidemic diarrhea or collective food poisoning (Chaisowwong et al., 2012; Dinu and Bach, 2013; Pasquaroli et al., 2014). It also includes some pathogens such as *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, and *Salmonella* that mainly infect human beings by first infecting other organisms (like poultry, livestock, and seafood) (Bates and Oliver, 2004; Albertini et al., 2006; Braden, 2006). **Table 1** also lists the main survival environment of foodborne pathogenic bacteria, including soil, freshwater, seawater, raw food, organisms, and even dust. Moreover, foodborne pathogens may enter the VBNC state and inhabit different stressful environments, including starvation, extreme temperature, chemical and UV-exposed environments. These findings allow readers to gain a better understanding of the various induction and resuscitation conditions of VBNC foodborne pathogens.

CHARACTERISTICS OF VBNC CELLS

Although VBNC bacteria lose culturability on normal culture medium, this does not mean that these cells are equivalent to dead cells. For example, the membrane of dead cells is damaged so that the genetic material in the cell cannot be preserved and expressed, while VBNC cells have a complete membrane structure that ensure the genetic information is not lost (Lahtinen et al., 2008).

TABLE 1 | The species of foodborne pathogenic bacteria with a proven VBNC state.

Genus	Name of strains	Survival environment	Induced condition	Resuscitation condition	Reference
Gram-negative bacteria					
Aeromonas	*A. hydrophila*	Aquatic environments (freshwater)	Starvation, low temperature, salt stress	Liquid media with sodium pyruvate, temperature upshift	Pianetti et al., 2012
Brucella	*B. melitensis*	Livestock	Metallic copper surfaces	–	Borkow, 2014
Burkholderia	*B. pseudomallei*	Soil, water	High temperature, low pH, osmotic stress, UV exposure	–	Inglis and Sagripanti, 2006
Campylobacter	*C. coli*	Intestinal tract of chicken	Starvation, low pH, low temperature	Embryonated chicken eggs	Chaveerach et al., 2003
	C. jejuni	Untreated water, raw milk, poultry meat	Oxygen-rich conditions, low temperature in nutrient-rich conditions	Mouse model, embryonated eggs	Chaveerach et al., 2003; Chaisowwong et al., 2012; Patrone et al., 2013; Magajna et al., 2015; Oh et al., 2015
Citrobacter	*C. freundii*	–	–	–	Rowan, 2004
Edwardsiella	*E. tarda*	Freshwater and marine fish	Starvation seawater, low temperature	Chick embryos, nutrition with temperature upshift	Du et al., 2007b
Cronobacter	*C. sakazakii*	Milk product	Dry Stress	–	Barron and Forsythe, 2007
Escherichia	*E. coli* O157:H7 EHEC	Vegetables and drinking water	Starvation, low temperature, UV exposure, high pressure carbon dioxide HPCD	Fresh Tryptic Soytone Broth, temperature upshift	Dinu and Bach, 2013; Zhao F. et al., 2013; Zhang et al., 2015
	E. coli O104:H4	Enugreek sprouts and seeds	Starvation copper ions or tap water	Stress relief and plated on rich agar medium	Aurass et al., 2011
	E. coli ST64111 ETEC	Water environments ponds, rivers, lakes and sea	Starvation seawater and freshwater	–	Lothigius et al., 2010
	E. coli H10407 EPEC	Marine environment	Starvation seawater	–	Pommepuy et al., 1996
Pseudomonas	*P. fluorescens*	Soil, water, poultry meat	Starvation, chemicals chlorination, high temperature	–	Peneau et al., 2007
	P. putida	–	–	–	Rowan, 2004
Salmonella	*S. agona*	Feed processing environment	Desiccation	–	Habimana et al., 2014
	S. choleraesuis	Intestines of pigs	Starvation	–	Bogosian et al., 1998; Hsueh et al., 2004
	S. enteritidis	Poultry and eggs	Rich medium with H_2O_2	M9 liquid medium with pyruvate	Braden, 2006; Morishige et al., 2013
	S. enterica serovar Oranienburg	Pickled foods	Starvation 7% NaCl at 37°C	Recombinant Rpf rRpf proteins	Panutdaporn et al., 2006
	S. typhi	Groundwater, vegetable surface	Low temperature, copper ions	Rich medium with 1% catalase or 3% Tween 20	Zeng et al., 2012
	S. typhimurium	Intestines of poultry, livestock and mouse	Starvation, low temperature, desiccation	Rich medium, temperature upshift	Gupte et al., 2003; Habimana et al., 2014

(Continued)

TABLE 1 | Continued

Genus	Name of strains	Survival environment	Induced condition	Resuscitation condition	Reference
Shigella	*S. dysenteriae*	Utensils used in eating and drinking glasses	MacConkey agar with fomites, low temperature	Rich medium, temperature upshift	Rahman et al., 1996
	S. sonnei	Vegetable surfaces, raw ground beef	–	–	Oliver, 2005
Vibrio	*V. alginolyticus*	Seafood, seawater	Starvation, low temperature	Rich medium, temperature upshift	Albertini et al., 2006; Du et al., 2007a
	V. cholera	Estuarine and brackish water	Starvation, low temperature, oxygen	Temperature upshift, co-cultivation with HT-29 cells	Rao et al., 2014; Fernández-Delgado et al., 2015; Imamura et al., 2015
	V. parahaemolyticus	Estuarine water, seafood	Starvation, low temperature	Rich medium, temperature upshift	Bates and Oliver, 2004; Hung et al., 2013
	V. vulnificus	Raw and undercooked shellfish, seafood	Oxidative stress, low temperature, starvation	Rich medium, temperature upshift, quorum sensing QS signal molecule AI-2	Abe et al., 2006; Ayrapetyan et al., 2014; Rao et al., 2014
Yersinia	*Y. pseudotuberculosis*	Warm- blooded organisms domestic animals and birds	Starvation, low temperature	Rich medium	Pawlowski et al., 2011
	Y. enterocolitica	Natural water, soil, animal manure, refrigerated foods	–	–	Pawlowski et al., 2011
Gram-positive bacteria					
Bacillus	*B. cereus*	Soil, cereals	Pulsed electric field	–	Rowan, 2004
Clostridium	*C. perfringens*	Crops and vegetables	–	–	Rahman and Noor, 2012
Enterococcus	*E. faecalis*	Intestines of animals	Starvation, low temperature, high pH	Rich medium, temperature upshift	Lleo et al., 2001; Jiang et al., 2014
	E. faecium	Intestines of animals	Starvation, low temperature	Rich medium, broth with sodium pyruvate	Lleo et al., 2001
Listeria	*L. monocytogenes*	Raw food milk, meat, vegetables, sausages and seafood, ready-to-eat RTE food	Starvation, low pH, low temperature or not, chemicals food preservatives, pulsed electric field	NOT rich medium with/without sodium pyvurate, embryonated eggs	Rowan, 2004; Cappelier et al., 2007; Cunningham et al., 2009; Gião and Keevil, 2014; Xuan et al., 2017
Mycobacterium	*M. avium*	Drinking water distribution systems, household plumbing	–	–	Radomski et al., 2010
Staphylococcus	*S. aureus*	A wide range of environments water, dry dust and mammalian abscesses	Starvation, low temperature, antibiotic vancomycin or quinupristin/dalfopristin	Rich medium with sodium pyruvate, temperature upshift	Masmoudi et al., 2010; Pasquaroli et al., 2013, 2014; Li et al., 2016

"–" means unknown.

Moreover, while dead cells lose absorptive capacity and are metabolically inactive, Rahman et al. (1994) confirmed that VBNC *Shigella* can take up methionine for protein synthesis, thus demonstrating that bacteria in the VBNC state can exchange outside material.

Although VBNC bacterial cells have many common features with culturable cells, a series of physiological changes occur during the transition from the normal state to the VBNC state, including slowing down of the absorption of nutrients and reduction of the level of macromolecular synthesis and metabolism, the concentration of the cytoplasm and total proteins (Jeffreys et al., 1998). In summary, these variations encompass cellular morphology, metabolism, stress tolerance, gene expression and potential virulence, and formation mechanism of VBNC cells.

Cellular Morphology

Regarding cellular morphology, VBNC cells maintain apparent cell integrity but exhibit dwarfing (Costa et al., 1999). Apart from cell dwarfing, researchers have also observed cell rounding in VBNC state of many species, with a reduced size and varied spherical shape (Adams et al., 2003). VBNC cells of *Salmonella typhi* exhibiting metabolic activity were decreased in size and coccoid in shape compared with the normal rod-shaped cells (Zeng et al., 2012). *Edwardsiella tarda* changes from a 1.9 μm × 1.1 μm short rod to a coccoid shape with an average radius of 0.5 μm in the VBNC state (Du et al., 2007b). *V. parahaemolyticus* also changes from rods in the exponential phase to cocci in the VBNC state, and concurrently the cell walls become looser and more flexible at the initial stage, followed by the formation of a new thin wall (Su et al., 2013). Although most bacteria entering the VBNC state are reduced in size, some Gram-positive bacteria become larger, such as *Enterococcus faecalis*, in a low temperature and low nutrient environment and are slightly elongated (Signoretto et al., 2000). Moreover, it is worth noting that the morphological change from rod to ball does not necessarily appear in all VBNC state bacteria, some VBNC cells have been confirmed to remain intact or to exhibit a spiral morphology at lower temperatures (Lázaro et al., 1999). However, these changes in morphology do not reveal whether a bacterium is in the VBNC state because they also commonly occur in non-VBNC cells (Li et al., 2014).

The VBNC cell wall and membrane differ from normal cells, which could be a manifestation of the physiological state associated with maintaining viability. An increase in the percentage of short and long chain fatty acids and a reduction in the main membrane lipid content (C16, C16:1, C18) were observed in VBNC cells of *Vibrio vulnificus* by Linder K (Linder and Oliver, 1989). These results show that changes in fatty acids play an important role in the protection of cell membrane fluidity during environmental stress. In terms of proteins, an obvious reduction in membrane protein (6.3 kDa) was observed in VBNC *Staphylococcus aureus* (Trudeau et al., 2012). Moreover, after entering the VBNC state, the structure of the peptide in the cell wall displayed relatively large changes, and Signoretto et al. (2002) also discovered that peptidoglycan DAP–DAP cross-linking in VBNC cells of *E. coli* increased more than three times. These findings indicate that the ability of VBNC cells to resist external mechanical damage is greatly improved.

Metabolic Activity

Bacterial cells in the VBNC state maintain metabolic activity in harsh environments (Du et al., 2007a), and the energy of VBNC bacteria is mainly supplied by branched chain amino acids under starvation conditions (Ganesan et al., 2007). However, reductions in the percentage of total lipids, carbohydrates and poly-β-hydroxybutyrate were detected in starved *Vibrio cholera*, which indicated that these large molecules could be used as the primary energy source to maintain the survival of cells (Clements and Foster, 1998). A reduction in DNA was also observed in VBNC cells (Jeffreys et al., 1998). In addition, Trevors et al. (2012) found that the contents of nucleic acid molecules in the cytoplasm of starved bacteria and VBNC bacteria were lower than those in normal bacteria. Nevertheless, the mechanism underlying the decrease in DNA content in VBNC cells remains unclear and necessitates further research.

Although VBNC cells are similar to starved cells, their protein expression levels are different. Lai et al. (2009) showed an increase in protein content in VBNC cells of *V. parahaemolyticus* ST550, and the protein content in starved cells was significantly reduced. Either the upregulation of VBNC cell protein or the downregulation of starved cell protein can suppress the exponential phase of *V. parahaemolyticus* and lead to entry into the VBNC state.

Stress Tolerance

Compared with culturable cells, VBNC cells have greater physical, chemical, and antibiotic resistance, which might be due to their lower metabolic activity and stronger cell wall strengthened resulting from the increased peptidoglycan cross-linking (Signoretto et al., 2000). To study the VBNC state, Nowakowska and Oliver (2013) developed a model organism from *V. vulnificus*. Using this model, VBNC cells of *V. vulnificus* can withstand a variety of stresses while dormant, including high doses of antibiotics, toxic heavy metals, high temperatures, high salinity, ethanol, and acid. In terms of chemical stress, a similar study conducted in *V. parahaemolyticus* showed that VBNC cells were resistant H_2O_2 and low salinity but remained sensitive to bile salts (Wong and Wang, 2004). In terms of antibiotic stress, the VBNC state of several foodborne pathogens such as *E. coli* O157, *S. aureus*, *V. vulnificus,* and *C. jejuni* have been found to be resistant to several antimicrobials (Ramamurthy et al., 2014). *S. aureus* can enter the VBNC state in infectious biofilms and the presence of vancomycin or quinupristin/dalfopristin can inadvertently induce a true VBNC state or persistence in *S. aureus* cells embedded in biofilms, suggesting a role for *staphylococcal* biofilms in recurrent infections (Pasquaroli et al., 2013). During food pasteurization and preservation processes, certain bacterial cells can be induced to enter the VBNC state (Zhao F. et al., 2013; Kramer and Muranyi, 2014). In general, VBNC cells are resistant to multiple antimicrobials, which may cause treatment failure at times (Hu and Coates, 2012). In fact, a recent study found that foods subjected to antimicrobial treatment harbored considerable numbers of VBNC cells (Anvarian et al., 2016). The presence

of VBNC cells is considered a threat to human health and food safety due to the shortened shelf life that cause early spoilage of food products (Ayrapetyan and Oliver, 2016). Although VBNC cells of foodborne pathogens have been shown to resist a range of stresses, with respect to food safety, more research examined VBNC cell resistance to specific food treatments, such as special processing, preservation and packaging techniques, is warranted (Ayrapetyan and Oliver, 2016).

Gene Expression and Potential Virulence

Bacteria in the VBNC state retain the ability to express multiple genes. Yaron and Matthews (2002) discovered many genes that could be expressed in the VBNC state of *E. coli* O157:H7, including *mob*A, *rfb*E, *stx*1, *stx*2 and some genes related to the synthesis of 16s rRNA. Recently, Patrone et al. (2013) detected the expression of the protein gene *Cad* F in VBNC cells of *C. jejuni* ATCC 33291 and *C. jejuni* 241 by RT-PCR, and observed that the *C. jejuni* VBNC population maintained an ability to adhere intestinal cells. After entering the VBNC, *Helicobacter pylori* can continue to express *Mur* G, which is a kind of sugar-based transfer enzyme that has been confirmed to be necessary for the peptidoglycan recombination that occurs in VBNC state *E. coli* (Signoretto et al., 2002). These studies demonstrated that virulence genes in VBNC cells can be expressed and the synthesis of metabolites carried out normally.

Escherichia coli O157: H7 is one of the most important foodborne pathogenic bacteria, arising mainly from A/E damage on the surface of intestinal epithelial cells via the production of Shiga toxin, haemolysin and adhesin, which can cause diseases such as diarrhea, haemorrhagic colitis, and haemolytic-uremic syndrome. Yaron and Matthews (2002) found that the toxin genes (*stx1* and *stx2*) could still be expressed in the VBNC state of *E. coli* O157:H7 by reverse transcription PCR. Moreover, in a study examining food safety risk factors, Dinu and Bach (2011) also discovered that VBNC *E. coli* O157: H7 exhibited the potential virulence, and stable expression of the toxin gene (*hly*, *stx*1, and *stx*2) and the production of enterotoxin were observed in VBNC cells. In addition, the VBNC cells of *C. jejuni* also retained the ability to invade human intestinal epithelial cells (Chaisowwong et al., 2012). However, interestingly, expression of the virulence gene in VBNC cells does not necessarily indicate that the cells will produce toxins. Lothigius et al. (2010) found that although enterotoxigenic *E. coli* (ETEC) entering the VBNC state maintain the expression of virulence genes *eltB* and *estA* encoding the LT and STh enterotoxins, enterotoxins were not produced as determined using GM1-ELISA methods. Nonetheless, there is the potential danger in VBNC cells that are still pathogenic and even cause fatal diseases, which may be due to rapid resuscitation in suitable condition (Du et al., 2007b).

Mechanism of VBNC Cell Formation

Since the concept of the VBNC was proposed, a great deal of literature has been published on the VBNC state, although most of them have concentrated on biological characteristics. Thus, the mechanism by which the VBNC state occurs in bacteria is still not well understood (Pinto et al., 2015). The present formation mechanism of the VBNC state has been hypothesized as follows. First, the extreme conditions can lead to poor-quality cells, resulting in a loss of cell activity so that the VBNC cells cannot be cultured (Nyström, 2003). Second, the VBNC state exhibits a tendency not to die but exhibits a survival strategy, with procedural responses to adapt to adverse environments (Oliver, 2010). Third, this hypothesis is currently supported by the finding that non-culturable VBNC cells are the result of gene regulation (Ayrapetyan et al., 2015).

Although the molecular mechanism underlying the formation of the VBNC state is not fully understood, several genes that exhibit importance in the VBNC state have also been found to play integral roles in the formation of VBNC cells. Here, it is worth discussing the *rpoS* gene. The major stress regulator, RpoS (σ^S), which is expressed by the *rpoS* gene, is a stationary-phase sigma factor that allows bacteria to survive under different environmental stresses (such as acidic conditions, high osmotic pressure, oxidation, and starvation) (Bhagwat et al., 2006). This capability implies that RpoS can improve the ability of cells to adapt to the environment and thus hinder formation of the VBNC state. It has been shown that *rpoS* mutants lacking ppGpp more rapidly enter the VBNC state than normal strains (Boaretti et al., 2003). Additionally, Kusumoto et al. (2012) showed a reduced RpoS level during VBNC induction of *Salmonella*, and RpoS indeed delayed the formation of VBNC cells. Although RpoS is an obstacle for the formation of the VBNC state, it has been reported that VBNC cells continue to express the *rpoS* gene (Smith and Oliver, 2006). It is possible that VBNC cells must regulate RpoS, which is important for the maintenance of resistance and persistence under stresses. Boaretti et al. (2003) confirmed this hypothesis and found that the *rpoS* mutants lost culturability and died earlier than VBNC cells of their *E. coli* parental strains, suggesting that the *rpoS* gene is closely related to persistence in the VBNC state. These findings indicate that although the expression and regulation of the *rpoS* gene significantly hinder the formation of VBNC cells, long-term survival of VBNC cells is not possible in the absence of RpoS protein. However, a contrasting viewpoint has been proposed. This opposing perspective is that (p)ppGpp modulated by protein RelA may be an inducer of the VBNC state and that cells lacking ppGpp are less likely to enter the VBNC state (Ayrapetyan et al., 2015). It is known that (p)ppGpp is a regulatory signaling molecule that can regulate RpoS, and the elevated level of (p)ppGpp will lead to a several-fold increase in the amount of RpoS, which plays a crucial role in the accumulation of RpoS (Magnusson et al., 2005). Thus, the possibility that (p)ppGpp may be an inducer of the VBNC state suggests that RpoS may be an inducer that can facilitate the more rapid entry of cells into the VBNC state. Although these two views are conflicting, they both illustrate that RpoS significantly affects the formation of VBNC cells and also enhances stress resistance in VBNC cells.

The persister state was first described by Bigger (1944) as a multidrug-tolerant state, representing another dormancy state related to the VBNC state. In this state, the cells are not growing but can quickly regain culturability on medium. Ayrapetyan et al. (2015) argued that these two closely related states are part of a shared 'dormancy continuum,' suggesting that logarithmic-phase cells would enter the persister state before entering the

VBNC state. Orman and Brynildsen (2013) also found that the presence of a small number of persisters could cause an accumulation of VBNC cells, and believed that the persister state is a transitory state leading to the VBNC state. The above findings offer some hypotheses regarding the VBNC formation mechanism (**Figure 1**).

The above research about the formation mechanism of VBNC mainly focused on hypotheses and reasoning. At the molecular level, the research was mainly focused on the gene or protein level with some functions, which only partly reflected the formation mechanism of VBNC state. However, the study focused on the single and individual mechanisms, the lack of a comprehensive and systematic analysis. Most of the literatures to a single transcriptomics or proteomics approaches, the lack of integration analysis of the both, it is difficult to achieve information complement and functional verification (Capozzi et al., 2016; Feng et al., 2016). With the development and application of high-throughput sequencing technology and bioinformatics, in order to comprehensively and thoroughly understand the formation mechanism of VBNC state, researchers will use omics technology to study the formation of VBNC state mechanism.

DETECTION OF VBNC CELLS

At present, the method used to detect VBNC cells is mainly based on two key characteristics of VBNC cells: viability and non-culturability. Generally, if bacteria lose culturability but are still viable, they can be considered to have entered the VBNC state. Thus, using the conventional plate counting technique to confirm the cells in a non-culturable state is the first major step used to detect VBNC cells, followed by the estimation of viable cells using other methods.

One common method is based on microscopic enumeration with staining procedure to directly detect the viable cells, including the direct counting method of viable bacterial cells (DVC) based on the substrate absorption ability (Kogure et al., 1979), the respiration detection method (such as CTC or INT) based on the ability of the electron transport system (Albertini et al., 2006) and the LIVE/DEAD® BacLight™ fluorescence staining method based on the cell membrane structure integrity (Cunningham et al., 2009).

The second method is based on molecular diagnostic approaches to detect gene expression or gene amplification selectively, such as reverse transcription PCR, quantitative real-time polymerase chain reaction (qRT-PCR) and loop-mediated isothermal amplification (LAMP). Bacterial mRNAs have been proposed as markers for cell viability because they are very unstable molecules with very short half-lives inside the cell (Sheridan et al., 1998). Thus, it would be expected that as long as VBNC bacteria are alive, they should produce some mRNA molecules. Reverse transcription PCR is commonly used in many bacterial species to determine the viability of cells (Trevors, 2011). Selective gene amplification is an emerging approach to detect viable cells. Using this approach, viability is based on membrane integrity. Propidium monoazide (PMA) or ethidium monoazide (EMA) is a high-affinity photolysis DNA nucleic acid dye that can only enter cells with damaged membranes (considered 'dead') and bind covalently to cellular DNA through visible-light photocatalysis, whereas the intact membranes of 'live' cells pose a barrier to this molecule. The PMA treatment is followed by extraction of genomic DNA and its analysis by quantitative PCR or LAMP. The covalent cross-linkage of PMA to DNA has been shown to result in a strong inhibition of PCR amplification of the modified DNA. The result of treatment is that only unmodified DNA from intact cells containing DNA that is not cross-linked to PMA can be amplified, whereas PCR amplification of modified DNA from membrane-compromised cells is efficiently suppressed (Nocker and Camper, 2009). Dinu and Bach (2013) accurately detected *E. coli* O157:H7 VBNC cells on the surface of lettuce and spinach plants by PMA-qPCR, which provided a detection limit of 10^3 CFU/g leaf, suggesting that PMA-qPCR was an appropriate technique to detect the VBNC cells of foodborne pathogens in contaminated vegetables.

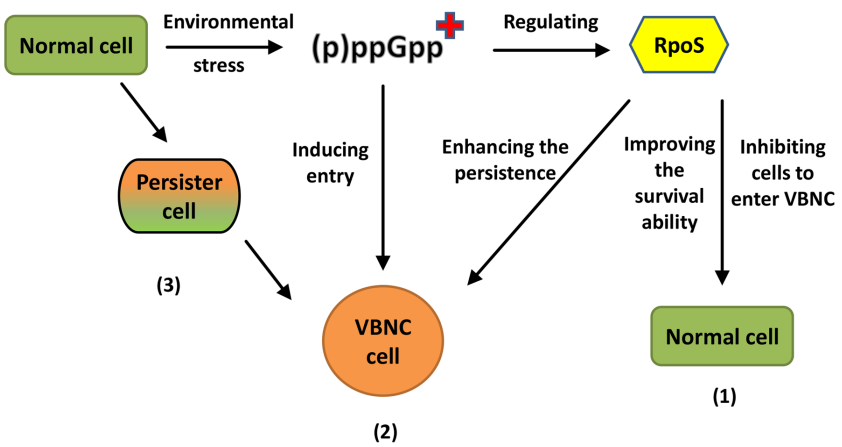

FIGURE 1 | Different views on the formation mechanism of VBNC cells. (1) RpoS proteins significantly hinder the formation of VBNC cells but enhance the persistence of VBNC. "+" means an accumulation of (p)ppGpp. (2) (p)ppGpp may be an inducer which can make cells more quickly into VBNC. (3) The normal cell will enter the persister state before entering VBNC state.

PMA can also be combined with LAMP. Zhao X. et al. (2013) developed a PMA-LAMP assay and selectively detected viable *E. coli* O157 cells within 1 h by PMA-LAMP. Zhong et al. (2016) also developed a real-time fluorescence LAMP technique combined with PMA, and applied it for the quantitative detection of VBNC *V. parahaemolyticus*. Lin et al. (2016) also adopted qRT-PCR and PMA-qPCR to observe the potential induction of VBNC cells by water reclamation processes.

The other methods focus on the identification of VBNC cells using biological sensors, especially gene sensors and receptor sensors that are biosensors based on DNA (Paniel et al., 2013). A DNA or RNA target gene sensor method is applied mainly to detect the hybridization reaction between DNA or RNA sensors and single-stranded DNA during sample identification. Regarding the receptor sensor method, DNA or RNA can be used as a receptor to achieve high affinity and specificity in combination with the target molecules. It is worth noting that one should first select the appropriate biological recognition elements and sensing format when adopting this method to achieve the desired objectives. However, less research has been conducted in this area, and further validation is needed.

Recently, novel detection methods have also developed for separating cells based on their physiological states (Ayrapetyan and Oliver, 2016). Fluorescence techniques combined with direct optical detection methods for the rapid assessment of bacterial viability have been increasingly followed for several years. Among these techniques, flow cytometry (FCM) has been shown to be a powerful tool for rapidly analyzing populations on a cell-by-cell basis and can be applied in many areas of food safety or medical microbiology (Léonard et al., 2016). The main principle is that particles in suspension are pumped into a narrow flow stream intersected by one or more laser beams. Single particles, such as microbial cells, are illuminated individually with the resulting light scatter and fluorescence emission detected at appropriate wavelengths (Bridier et al., 2015). Bridier et al. (2015) summarized the applications of FCM in food microbiology such as study of food bacteria function, detection of food microbial communities or detection and persistence of foodborne pathogens. Recently, Mathur et al. (2016) reviewed the advancement of FCM and the introduction of novel fluorochromes allow to study the viability of cells, the membrane structure and its integrity, and the membrane potential at a single-cell level. The ability to use FCM to visualize, enumerate and analyze a population of cells into subpopulations of varying physiological status is a valuable aid to understanding this intricate area for the microbiologists (Léonard et al., 2016). Indeed, the use of personalized probes and dyes for the detection of changes in specific targets and intracellular activities permits the targeted use of FCM to ascertain the structural and functional characteristics of a population of VBNC cells.

INDUCTION OF VBNC CELLS

During the process of food processing and storage, there are numerous factors that induce foodborne pathogens to enter into the VBNC state. In general, the factors (physical and chemical) create worse bacteria growth conditions that may stress the bacterial cells into the VBNC state. The physical factors that induce the VBNC state of bacteria mainly include low/high temperature (Dinu and Bach, 2013), drying (Barron and Forsythe, 2007), irradiation (Zhang et al., 2015), oxidative stress (Oh et al., 2015), starvation (Lothigius et al., 2010), a pulsed electric field (Rowan, 2004), pulsed light and high pressure carbon dioxide (HPCD) (Feng et al., 2016). The chemical factors include food preservatives and disinfectants (Oliver, 2010; Ding et al., 2016).

To explore the physical induction factors, many researchers have carried out relevant simulation experiments (Rowan, 2004; Barron and Forsythe, 2007; Dinu and Bach, 2013; Patrone et al., 2013; Zhao F. et al., 2013). For example, the major foodborne pathogens, such as *E. coli* O157, *C. jejuni*, *V. parahaemolyticus*, *L. monocytogenes,* and *S. aureus*, have been validated to enter the VBNC state under low temperatures conditions (Bates and Oliver, 2004; Masmoudi et al., 2010; Chaisowwong et al., 2012; Dinu and Bach, 2013; Patrone et al., 2013; Gião and Keevil, 2014; Liu et al., 2016). In addition to low temperature factors, cells entering the VBNC state have been detected during high temperature sterilization processes such as the pasteurization of milk (Gunasekera et al., 2002). Regarding other physical factors, the VBNC cells of *Cronobacter sakazakii* have been detected in milk products as a result of dry stress (Barron and Forsythe, 2007). *C. jejuni* (Oh et al., 2015) and *V. vulnificus* (Abe et al., 2006) have been confirmed to enter the VBNC state under oxidative stress. *L. monocytogenes* and *B. cereus* entered the VBNC state by treatment with a pulsed electric field (Rowan, 2004). The cells of *E. coli* O157:H7 were induced into a VBNC state by UV disinfection (Zhang et al., 2015). A large number of *E. coli* were also observed to enter the VBNC state after pulsed light treatment, and some of them exhibited metabolic loss and cell membrane damage (Kramer and Muranyi, 2014). These findings all support the idea that various kinds of physical stress factors during food processing may induce foodborne pathogens to enter the VBNC state.

Some chemical reagents are commonly used in the process of food processing, such as the addition of food preservatives to extend the shelf life of food or of disinfectants in the processing plant and equipment for disinfection. In the food industry, pathogens with a certain degree of resistance are created by the indiscriminate use of disinfection solution (Meyer, 2006). Some pathogens leave behind and build up a resident flora on surfaces after cleaning and disinfection, and partial cells from the gradual accumulation of resident flora can be induced to enter a VBNC state (Peneau et al., 2007). Such chemical reagents were once used indiscriminately, not only cannot improve food safety but also to induce bacteria to enter the VBNC state. Researchers have shown that the indiscriminate use of preservatives may be a threat to public health. For example, although potassium sorbate is a kind of commonly used broad-spectrum antimicrobial agent, Cunningham et al. (2009) found that the cells of *L. monocytogenes* grown in the presence of potassium sorbate at pH 4.0 entered a VBNC state within 24 h. They also indicated that temperature had a significant impact on the ability of potassium sorbate to induce VBNC cells, which were observed at 37°C but not at 4°C or

21°C. However, the wide use of chlorinating disinfectants in food processing workshops may also cause bacteria to enter the VBNC state. Peneau et al. (2007) simulated meat processing plants in the laboratory and adopted the same disinfection methods used in the factory to disinfect production equipment. They found VBNC cells of *Pseudomonas fluorescens* on the production equipment. It has also been reported that excessive use of disinfectants induce pathogenic bacteria, such as *E. coli* and *C. jejuni*, to enter the VBNC state (Meyer, 2006). Moreover, because of the use of chlorinating disinfectants, bacteria that are present in tap water may also enter the VBNC state. Al-Qadiri et al. (2011) confirmed the presence of VBNC state *E. coli* O157:H7 and *C. jejuni* in tap water by molecular biological detection. Therefore, it is important to pay attention to the rational use of disinfectants during the process of food safety production.

RESUSCITATION OF VBNC CELLS

What Is Resuscitation?

The term "resuscitation" was first presented by Roszak et al. (1984) to describe the recovery of VBNC cells of *Salmonella enteritidis*. It is an important feature of VBNC cells, and the recovered cells display improved metabolic activity and restored culturability. In fact, resuscitation is a complicated process and may not be performed by only direct removal of inducing factors. In addition, not all VBNC strains can be recovered (Rowan, 2004). The resuscitation conditions differ for different bacteria, and thus only under suitable conditions can VBNC cells achieve resuscitation. **Table 1** presents the various resuscitation conditions that have been reported for different foodborne pathogens, such as *C. jejuni*, which could be resuscitated by incubation in embryonated chicken eggs but not in rich medium. In addition, the same resuscitation method had different effects on different bacteria and even on different strains of the same species. For example, Pinto et al. (2011) discovered amino acids could resuscitate VBNC cells of haemolytic *E. coli* but not *E. coli* O157:H7.

The biggest challenge associated with the resuscitation of VBNC cells has existed for a long time: whether the culturable state of bacteria is caused by a real recovery of VBNC cells or the regrowth of residual culturable cells that are not undetected by the plate counting method (Whitesides and Oliver, 1997). The difference between residual undetected culturable cells and VBNC cells is that the former retains culturability. To date, there is no effective method to distinguish culturable cells from resuscitation cells or normal cells, and thus different views are apparent regarding the resuscitation of VBNC cells. In the resuscitation experiment of VBNC *V. cholerae*, Ravel et al. (1995) found that the number of resuscitated cells was similar to the samples diluted 10 times and 100 times, which was also approximately 2.2×10^5 cfu/mL, rather than the expected decrease in presentation. Consequently, they believed that the increased number of *V. cholerae* was caused by the regeneration of residual bacterial cells but not the resuscitation of VBNC cells. Nevertheless, Whitesides and Oliver (1997) demonstrated the resuscitation of VBNC cells after 2 years by further reducing the proportion of culturable cells by serial dilutions. Thus, the resuscitation has been widely recognized.

To date, the virulence of VBNC pathogens has been proven to be recovered or maintained after resuscitation. Resuscitated VBNC cells such as *L. monocytogenes* (Cappelier et al., 2007) and *S. typhi* (Zeng et al., 2012) retain their virulence and cause varying degrees of damage to mice, leading even to death. Moreover, the resuscitated VBNC pathogens may be involved in several foodborne outbreaks, such as *E. coli* O157:H7 (Makino et al., 2000), *E. coli* O104:H4 (Aurass et al., 2011), and *Salmonella* (Asakura et al., 2002). Although there is no clear evidence to prove that resuscitated foodborne pathogens can directly cause human diseases, their security risks to public health cannot be ignored. Therefore, it is necessary to understand the factors that can promote resuscitation and thus taking effective measures to prevent the occurrence of food hazards.

Factors That Stimulate Resuscitation

The resuscitation of VBNC cells can be triggered by a variety of stimuli factors, such as an increase in the nutrient concentration, increases or decreases in temperature, the presence of chemical stimuli and even co-cultivation with host cells. In 1984, rich medium was first used to resuscitate VBNC cells in *S. enteritidis* by Roszak et al. (1984). Since then, to identify the factors that stimulate the recovery of VBNC cells, a number of researchers have successively performed resuscitation experiments under different conditions.

An increase in temperature is a common physical stimulus to resuscitate most VBNC cells induced by low temperature, such as *E. coli* O157:H7, *A. hydrophila*, *S. typhimurium*, *S. dysenteriae*, *Vibrio* spp., *E. faecalis,* and *S. aureus* (**Table 1**). Resuscitation can also be mediated by different kinds of chemical stimuli, including sodium pyruvate (Lleo et al., 2001; Pinto et al., 2011; Morishige et al., 2013; Pasquaroli et al., 2013), amino acids (Pinto et al., 2011), and Tween 80 (Trinh et al., 2015). It is worth mentioning that researchers have different views on the resuscitation role of pyruvate. On the one hand, it is deliberated whether pyruvate cannot recover VBNC cells. Li et al. (2014) reported that the VBNC cells of *S. typhimurium* could not be recovered by supplementation with antioxidants such as pyruvate, catalase or oxyrase, but could be resuscitated by an autoinducer. On the other hand, some people insist that pyruvate has a significant effect on the resuscitation of VBNC strains. For example, Pinto et al. (2011) found that starving cells of *E. coli* could easily enter the VBNC state after the addition of pyruvate. Morishige et al. (2013) also discovered that the VBNC cells of *S. enteritidis* caused by H_2O_2 stress could regain culturability by the addition of sodium pyruvate but not pyruvate analogs (like phenyl pyruvate or bromoacetone), thus confirming that pyruvate was one of the key molecules in the process of resuscitation by triggering the synthesis of macromolecules such as DNA and protein.

In addition, VBNC cells can also be resuscitated by biological stimuli such as eukaryotic cells. Senoh et al. (2010) found that the VBNC cells of *V. cholerae* could be converted into a culturable state after co-cultivating with eukaryotic cells. However, another study showed that VBNC *C. jejuni* cells could form colonies on agar plates after co-cultivation with Caco-2 cells, but most

VBNC cells could not be resuscitated (Chaisowwong et al., 2012), which indicated that the presence of host cells was a biological stimulus factor that can trigger the resuscitation of a fraction of VBNC cells. Additionally, Imamura et al. (2015) discovered a phenomenon in which VBNC cells of *V. cholerae* were initially converted into a culturable state by treatment with HT-29 cell extract or catalase but subsequently entered a state from which they could not be resuscitated. These non-resuscitated cells were verified to be viable by fluorescence microscopy and could be resuscitated by co-cultivation with HT-29 human colon adenocarcinoma cells. However, all cells entered a state from which they could not be resuscitated, even by co-cultivation with HT-29. Thus, the VBNC cells that were resuscitated by biological factors could not be maintained for a long time because the requirements for resuscitated VBNC cells changed over time such that the HT-29 cells could not always maintain resuscitation of the VBNC cells.

Resuscitation Mechanism of VBNC Cells

Although the resuscitation mechanism of VBNC cells remains largely unknown, studies examining resuscitation from the VBNC state have provided many promising results, and people have gradually acquired a greater understanding of the resuscitation mechanism with the development of molecular biology. Resuscitation promoting factor (Rpf), a highly conserved protein composed of 220 amino acids that is directly related to the resuscitation of VBNC cells, has been demonstrated to restore the growth and reproductive ability of VBNC cells (Mukamolova et al., 1998). Rpf proteins have been shown to act as cytokines that, when secreted into the medium by growing cells, bind to the surface receptors of dormant cells and trigger resuscitation (Pinto et al., 2015). Panutdaporn et al. (2006) also showed that the growth of *S. enterica* serovar Oranienburg cells could be enhanced by a certain concentration of rRpf protein. Moreover, Pinto et al. (2011) observed that Rpf supernatant fluid treated with proteinase K could resuscitate the VBNC cells of *E. coli*, suggesting that the breakdown products of Rpf could also restore VBNC cells. In addition, Pinto et al. (2013) validated the hypothesis that two Rpfs of *L. monocytogenes*, Lmo0186 and Lmo2522, could promote resuscitation via a mechanism analogous to actinobacteria Rpf proteins. Although the mechanism of Rpf in VBNC cell resuscitation is still not well understood, most researchers believe that the mechanism of Rpf is similar to that of lysozyme, both of which play a role in hydrolysis to divide the peptidoglycan in the cell wall (Keep et al., 2006a). There are two viewpoints regarding the mechanism of Rpf (**Figure 2**): one is that the breakdown product(s) of peptidoglycan by Rpf may interact with other factors and function as 'second messengers' to stimulate the resuscitation and growth of VBNC cells; the other is that Rpf is required to cleave peptidoglycans with inhibitory properties that are distributed in specific areas of the dormant cell wall and thus promote cell division and growth resumption (Keep et al., 2006b). To provide insights into the regulatory mechanism of Rpf protein, Aydin et al. (2011) obtained high-level expression of recombinant *V. parahaemolyticus* YeaZ in *E. coli* to determine the atomic structure and elucidate the three-dimensional structural conservation in YeaZ homologs, which may broaden perspectives regarding the mechanism of Rpf. However, the mechanism of Rpf and its breakdown products are still not clear and require further study.

In contrast, Moorhead and Griffiths (2011) found that *C. jejuni* could respond to quorum-sensing (QS) signaling molecules (such as C4-HSL, 3OH-C4-HSL, C12-HSL, and HSL), which indicated that biofilm formation was blocked and the entry of cells into the VBNC state was delayed. It was speculated that QS signaling molecules were related to formation of the VBNC state. This conjecture was further confirmed by Ayrapetyan et al. (2014), who only found that the QS signaling molecule autoinducer-2 (AI-2) could directly awaken the VBNC cells of *V. vulnificus* but also that the AI-2 deletion mutant lost resuscitation ability from the VBNC state. Bari et al. (2013) also demonstrated that the resuscitation of dormant *V. cholera* was dramatically improved by the addition to the enrichment medium of biologically synthesized AIs, suggesting that these molecules might signal to dormant cells and then improve conditions for better growth. Furthermore, *rpoS* deletion mutant strains could not be resuscitated even with the addition of exogenous AI-2. This result shows that RpoS is not only a significant protein for VBNC formation but also an important participant in the resuscitation process mediated by AI-2.

PUBLIC HEALTH AND FOOD SAFETY OF VBNC FOODBORNE PATHOGENS

It is worth discussing whether VBNC pathogens maintain their pathogenicity if they are unable to be resuscitated. Although there is no relevant information to confirm that pathogenic bacteria that remain in the VBNC state can cause human disease, it has been reported that some VBNC pathogens retain pathogenic effects. For example, Oliver and Bockian (1995) described mice that were in a lethal state after inoculation with VBNC cells of *Vibrio vulnificus*. Amel et al. (2008) observed a fluid accumulation in the rabbit ileal loop assay (RICA) in response to VBNC *V. cholerae* O1. VBNC *Legionella pneumophila* that retained the capacity to infect the amoeba, which is its natural host (Al-Bana et al., 2014). Furthermore, one study demonstrated that VBNC *enteropathogenic E. coli* showed pathogenicity due to the continual production of enterotoxin (Pommepuy et al., 1996), and another study showed that *uropathogenic E. coli* that remained in the VBNC state may be the major causative agent of recurrent urinary tract infections in many individuals (Anderson et al., 2004). However, some VBNC pathogens have been confirmed to be non-pathogenic. Compared with culturable cells, the VBNC cells of *L. monocytogenes* were avirulent because of a failure to colonize the spleen of mice or adhere to HT-29 cells (Cappelier et al., 2007), VBNC *C. jejuni* cells were unable to colonize the caecum of newly hatched leghorn chicks (Ziprin et al., 2003) and VBNC *Salmonella typhimurium* could not infect all mice in the experiment (Habimana et al., 2014). In a word, when VBNC cells are unable to resuscitate in animals, some of them are still pathogenic and others are avirulent. The pathogenicity of VBNC cells in animals may differ due to

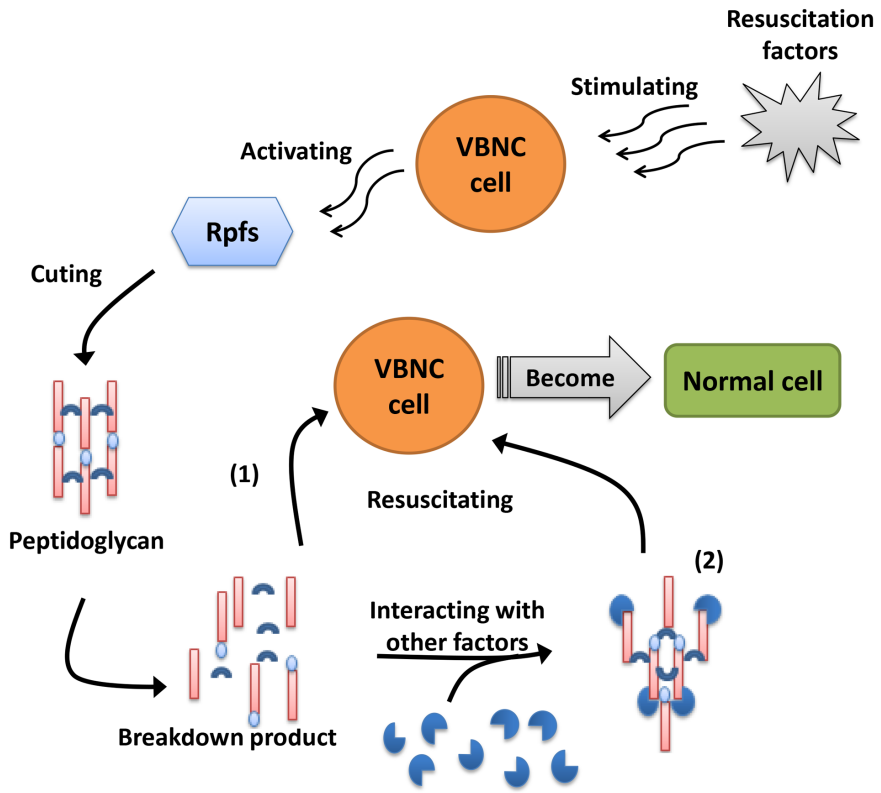

FIGURE 2 | Two viewpoints about the mechanism of Rpfs. (1) Rpfs are required to cleave the peptidoglycans with inhibitory properties distributed in specific area of dormant cell wall and thus promote cell division and growth again. (2) The breakdown product(s) of peptidoglycan divided by Rpfs may interact with other factors and function as "second messengers" to stimulate the resuscitation and growth of VBNC cells.

different strains or the type of animals. To better understand the pathogenicity of VBNC cells, we propose to expand the research to include assessments of the pathogenicity of different VBNC cells and to attempt to distinguish the VBNC cells that can directly lead to disease.

Interestingly, Amel and Amina (2008) found that VBNC *S. typhimurium* cells were only recovered into culturable cells by oral administration but not by intraperitoneal injection in mice, indicating that the intestinal environment might be an essential condition for resuscitation. However, Habimana et al. (2014) demonstrated that *S. typhimurium* failed to resuscitate during passage through the gastrointestinal tract. We speculate that there may be several reasons for the different pathogenic characteristics of the same strain in mice. First, the strains of *S. typhimurium* were not the same in the two experiments; the former was LT4 and the latter was ATCC 14028. Second, the differences between the experimental animals in the two experiments may have affected the results of the experiment, such as the different genders and ages. Third, the pressure that caused the bacteria to enter the VBNC state and the induction method may determine the resuscitation ability of VBNC cells. As shown in **Table 1**, most of the VBNC cell resuscitation methods are based on the method of induction, mainly stress relief, such as *E. coli* O157:H7 induced by low temperature and the corresponding increase in temperature for resuscitation. A similar hypothesis was also proposed by Habimana, who proposed that the phenomenon was dependent on how the pathogens were originally induced into a non-culturable state (Habimana et al., 2014). The specific cause of this phenomenon is still uncertain, requiring further experiments to reach a definitive conclusion.

In the previous section (Introduction), we have described the role of VBNC pathogens in public health and food safety, and even their involvement in many foodborne outbreaks; however, there is no evidence to show that VBNC pathogens directly caused the outbreak. We propose that one of the most possible reasons is the undetectability of VBNC cells. Recent findings showed that 20% of illnesses can be linked to known pathogens, but the remaining 80% are due to unspecified or unidentified agents (Nicolò and Guglielmino, 2012), indicating that VBNC pathogens may be ignored during most outbreaks due to undetectability. For example, in 2011 in Germany, there was a large outbreak caused by an *E. coli* O104:H4 strain expressing genes characteristic of enterohemorrhagic (EHEC) and enteroaggregative *E. coli* (EAEC), involving more than 3000 cases of bloody diarrhea and haemolytic uremic syndrome. Unfortunately, the local detection department failed to detect or isolate the *E. coli* O104:H4 strain from the source of contamination until a small amount of the pathogens were

isolated from the patients (Aurass et al., 2011; Scheutz et al., 2011). We suggest that the main reason for the failure to isolate the *E. coli* O104:H4 outbreak strain is that this outbreak strain entered a non-culturable state. Aurass et al. (2011) also confirmed that the *E. coli* O104:H4 outbreak strain induced by copper ions or tap water of outbreak area entered the VBNC state and was resuscitated to become a potentially pathogenic bacterium by stress relief. This finding implies that VBNC *E. coli* O104:H4 may invade the human body through contaminated food, undergo resuscitation and thus lead to disease.

The presence of VBNC cells in food is widely documented (Rowan et al., 2015). Food is frequently exposed to a complex environmental system, in which physiochemical characteristics (pH, a_w, disinfectant and chemical composition) and environmental factors (high pressure CO_2, elevated temperatures, storage temperature and time, decontamination treatments, pasteurization and packaging under modified atmosphere) act simultaneously on contaminating bacteria leading to the VBNC state. This alone poses a significant risk to the public health and food safety, as these bacteria cannot be detected by commonly used techniques (Fakruddin et al., 2013). This risk is made even greater by the fact that VBNC cells can resuscitate within the human host (Ayrapetyan and Oliver, 2016). Furthermore, studies have proven that VBNC cells of foodborne pathogens, continue to produce virulence factors in food (Dinu and Bach, 2011). These studies indicate that more effective methods for detection of foodborne pathogen must be employed, to tackle the threat posed by VBNC bacteria with regard to public health and food safety.

CONCLUSION

After decades of research, the VBNC concept has attracted great attention for a variety of foodborne pathogens and the corresponding adaptation mechanisms. It seems clear that the conditions, factors and regulators during the induction and resuscitation of the VBNC state play prominent roles in some strains. However, the formation and resuscitation mechanism of the VBNC state remain unclear and thus require further study. The abilities of VBNC cells to evade detection by conventional plate counting techniques, to tolerate stressful environments including food pasteurization processes and antibiotics, and to resuscitate with virulence and cause disease could pose a great threat to food safety and infectious disease prevention. Therefore, the development of rapid, sensitive, cost-effective, and easy-to-operate methods for detection of the VBNC state is an urgent need. In conclusion, the potential application of fundamental research examining the VBNC state is very important to prevent foodborne infections, protect food safety and identify new treatments to reduce the risk of disease caused by foodborne pathogens.

AUTHOR CONTRIBUTIONS

XZ, JZ, and TD wrote the manuscript. CW and C-WL participated in its organization and helped to draft the manuscript. XZ, JZ, and TD revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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frontiers in Microbiology

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Effect of Dietary Minerals on Virulence Attributes of *Vibrio cholerae*

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Vibrio cholerae is a water-borne pathogen responsible for causing a toxin-mediated profuse diarrhea in humans, leading to severe dehydration and death in unattended patients. With increasing reports of antibiotic resistance in *V. cholerae*, there is a need for alternate interventional strategies for controlling cholera. A potential new strategy for treating infectious diseases involves targeting bacterial virulence rather than growth, where a pathogen's specific mechanisms critical for causing infection in hosts are inhibited. Since bacterial motility, intestinal colonization and cholera toxin are critical components in *V. cholerae* pathogenesis, attenuating these virulence factors could potentially control cholera in humans. In this study, the efficacy of sub-inhibitory concentration (SIC, highest concentration not inhibiting bacterial growth) of essential minerals, zinc (Zn), selenium (Se), and manganese (Mn) in reducing *V. cholerae* motility and adhesion to intestinal epithelial cells (Caco-2), cholera toxin production, and toxin binding to the ganglioside receptor (GM1) was investigated. Additionally, *V. cholerae* attachment and toxin production in an *ex vivo* mouse intestine model was determined. Further, the effect of Zn, Se and Mn on *V. cholerae* virulence genes, *ctxAB* (toxin production), *fliA* (motility), *tcpA* (intestinal colonization), and *toxR* (master regulon) was determined using real-time quantitative PCR. All three minerals significantly reduced *V. cholerae* motility, adhesion to Caco-2 cells, and cholera toxin production *in vitro*, and decreased adhesion and toxin production in mouse intestine *ex vivo* ($P < 0.05$). In addition, Zn, Se, and Mn down-regulated the transcription of virulence genes, *ctxAB, fliA,* and *toxR.* Results suggest that Zn, Se, and Mn could be potentially used to reduce *V. cholerae* virulence. However, *in vivo* studies in an animal model are necessary to validate these results.

Keywords: ***Vibrio cholerae,*** **virulence, adhesion, motility, cholera toxin, essential minerals, gene expression**

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INTRODUCTION

Vibrio cholerae, the causative agent of cholera, is a motile, Gram-negative, comma shaped bacterium responsible for producing profuse diarrhea in humans. Of the 150 serogroups of *V. cholerae,* O1 and O139 are important because they are responsible for causing pandemics across Asia, Africa, and Latin America (Faruque et al., 1998; Sack et al., 2006). The O1 serogroup is further subdivided into classical and El Tor biotypes. The classical biotype was responsible for six cholera pandemics before 1961, whereas the El Tor biotype was implicated in pandemics after 1961 (Ramamurthy et al., 2003).

The pathogenesis of cholera is primarily mediated by *V. cholerae* motility, followed by binding to the intestinal epithelial cells and production of cholera toxin in the intestinal lumen. Cholera toxin is an oligomeric protein complex composed of two subunits, subunit A (28 kDa) and subunit B (11 kDa). Once attached to the intestinal epithelium, *V. cholerae* releases the exotoxin, which by means of its pentameric subunit B binds to the GM1 ganglioside receptor present on the surface of intestinal epithelial cells, thereby leading to endocytosis of the toxin into the cell cytoplasm. Following the dissociation of the toxin in the endoplasmic reticulum, the monomeric subunit A causes an increase in the cytoplasmic adenylate cyclase activity leading to an ATP-mediated efflux of sodium and potassium ions in the intestinal lumen. To counter the increase in solute concentration in the lumen, the cells secrete water, and this accumulation of water in the lumen causes dehydrating diarrhea in patients affected by *V. cholerae* (Ichinose et al., 1987; Ikigai et al., 1996; Olson and Gouaux, 2005; O'Neal et al., 2005; Sánchez and Holmgren, 2011). Thus, reducing *V. cholerae* motility, intestinal attachment and toxin production could potentially control cholera in humans.

Currently, the most common treatment strategy against cholera is oral rehydration therapy (Iwu et al., 1999; Sack et al., 2006), which only restores fluids to patients and aids in recovery from dehydration. Although antibiotics are frequently administered to reduce the severity and duration of the disease, a majority of *V. cholerae* strains from cholera endemic countries have been reported to be resistant to multiple antibiotics (Garg et al., 2000; Chakraborty et al., 2001). Moreover, oral vaccines against *V. cholerae* are not completely effective in providing protective immunity (World Health Organization [WHO], 2011 Guidelines). Thus, there is a need for effective and easily implementable approaches that primarily target the pathogen for controlling cholera.

Since ancient times, metals have been known to exert antimicrobial effect against various microorganisms. Following the discovery of antibiotics in the 1920s, the use of metals as antimicrobial agents diminished rapidly in human medicine. However, with the emergence of antibiotic resistant strains of pathogens and a lack of new and effective antibiotics, interest in the use of metals as antimicrobial agents is renewed. Zinc, selenium, and manganese are naturally occurring essential microelements recommended for daily intake by the United States Food and Drug Administration. These minerals are present in a wide range of foods in addition to their presence in dietary supplements. Selenium plays structural and enzymatic roles in many biological processes in humans (Rayman, 2000; Kryukov et al., 2003). Manganese (Mn) is an essential micronutrient critical for the activity of a variety of enzymes, and is required for proper immune function, regulation of blood sugar and cellular energy, reproduction, and blood coagulation (Horning et al., 2015). Manganese (Mn) possesses potent anti-inflammatory and anti-oxidant properties (Kovala-Demertzi et al., 2009). Zinc (Zn) is important for cell division and growth, and proper functioning of the immune system. The antimicrobial property of Zn is well documented (Espitia et al., 2012; Singh et al., 2012), and its supplementation has been reported to exert a beneficial effect in controlling diarrhea in children (World Health Organization [WHO], 2009, 2011). A study conducted in Bangladesh found that zinc supplementation significantly reduced the duration of diarrhea and stool output in children with cholera (Roy et al., 2008).

The objective of this study was to investigate the effect of sub-inhibitory concentration (SIC, highest concentration that does not affect bacterial growth) of Zn, Se, and Mn in reducing *V. cholerae* motility, adhesion to intestinal epithelial cells, cholera toxin production, and toxin binding to GM1 ganglioside receptor *in vitro*. Since intestinal attachment and toxin production play a key role in the pathogenesis of cholera, an *ex vivo* mouse intestine model was also used to determine the effect of metals *V. cholerae* adhesion and cholera toxin production. In addition, the effect of Zn, Se, and Mn on the transcription of genes associated with aforementioned virulence factors was studied.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

All bacteriological media were purchased from Difco (Becton Dickinson, Sparks, MD, United States). Three strains of *V. cholerae*, including BAA-25870, BAA-2163, and N16961 (denoted as VC 569b, VC 2163 and VC N16961, respectively) were used. Each strain was individually cultured in sterile 10 mL tubes containing alkaline peptone broth supplemented with 0.5% sodium bicarbonate, and incubated at 37°C for 24 h (Tran et al., 2012). Following incubation, the cultures were sedimented into a pellet by centrifugation (3600 × *g* for 15 min), washed twice and resuspended in 10 mL of sterile phosphate buffered saline (PBS, pH 7.0). Serial 10-fold dilutions were plated onto Tryptic Soy Agar (TSA) and Thiosulfate Citrate Bile Salt Sucrose agar (TCBS) plates, and *V. cholerae* colonies were enumerated after incubation at 37°C for 24 h.

SIC Determination

The SIC of Zn (Zinc chloride, Sigma–Aldrich, St. Louis, MO, United States), Se (Sodium selenite, Sigma–Aldrich) and Mn (Manganese chloride, Sigma–Aldrich) was determined, as previously described (Johny et al., 2010; Amalaradjou et al., 2011; Upadhyay et al., 2013). Briefly, ∼ 5 log *V. cholerae* was added to sterile 10 mL tubes containing alkaline peptone broth containing 0.5% Sodium bicarbonate, followed by the addition of tapering concentrations of Zn, Se, and Mn from 0.2% to 0.005% (wt/vol) in increments of 0.05%. The tubes were incubated at 37°C for 24 h, and bacteria were enumerated as described before on TSA and TCBS agar. The highest concentration of each metal that did not inhibit *V. cholerae* growth at 24 h was selected as the respective SIC for this study.

Motility Assay

The effect of Zn, Se, and Mn on *V. cholerae* motility was determined according to a published protocol (Niu and Gilbert, 2004). Briefly, Luria Bertani (LB) agar (0.3%) containing the respective SIC of Zn, Se, and Mn was prepared. LB agar without

the metals served as control. An overnight culture of *V. cholerae* was centrifuged at 3600 × *g* for 15 min and was washed three times with sterile PBS. Twenty μL of the resuspended culture (∼8 log CFU/mL) was spot inoculated at the center of the LB agar plates, incubated at 37°C for 16 h, and the zone of motility was measured (cm).

Cell Culture

Human intestinal epithelial cells (Caco-2, ATCC-HTB37, Manassas, VA, United States) were maintained in DMEM (GIBCO, Invitrogen, Carlsbad, CA, United States) containing 10% Fetal Bovine Serum (FBS, Invitrogen). Trypsin-treated cells were passaged five times and were seeded onto 12-well cell culture plates (∼6×10^5 cells per well), and grown at 37°C in the presence of 5% CO_2 for 48 h to form a monolayer.

Adhesion Assay

The effect of Zn, Se, and Mn on *V. cholerae* adhesion to Caco-2 was investigated by following a published protocol (Krebs and Taylor, 2011). Briefly, the cells were seeded in 24-well tissue culture plates and incubated at 37°C in a humidified, 5% CO_2 incubator for 18 h. Overnight culture of each *V. cholerae* was washed and resuspended in Caco-2 culture medium supplemented with SIC of Zn, Se, or Mn. Approximately 6 log CFU (MOI of 10:1) of *V. cholerae* suspension was added to the monolayer either in the presence or absence of Zn, Se, and Mn. Following a 2 h incubation at 37°C, the infected monolayer was rinsed three times with sterile PBS and the cells were lysed with 0.1% Triton X-100. Viable adherent bacteria were enumerated by serial 10-fold dilution in PBS and plating on TCBS agar following incubation at 37°C for 24 h.

Cholera Toxin Quantification

The effect of Zn, Se, and Mn on toxin production by *V. cholerae* strains was determined using Enzyme linked Immunosorbent assay (ELISA) that was customized for Cholera toxin using a published method with alterations (Upadhyay et al., 2013). Protein detector ELISA kit (Kierkegaard and Perry Laboratories, Gaithersburg, MD, United States) was standardized using pure cholera toxin (Sigma–Aldrich) and Cholera toxin b-subunit (Sigma–Aldrich). *V. cholerae* was grown for 24 h at 37°C in sterile 10 mL alkaline peptone broth with the SIC of Zn, Se, or Mn. The cultures were centrifuged at 4000 × *g* for 20 min and the supernatants were collected. Polystyrene 96-well ELISA plate (Fisher Scientific, Pittsburg, PA, United States) was coated with anti-Cholera toxin B-subunit (Sigma–Aldrich) that was diluted 1:500 in coating buffer. After 1 h incubation, the wells were washed five times with a washing buffer followed by a 15 min blocking step with 1% bovine serum albumin (BSA, Sigma–Aldrich) to prevent non-specific binding. Following the blocking step, the sterile filtered supernatant of *V. cholerae* cultures was incubated in the wells for 2 h. After washing, the wells were incubated with a HRP-conjugated antibody against the B-subunit (1:2500 dilution) of the toxin. Peroxide solution was added and the colorimetric analysis was done using a spectrophotometer at 405 nm. As a positive control, 100 ng/mL of cholera toxin was incubated in a separate well. The results were represented as ng/mL of the toxin in the supernatant based on a standard curve.

Cholera Toxin-GM1 Binding Assay

The effect of Zn, Se, and Mn on the binding of Cholera toxin to GM1 receptor was determined using an ELISA protocol (Lindholm et al., 1983). Briefly, 0.5 μg/mL of GM1 was coated onto 96-well polystyrene plates. The wells were then blocked with 1% BSA to prevent non-specific binding. The culture free broth containing the pure toxin treated without (control) and with SIC of Zn, Se, or Mn was then added to the wells, and incubated for 2 h. Following a 5X wash step, HRP-conjugated secondary antibody was added and incubated for 1 h. Peroxide solution was subsequently added to the wells and the optical density was measured at 405 nm.

Ex Vivo Mouse Intestine Adhesion and Toxin Production Assay

Ex vivo V. cholerae adhesion assay was performed following a published protocol (Liu et al., 2011; Ajjampur et al., 2016). Briefly, from euthanized 5-week old BL6 mice (procured from the Department of Physiology and Neurobiology, University of Connecticut), small intestine was cut open, trimmed to equal-sized pieces, and placed in 6-well dishes containing DMEM, supplemented with 10% FBS. Approximately 6 log CFU of *V. cholerae* were inoculated to wells with or without the SIC's of Zn, Se, and Mn. Viable adherent *V. cholerae* to mouse intestine were enumerated after 24 h by homogenization and serial 10-fold dilutions and plating on TSA+1.5% agar plates followed by incubation at 37°C. In addition, cell free supernatant obtained from each well and subjected to centrifugation at 4000 × *g* for 20 min was used to estimate the toxin concentration.

RNA Isolation and Real-Time Quantitative PCR (RT-qPCR)

The effect of Zn, Se, and Mn on the expression of *V. cholera* virulence genes was investigated using real-time quantitative PCR (RT-qPCR) (Coutard et al., 2007). Each *V. cholerae* strain was grown separately with the SIC of Zn, Se, or Mn at 37°C in alkaline peptone broth, and total RNA was extracted after 24 h using a RNeasy RNA isolation kit (Qiagen, Valencia, CA, United States). The complementary DNA (cDNA) synthesized using the IScript cDNA synthesis kit (Bio-Rad, Hercules, CA, United States) was used as the template for RT-qPCR. The amplification product was detected using Power® SYBR green reagent (Bio-Rad). The primers of each gene used in this study (*ctxAB, fliA, tcpA,* and *toxR*) were designed from published *V. cholerae* sequences using NCBI Primer Blast. Relative gene expression was determined using comparative critical threshold (ct) method using a 7500 Step one real Time PCR system (Applied Biosystems, Carlsbad, CA, United States). Data were normalized to the endogenous control (16S rRNA), and the level of the candidate virulence gene expression between treated and control samples was compared by analyzing the RQ values.

Statistical Analysis

A completely randomized design was used for the study. All experiments had duplicate samples and were repeated three times. The data from independent, replicate trials were pooled and analyzed using the PROC MIXED subroutine of the Statistical Analysis Software (SAS ver. 9.2). The least significant difference test was used to determine significant differences ($P < 0.05$) due to treatments on bacterial counts. Data comparisons for the gene expression study were made by using Student's *t*-test. Differences were considered significant when the *P*-value was < 0.05.

RESULTS

Sub-inhibitory Concentration and Effect of Zn, Se, and Mn on *V. cholerae* Motility

The SIC of Zn, Se, and Mn that did not inhibit the growth of *V. cholerae* strains at 24 h of incubation were 0.6, 3, and 4.5 mM, respectively. The effect of SIC of Zn, Se, and Mn on *V. cholerae* motility is shown in **Figure 1**. The average zone of motility produced by VC 569b in the absence of metals (control) was ∼8.5 cm. However, in the presence of SIC of Zn, Se, and Mn, the zone of motility was reduced to 4.0, 0.9, and 0.85 cm, respectively ($P < 0.05$). Similarly, the average zone of motility of untreated VC 2163 and VC N16961 was 8.5 cm. In the presence of Zn, the motility zone of these isolates was decreased to ∼50%, whereas Se and Mn reduced the motility by ∼90% ($P < 0.05$). Taken together, these results show that Zn, Se, and Mn have a significant inhibitory effect on *V. cholera* motility.

Effect of SIC of Zn, Se, and Mn on *V. cholerae* Adhesion to Caco-2 Cells

The SIC of Zn, Se, and Mn significantly reduced the number of adherent *V. cholerae* to Caco-2 cells **Figure 2**. Approximately 6.0 log CFU/mL *V. cholerae* attached to the intestinal epithelial cells in the control wells for all three strains used in this study. However, Zn, Se, and Mn decreased Caco-2 cell adhesion of all *V. cholerae* isolates by ∼2 log CFU/mL ($P < 0.05$), indicating that these minerals have a significant inhibitory effect on *V. cholerae* adhesion to intestinal epithelial cells.

Effect of SIC of Zn, Se, and Mn on Cholera Toxin Production

The toxin concentration in the filtered supernatant was determined using ELISA, and the results are shown in **Figure 3**. In control samples, the concentration of toxin produced by the three isolates ranged from 2000 ng/mL (by VC 569b) to 4500 ng/mL (by VC N16961). However, at the respective SIC, Zn, Se, and Mn significantly reduced the toxin production in all three *V. cholerae* isolates ($P < 0.05$). For example, Zn decreased the toxin concentration to 14.5, 8.8, and 150 ng/mL in isolates 569b, 2163, and N16961, respectively. Similarly, the total concentration of the toxin detected the culture supernatant of Se-treated 569b, 2163, and N16961 were 11.8, 9.4, and 150 ng/mL and in Mn-treated culture supernatant 569b, 2163, and N16961 were 24, 7.458, and 97.25 ng/mL, respectively. Collectively, these results show that Zn, Se, and Mn reduced the production of Cholera toxin by more than 95% compared to control.

Effect of Zn, Se, and Mn on the Binding of Cholera Toxin to GM1

Since the binding of Cholera toxin to the GM1 receptor in the human intestinal epithelial cells is a critical step *V. cholerae* pathogenesis, we investigated the effect of SIC of Zn, Se, and Mn on the binding of toxin to the receptor. Results indicate that the metals did not exert any effect on toxin binding to GM1 receptor.

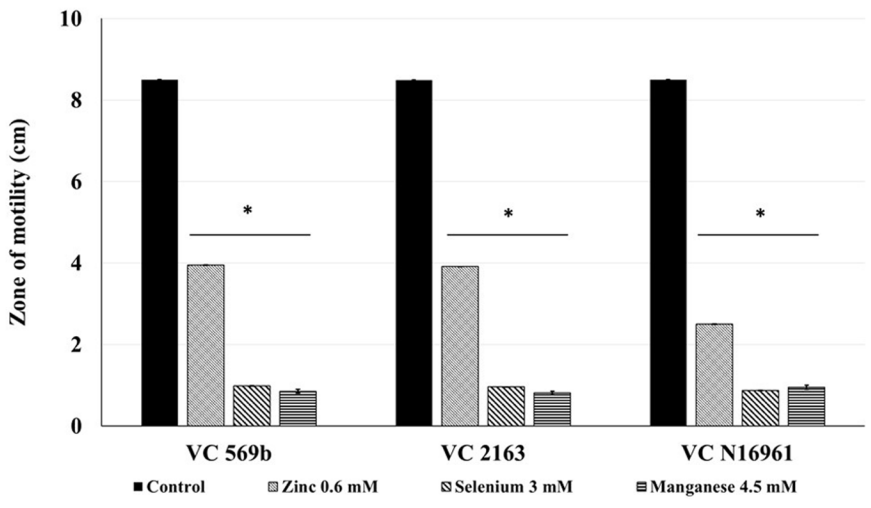

FIGURE 1 | Effect of SIC's of Zn, Se, and Mn on motility of *Vibrio cholerae* (Strains VC 569b, VC 2163, and VC N16961). Error bars represent SEM ($n = 6$; $P < 0.05$). *Treatment significantly different from control.

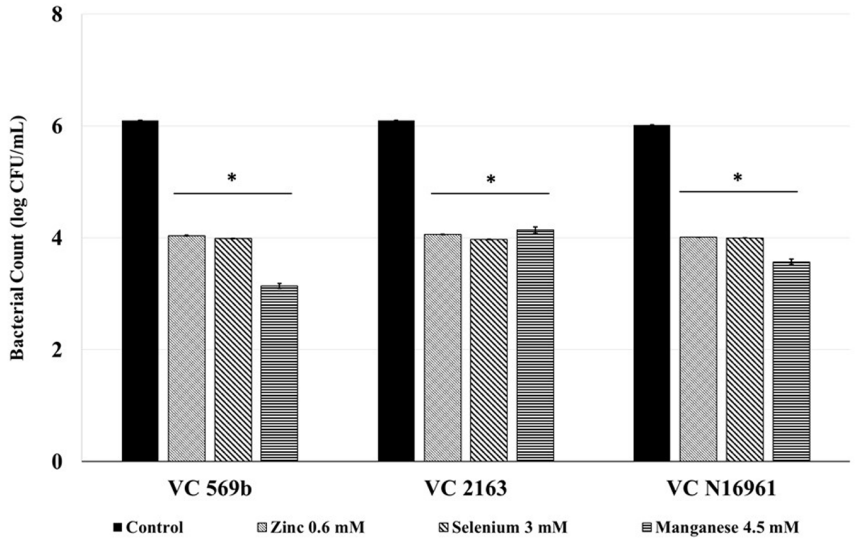

FIGURE 2 | Effect of SIC's of Zn, Se, and Mn on *V. cholerae* adhesion (Strains VC 569b, VC 2163, and VC N16961) to Caco-2 intestinal epithelial cells. Error bars represent SEM ($n = 6$, $P < 0.05$). *Treatment significantly different from control.

Ex Vivo Mouse Intestine Adhesion and Toxin Production Assay

Following a 24 h incubation period, the count of adhered *V. cholerae* in control was approximately 6.0 log CFU/mL. Compared to control, Zn, Se, and Mn (**Figure 4**) reduced bacterial attachment by ∼2.0 log CFU/mL ($P < 0.05$). Similarly, the amount of toxin produced by the three *V. cholerae* isolates in control samples ranged from 700 to 800 ng/mL. Similar to the *in vitro* results, Zn, Se, and Mn reduced toxin production by more than 90% (<100 ng/mL) in all the three strains tested (**Figure 5**).

Effect of Zn, Se, and Mn on the Transcription of *V. cholerae* Virulence Genes

The effect of SIC of Zn, Se, and Mn on the expression of virulence genes in strains VC569b, VC 2163, and VC N16961 is shown in **Figures 6A–C**, respectively. As evident from the RQ values, Zn, Se, and Mn significantly down-regulated the expression of *ctxAB, fliA, tcpA,* and *toxR* in all three *V. cholerae* strains ($P < 0.05$) although the magnitude of reduction was greatest in VC N16961. Among the three genes tested, the down-regulation of *ctxAB* was found to be generally greater in all VC strains compared to other genes.

DISCUSSION

Human cholera continues to pose a significant public health concern in the developing world, with a stable increase in the number of cases reported since 2007 (World Health Organization [WHO], 2012). Currently the most common treatment strategy against cholera is oral rehydration therapy, which primarily restores fluids to patients and aids in recovery from dehydration (Guerrant et al., 2003). In addition, antibiotics are frequently administered to reduce the severity and duration of the disease (Yamasaki et al., 2011; Anthouard and DiRita, 2013). However, the development of bacterial antibiotic resistance is a concern, and a majority of *V. cholerae* strains involved in cholera endemic countries have been reported to be resistant to multiple antibiotics (Garg et al., 2000; Chakraborty et al., 2001; Das et al., 2008). This has triggered renewed interest in identifying effective alternate approaches to control cholera in humans (Fazil and Singh, 2011; Hung et al., 2005; Yamasaki et al., 2011). Treatments that target *V. cholerae* toxin production and/or colonization alone or in combination with existing therapies represent a potential strategy for controlling this pathogen (Anthouard and DiRita, 2013).

A new approach that is increasingly being explored for controlling infectious diseases involves inhibiting bacterial virulence rather than growth, where a pathogen's specific mechanisms critical for causing infection or disease symptoms in hosts are targeted (Rasko and Sperandio, 2010; Anthouard and DiRita, 2013; Khodaverdian et al., 2013). Since anti-virulence agents are neither bacteriostatic nor bactericidal, they exert a reduced selection pressure for the development bacterial drug resistance (Hung et al., 2005; Clatworthy et al., 2007; Cegelski et al., 2008; Mellbye and Schuster, 2011; Maeda et al., 2012), besides being minimally deleterious on the host endogenous microbiota.

Vibrio cholerae is a highly motile organism, where motility as a first step in its pathogenesis helps the bacterium for traversing through the intestine, especially to penetrate through the mucus layer and reach the intestinal cells (Guentzel and Berry, 1975), where it colonizes. Several studies have reported motility as a virulence factor critical for *V. cholerae* colonization and pathogenesis (Guentzel and Berry, 1975; Yancey et al., 1978; Gardel and Mekalanos, 1996; Syed et al., 2009; Millet et al.,

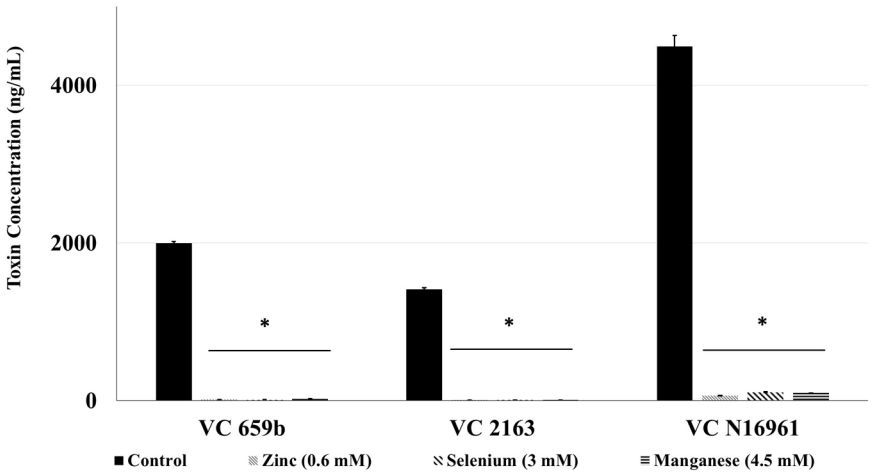

FIGURE 3 | Effect of SIC's of Zn, Se, and Mn on cholera toxin production in *V. cholerae* (VC 569b, VC 2163, and VC N16961). Error bars represent SEM ($n = 6$; $P < 0.05$). *Treatment significantly different from control.

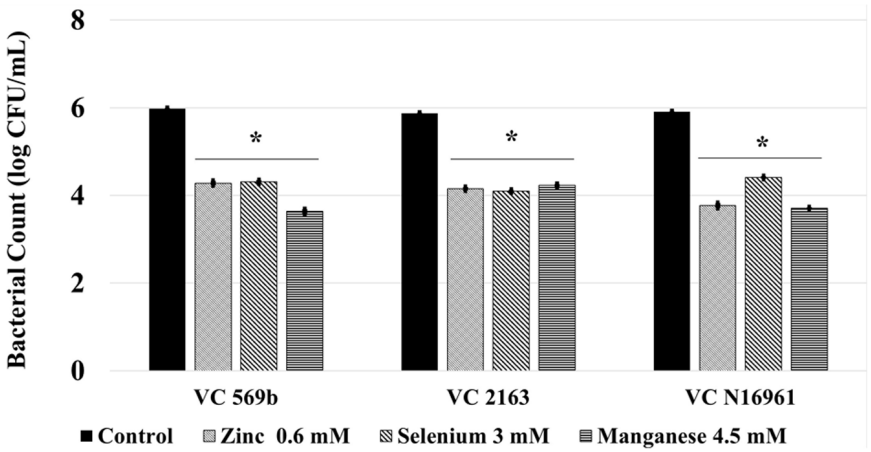

FIGURE 4 | Effect of SIC's of Zn, Se, and Mn on *V. cholerae* adhesion to mice intestine *ex vivo*. Error bars represent SEM ($n = 6$; $P < 0.05$). *Treatment significantly different from control.

2014). The results from the motility assay revealed that Zn, Se, and Mn substantially reduced motility in *V. cholerae*, although the mechanism behind the antimotility effect of the metals is not clear. Previous studies done in *Escherichia coli* O157:H7 and *Campylobacter jejuni* propose that minerals could alter the membrane integrity and cellular morphology thereby affecting the flagellar structures, which are essential for bacterial motility (Catrenich and Makin, 1991; Adams et al., 2003). However, further studies have to be done to elucidate the exact mechanism of action.

Since bacterial attachment to the small intestinal epithelium is essential for *V. cholerae* colonization and toxin production (Krebs and Taylor, 2011), we investigated the effect of Zn, Se, and Mn on its adherence to cultured Caco-2 cells. All three minerals were found to reduce bacterial attachment to the intestinal epithelial cells in all the three tested isolates of *V. cholerae*. Further Zn, Se, and Mn were very effective in inhibiting cholera toxin production, and reduced total toxin concentration by greater than 95% in the three *V. cholerae* isolates. Concurring with the *in vitro* results, Zn, Se, and Mn reduced both *V. cholerae* adhesion and toxin production *ex vivo*. This is important since Cholera toxin produced by the bacterium constitutively activates adenylate cyclase in host cells, and leads to a decrease in sodium uptake with a concurrent increase in chloride influx into the intestinal lumen, thereby resulting in profuse diarrhea and dehydration (Sánchez and Holmgren, 2011). However, Zn, Se, and Mn were not found to affect binding of cholera toxin to GM1 receptor on the intestinal epithelial cells, as revealed by the results from binding assay. These results suggest that Zn, Se, and Mn do not exert an inhibitory effect against the toxin on the host cells, but primarily act through the virulence machinery of the bacterium.

Since the minerals were used at their respective SIC in this study, the attenuation of the *V. cholerae* virulence observed is

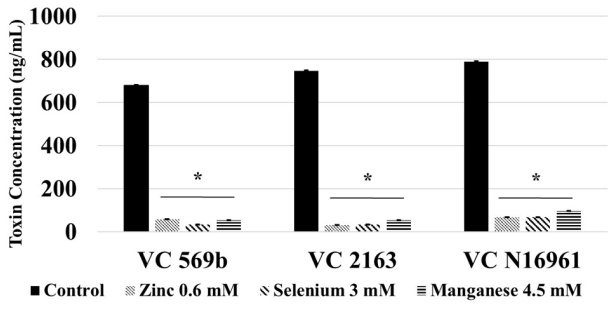

FIGURE 5 | Effect of SIC's of Zn, Se, and Mn on cholera toxin production in *V. cholerae* in mouse intestine *ex vivo* (VC 569b, VC 2163, and VC N16961). Error bars represent SEM ($n = 6$; $P < 0.05$). *Treatment significantly different from control.

not due to growth inhibition, but could be attributed to the effect of metals in modulating the transcription of respective bacterial genes associated with virulence. Therefore, we performed a RT-qPCR to determine the effect of Zn, Se, and Mn on major genes that are known to play a vital role in *V. cholerae* virulence in humans. Of the genes tested, *toxR* is transcription activator that controls many virulence factors, including cholera toxin, pilus colonization factor and outer membrane protein expression in *V. cholerae* (Krukonis et al., 2000; Bina et al., 2003). Similarly, *fliA* is a RNA polymerase sigma factor in *V. cholera* that controls flagella-related genes and motility in the bacterium (Guentzel and Berry, 1975), whereas *ctxAB* encodes the cholera toxin (DiRita et al., 1991). In addition, *tcpA* encodes co-regulated pilus that is responsible for intestinal colonization of *V. cholerae* (Herrington et al., 1988). Concurring with the results from phenotypic tests, it can be observed that Zn, Se and Mn exerted a significant inhibitory effect on the virulence genes in *V. cholerae* (**Figure 4**). However, the magnitude of inhibition of the genes differed in the three strains, with highest down-regulation of all the genes observed in VC N16961. Among the various tested genes, *ctxAB* was generally more sensitive to the inhibitory effect of the minerals, which concurred with the results from phenotypic tests, where cholera toxin production was found to be decreased by more than 95% in all three *V. cholerae* strains. In conclusion, this study indicated that Zn, Se, and Mn significantly reduced the major virulence properties in *V. cholerae*, especially the toxin production. The mechanism by which these minerals attenuate *V. cholerae* virulence is not known; however, emerging evidence suggests metal induced reactive oxygen species (ROS) and genotoxicity could be potential mechanisms by which metals exert antibacterial activity. For example, in enteric pathogens such as *E. coli,* production of ROS and superoxide anions leads to significant damage of DNA, membrane and cellular proteins (Imlay et al., 1988; Critchfield et al., 1993; Imlay, 2003; Parvatiyar et al., 2005; Teitzel et al., 2006; Pérez et al., 2007; Harrison et al., 2009; Warnes et al., 2012). However, transcriptomic analysis of *V. cholerae* exposed to Zn, Se, and Mn could potentially identify critical genome-wide pathways modulated by these essential minerals.

The SIC of Se at which the down-regulation of virulence observed in this study is approximately at the recommended upper tolerable levels (400 μg) and well below the no-observed-adverse-effect level (NOAEL, 800 μg/day) of Se (National Institute of Medicine, 2000). This is also the same for the other two metals, where the upper tolerable limit of Zn is 40 mg and the NOAEL is reported at 50 mg and for Mn, both the upper tolerable limit and the NOAEL have been reported at 11 mg

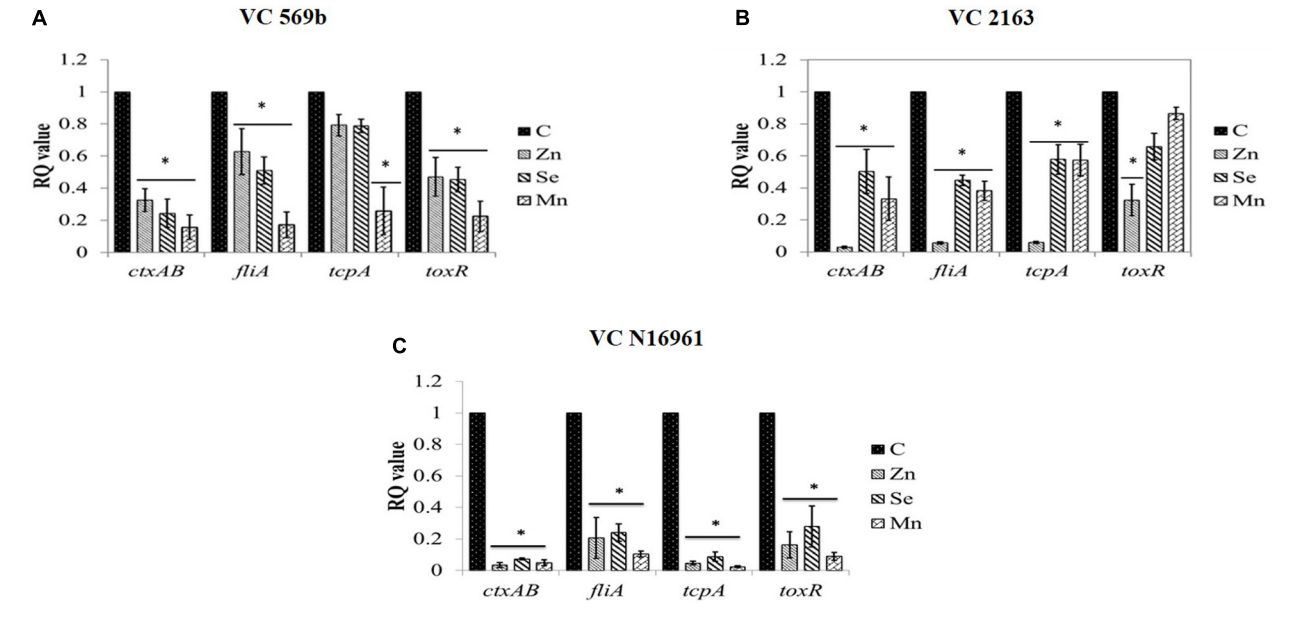

FIGURE 6 | Relative fold change in the expression level of *V. cholerae* (A) Strains VC 569b, **(B)** VC 2163, and **(C)** VC N16961 virulence genes in response to SIC's of Zn, Se, and Mn. *RQ value of treatment significantly different from control.

(Scientific Committee on Food of European Commission [SCFEC], 2002; Valko et al., 2005; De et al., 2006). Since these minerals are soluble in water, they could be incorporated as ingredients in the oral rehydration solution. Follow up *in vivo* studies are needed to validate the safety and efficacy of Zn, Se, and Mn in controlling cholera.

AUTHOR CONTRIBUTIONS

VB, AU, SM, and KV designed the study. VB, AU, H-BY, and SM conducted the experiments. AU and H-BY analyzed the data and VB wrote the manuscript. AU and KV critically analyzed and revised the manuscript.

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Removal of Foodborne Pathogen Biofilms by Acidic Electrolyzed Water

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Biofilms, which are complex microbial communities embedded in the protective extracellular polymeric substances (EPS), are difficult to remove in food production facilities. In this study, the use of acidic electrolyzed water (AEW) to remove foodborne pathogen biofilms was evaluated. We used a green fluorescent protein-tagged *Escherichia coli* for monitoring the efficiency of AEW for removing biofilms, where under the optimal treatment conditions, the fluorescent signal of cells in the biofilm disappeared rapidly and the population of biofilm cells was reduced by more than 67%. Additionally, AEW triggered EPS disruption, as indicated by the deformation of the carbohydrate C-O-C bond and deformation of the aromatic rings in the amino acids tyrosine and phenylalanine. These deformations were identified by EPS chemical analysis and Raman spectroscopic analysis. Scanning electron microscopy (SEM) images confirmed that the breakup and detachment of biofilm were enhanced after AEW treatment. Further, AEW also eradicated biofilms formed by both Gram-negative bacteria (*Vibrio parahaemolyticus*) and Gram-positive bacteria (*Listeria monocytogenes*) and was observed to inactivate the detached cells which are a potential source of secondary pollution. This study demonstrates that AEW could be a reliable foodborne pathogen biofilm disrupter and an eco-friendly alternative to sanitizers traditionally used in the food industry.

Keywords: biofilm, AEW, eradication, EPS, foodborne pathogens

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INTRODUCTION

Foodborne pathogens which persist in food processing facilities grow predominantly as biofilms rather than in planktonic mode (Barnes et al., 1999; Bae et al., 2012). Biofilms are complex communities of microorganisms attached to biotic or abiotic surfaces and protected them by providing firm three-dimensional, multicellular, complex, self-assembled structures that contain extracellular polymeric substances (EPS) (exopolysaccharides, proteins, and extracellular DNA, etc.) (Costerton et al., 1999; Hall-Stoodley et al., 2004). More than 80% of the bacterial infections in the human population are associated with biofilms and approximately 60% of foodborne outbreaks are caused by biofilms (Wolcott and Ehrlich, 2008; Simoes et al., 2010; Bridier et al., 2015). Furthermore, compared to planktonic cells, biofilm-associated cells are more resistant to external stresses such as antibiotics and detergents, thus they are extremely difficult to eliminate resulting in the onset of foodborne illness (Costerton et al., 1987; Hoiby et al., 2010).

It is well documented that biofilm development is one of the most complex physiological processes. Importantly, in the dynamic process, EPS facilitates the trapping of nutrients and maintenance of the structure integrity of the biofilm while also providing a sanctuary for the encased bacterial cells (Li and Yu, 2011; Bassin et al., 2012). Pathogens encased in the EPS-rich matrix, therefore provide a source of contamination when the biofilm interacts with food materials (Carpentier and Cerf, 1993). In addition, the structure of EPS reduces disinfectants access and possibly triggers bacterial tolerance to commonly used sanitizers (Flemming and Wingender, 2010; Xiao et al., 2012; Koo et al., 2013; Lebeaux et al., 2014).

The mode of action of conventional sanitizers to control of foodborne pathogen biofilms is antibacterial rather than EPS matrix disruption (Gao et al., 2016), and any bacteria in the biofilms that survives the sanitizer treatment may initiate biofilm regrowth (Bridier et al., 2015). Thus, novel approaches that include disruption of EPS formation and at the same time kills or removes biofilm cells would be highly desirable (Allaker and Memarzadeh, 2014; Wang et al., 2016). A recent candidate is acidic electrolyzed water (AEW) which has attracted attention in recent years as a promising sanitizing agent in the food, medical, and agricultural industries (Wang et al., 2014). AEW is generated by anodic electrolysis of dilute NaCl solutions and the physicochemical properties include low pH, available chlorine concentration (ACC) and oxidation reduction potential (ORP) (Kim et al., 2000; Xiong et al., 2010; Hao et al., 2012).

Acidic electrolyzed water has been documented to be an effective disinfectant for inactivating foodborne pathogens including *Escherichia coli*, *Vibrio parahaemolyticus,* and *Listeria monocytogenes* (Kim et al., 2000; Wang et al., 2014). The postulated mode of action is reduction of cell wall, nucleus, and outer membrane integrity which leads to the rapid leakage of intracellular DNA and proteins (Zeng et al., 2010, 2011; Ding et al., 2016). Additionally, AEW is an environmental friendly sanitizer and poses minimal risk to human health (Mori et al., 1997; Wang et al., 2014).

Above all, many studies have shown the bactericidal effect of AEW on planktonic pathogens, but study on the applying the AEW for removing foodborne pathogen biofilms is still lacking. Therefore, this study attempted to use the AEW as a novel scavenger to control foodborne pathogen biofilms, and evaluated the eradication effect of AEW on biofilms and EPS disruption.

MATERIALS AND METHODS

Bacterial Strain and Culture Preparation

Escherichia coli K-12 strain ATCC 25404 was used as a model biofilm-forming strain. To generate a fluorescent variant, *E. coli* was transformed with the GFP plasmid pCM18 (Hansen et al., 2001), which conferred resistance to erythromycin. *E. coli* were grown overnight in Luria Bertani (LB, Land Bridge Technology, Beijing, China) broth containing 100 μg/mL ampicillin and IPTG with shaking (250 rpm) at 37°C. *V. parahaemolyticus* S36 and *L. monocytogenes* WaX12 used this study were isolated and stored in our laboratory. *V. parahaemolyticus* S36 and *L. monocytogenes* WaX12 were isolated from shrimp and pork samples by using specific selective media, species-specific gene and API system tests (BioMérieux, Marcyl'Etoile, France). *V. parahaemolyticus* S36 was cultured in tryptic soy broth (TSB, Beijing Land Bridge Technology Company Ltd, Beijing, China) plus 3% NaCl. *L. monocytogenes* WaX12 serotype 1/2a was grown in brain heart infusion (BHI, Land Bridge Technology, Beijing, China). The cultures were diluted to obtain a bacteria population of 9 log CFU/mL.

Biofilms Formation

Biofilm formation experiments were carried out as described previously (Krom and Willems, 2016; Song et al., 2016) with minor modifications. Static biofilms were grown in 24 well polystyrene microtiter plates (Sangon Biotech Co., Ltd, Shanghai, China). The test bacteria cultures were diluted in fresh culture medium (1:100) and aliquoted into wells. *E. coli* was incubated statically to form biofilms for various time (2, 4, 6, 8, 10, 12, 24, and 48 h). *L. monocytogenes* and *V. parahaemolyticus* were incubated statically to form biofilms for 48 h (Ayebah et al., 2006; Song et al., 2016).

Preparation of Acidic Electrolyzed Water

Acidic electrolyzed water was prepared according to Wang et al. (2014). The AEW generator model FW-200 (AMANO Corporation, Kanagawa, Japan) was ran for 15 min with the amperage set as 10 A before collection of a sample for testing. The pH and ORP were determined using a pH/ORP meter (model pH 430, Corning Life Sciences, New York, United States). The ACC in AEW was determined by a colorimetric method using a digital chlorine test kit (RC-2Z, Kasahara Chemical Instruments Corp., Saitama, Japan). All measurements were carried out in triplicate. The physicochemical properties of each AEW are shown in **Table 1**.

Crystal Violet Staining Method and MTT Assay

After incubation, biofilm production was quantified using a crystal violet staining method as described previously by Antoniani et al. (2010). Biofilms in the wells of the polystyrene microtiter plates were air-dried for 10 min, then stained with 1 mL of 0.1% (w/v) crystal violet (Sangon Biotech Co., Ltd, Shanghai, China) for 30 min. The wells were then washed three times with 0.1 M phosphate-buffered saline (PBS) (Sangon Biotech Co., Ltd, Shanghai, China). Biofilm was solubilized using 1 mL of 95% ethanol (Sinopharm Chemical Reagent Co., Ltd.,

TABLE 1 | Physicochemical properties of AEW electrolyzed by the different NaCl concentration.

NaCl concentration (g/L)	pH	ORP (mV)	ACC (mg/mL)
0.1	2.94 ± 0.05	1087.07 ± 1.85	8.67 ± 0.58
1	2.23 ± 0.01	1172.60 ± 3.47	48.33 ± 2.89
3	2.30 ± 0.03	1175.50 ± 3.84	136.33 ± 2.08
5	2.46 ± 0.05	1173.37 ± 3.93	173.67 ± 1.15

Shanghai, China) for 30 min. The optical density of each well was measured at wavelength of 600 nm.

The viability of the biofilm cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay which has been attributed to Mshana et al. (1998) and Krom et al. (2007). One milliliter of culture medium and 0.1 mL of 5 mg/ml MTT solution were added to each well, then incubated at 37°C for 2 h. The culture supernatant was then discarded and 1 mL of dimethyl sulfoxide was added to each well to solubilize the MTT for 2 h. The optical density of each well was measured at wavelength of 570 nm. All measurements were carried out in triplicate and the mean data are presented.

AEW Treatment on Established Biofilms

Following biofilm formation, the suspension was gently aspirated from the plate and the wells were rinsed three times with PBS to remove non-adherent cells. The biofilms were then exposed to 1 mL sterile deionized water (SDW) or AEW produced by different NaCl concentration (0.1, 1, 3, and 5 g/L) marked AEW-1, AEW-2, AEW-3, AEW-4, respectively, at room temperature (25 ± 1°C). Subsequently, 1 mL neutralizing agent (PBS containing 0.8% $Na_2S_2O_3$) was added to stop the bactericidal effects of AEW after a 30 s treatment (Luppens et al., 2002; Wang et al., 2014). Surviving cells were collected by vortexing and scraping of the wells and transferred to tubes containing sterile 0.85% NaCl solution. Serially dilution of the bacterial population was plated onto correspondent agar and incubated at 37°C for 24 h. Three replicates were tested for each treatment. According to Wang et al. (2016), the percentage of reduction biofilm cells (%) = (the cells numbers in control group − the cells numbers in treatment group)/the cells numbers in control group × 100. The percentage of reduction biofilm cells represents the removal efficiency.

Visualization of the Biofilms Using Epifluorescence and Scanning Electron Microscopy

Biofilms which were treated with AEW-3 and SDW and untreated control, were then fixed with 4% glutaraldehyde overnight, and dehydrated in an ascending acetonitrile series (30, 50, 70, 80, 90, and 100% twice for 10 min each). Samples were sputtered with gold and observed with a Nova 450 scanning electron microscope (FEI, Hillsboro, OR, United States). Epifluorescence visualizations were carried out without previous fixation or dehydration and directly observed in a EVOS® FL Auto Cell Imaging System (AMG, Thermo Fisher Scientific, Waltham, MA, United States). We initially observed different areas in one sample at low magnification. Then we choose one area in each sample based on similarity of all images and use high magnification of this area for analysis. The images from three independent experiments with three replications were used for analysis. Pictures were obtained using the same settings for each picture. The fluorescent density in the biofilm cells were quantified by the ImageJ software (National Institutes of Health, Bethesda, MD, United States)[1].

[1]http://rsb.info.nih.gov/ij/

EPS Chemical Analysis

Extracellular polymeric substance in a biofilm was extracted using the sonication method (Liu et al., 2007; Gong et al., 2009; D'Abzac et al., 2010). The density of suspended cultures was initially measured at $OD_{595\ nm}$. The biofilm cells were then collected by vortexing and scraping in 1 mL 0.01 M KCl solution. The cells were pretreated with a sonicator (VCX 500, SONICS, Newtown, CT, United States) for four cycles of 5 s of operation and 5 s of pause at a power level of 3.5 Hz. The sonicated suspension was centrifuged (4,000 rcf, 20 min, 4°C), and the supernatant was then filtered through a 0.22 μm membrane filter (Sangon Biotech Co., Ltd., Shanghai, China). The amounts of protein and carbohydrate in the filtrate were analyzed. The amounts of carbohydrate and protein were quantified by the phenol–sulfuric acid method and Lowry method (Kim and Park, 2013; Nakamura et al., 2013). The amount of protein and carbohydrate were quantified by $OD_{750\ nm}/OD_{595\ nm}$ and $OD_{490\ nm}/OD_{595\ nm}$, respectively. Each experiment was carried out at least three times.

Raman Spectroscope

Extracellular polymeric substance was extracted as described in EPS chemical analysis. All Raman spectrum were obtained by a Senterra R200-L Dispersive Raman Microscope (Bruker Optics, Ettlingen, Germany) at room temperature. Raman spectrum of each sample was determined as the average of fifteen measurements at different random sites on the biofilm. The Raman measurements were recorded with an accumulation time of 60 s in the 500–1250 cm^{-1} range. Raman spectral acquisition and preprocessing of preliminary data were carried out using the Bruker OPUS software.

Statistical Analysis

The percentage of reduction biofilm cells, carbohydrate and protein content in EPS and Raman intensity were analyzed by analysis of one-way ANOVA using the SPSS statistical software (version 19.0; SPSS Inc., Chicago, IL, United States). The level of statistical significance was $p < 0.05$.

RESULTS

The Dynamic Development of *E. coli* Biofilm Formation

Bacterial biofilm formation is a dynamic process with distinct phases of development (Hall-Stoodley et al., 2004). In principle, there are three stages of biofilm development: an initial attachment and growth phase, a mature phase and a dispersal phase. In our model system, stage one was evident when the number of small homogeneous green-fluorescent colonies of biofilm cells gradually increased (**Figures 1BI–III**). **Figure 1A** shows the corresponding increase in cell viability and biomass of the biofilm from 2 h to 6 h. Stage two of the biofilm developmental process could be seen from 8 h to 24 h where we observed the aggregation of cells into mature biofilm (**Figures 1A,BIV–VII**). The mature biofilm showed green fluorescence exclusively in their border regions, resulting

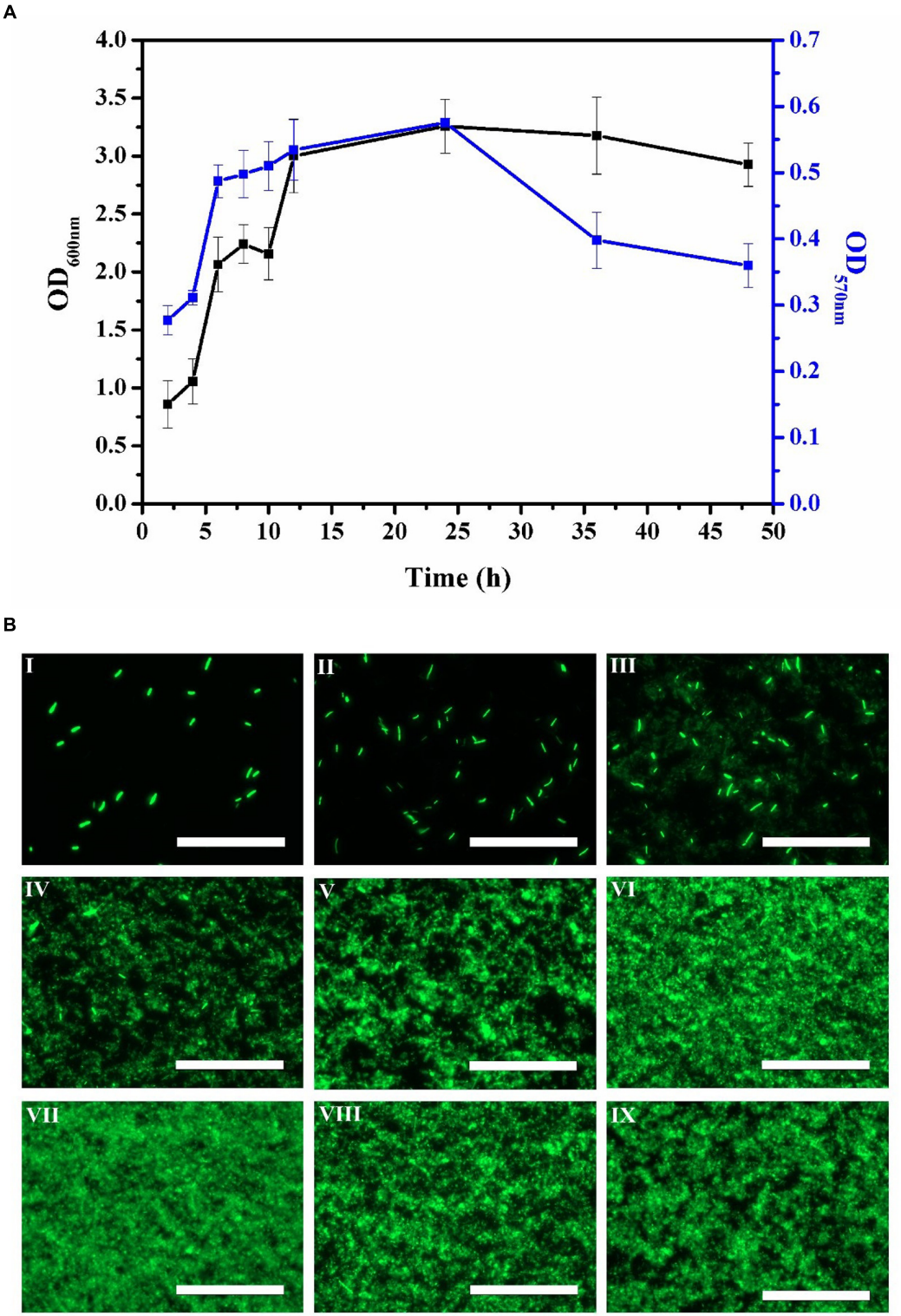

FIGURE 1 | Time course of *E. coli* biofilm production for 2, 4, 6, 8, 10, 12, 24, 36, and 48 h. **(A)** Biofilm biomass ($OD_{600\ nm}$) by crystal violet staining method and biofilm viability ($OD_{570\ nm}$) by MTT assay. Error bars indicated standard deviations of triplicate experiments. **(B)** Fluorescence images during *E. coli* biofilm development process by epifluorescence microscopy, **I-IX** represented 2, 4, 6, 8, 10, 12, 24, 36, and 48 h, respectively. The scale bar represented 100 μm. Pictures were representative of three independent experiments with three replicates each.

in multilayer films of bacterial cells. This indicated that the biofilm had been established and the biomass within the biofilm was relatively constant. After 24 h of cultivation, stage three commenced and the biomass appeared disaggregated as transient motility of the biofilm cells led to dispersal (**Figures 1BVIII,IX**).

The Eradication Effect of AEW on *E. coli* Biofilm

The numbers of *E. coli* cells in the biofilm after 24 h were approximately 6.77 log CFU/mL as confirmed by the plate count method. **Figure 2A** showed the effect of the different AEW treatments to cell numbers in the mature biofilms in our model system. AEW-1, AEW-2, AEW-3, and AEW-4 were produced using increasing concentrations of NaCl and the reduction in cell number was positively correlated to the NaCl concentration. The bactericidal activity of the AEW-3 treatment was optimal and the trend in reduction of biofilm cell number was confirmed by the visualization of *E. coli* biofilms using epifluorescence microscopy (**Figure 2C**). These images showed that only a few scattered viable cell aggregates were observed in the biofilm after 2 min exposure to AEW. The fluorescent intensity, an indication of cell density and viability, was still at maximum intensity after the SDW treatment and then decreased progressively after the AEW treatments. The fluorescent intensity was minimal after AEW-3 treatment and we selected this treatment for further analysis.

Increasing the contact time with AEW-3 from 2 to 10 min increased cell death and removal, minimally (**Figures 2B,D**). Taken together, it was observed that increasing the potency of AEW using increasing NaCl concentration was more important than treatment time for the eradication of *E. coli* biofilm cells. In addition, the number of viable cells which escaped from the biofilm after AEW-3 treatment was below the limit of detection (<1.4 log CFU/mL). However, the residual viable cells after SDW treatment were as much as 6 log CFU/mL. We chose AEW-3 exposure for 5 min for further experimentation.

EPS Analysis

To test the effect of AEW on EPS production, we analyzed the total carbohydrate and protein content of EPS in a 24 h *E. coli* biofilm. As shown in **Figures 3A,B**, both total carbohydrate and total protein were reduced after exposure to SDW and AEW-3 for 5 min. However, total protein was reduced more than total carbohydrate. The total protein with AEW was 65% of the control, whereas total carbohydrate with AEW was 72% of the control. However, SDW treatment resulted in only little reduction of carbohydrate and protein, and had no significant difference compared to control.

Representative Raman spectra of EPS in the spectral fingerprint range of 500–1250 cm^{-1} are presented in **Figure 3C**. The tentative peak assignments of the bands are summarized in **Table 2**. The prominent Raman bands of EPS belonged to carbohydrates: 561 cm^{-1} (C-O-C glycosidic ring def polysaccharide) and 1090–1095 cm^{-1} (C-O-C glycosidic link). The band intensity weakened dramatically with AEW treatment. Besides, EPS after AEW treated showed a shift in 561 cm^{-1} toward 581 cm^{-1}, 1095 cm^{-1} toward 1106 cm^{-1}. Two broad Raman bands at 1020–1085 cm^{-1} and 855–899 cm^{-1} were assigned to C-C stretching of carbohydrates (polysaccharides). After AEW-3 treatment, these intensities were significantly weakened as shown in **Figure 3D**.

Extracellular polymeric substance showed a decrease in the magnitude of Raman intensity at 637–695 cm^{-1}, 830–850 cm^{-1}, 1003 cm^{-1}, and 1005 cm^{-1}, the bands which corresponds to proteins. For example, bands at 1005 cm^{-1} could be observed in the spectrum of the control group and SDW group. However, these bands were not present in the spectra of EPS after AEW treatment.

The reduction of Raman intensity corresponding to DNA, such as the bands at 788 cm^{-1} and 830–850 cm^{-1}, arises from the destruction of the ring structure, indicating degradation of the DNA. This reduction provides further evidence for cell death or removal.

SEM Analysis

To gain further insight into the mode of action of AEW in eradicating biofilms, SEM images of *E. coli* biofilm treated with AEW were performed. Representative SEM images of *E. coli* biofilm are shown in **Figure 4**. Overall, the untreated samples revealed biofilms had well organized network structures encased in a protective EPS. After SDW treatment, there were still aggregates of cells held together by EPS. However, there were significant differences after AEW treatment, where only a few cells were scattered sporadically and cell lysis was evident when compared to control. The SEM images revealed that most of the biofilm cells were detached from the biofilm matrix and suggested that EPS was disrupted after AEW treatment.

Effect of AEW on Other Foodborne Pathogen Biofilms

While AEW demonstrated activity against *E. coli* biofilms, it was of interest to explore whether biofilms formed by other foodborne pathogens such as *L. monocytogenes* and *V. parahaemolyticus*, were also sensitive to AEW.

The efficacy of AEW in eradicating biofilm cells of *L. monocytogenes* and *V. parahaemolyticus* are showed in **Figure 5**. The populations of *L. monocytogenes* and *V. parahaemolyticus* biofilm cells were decreased by 82 and 52% after AEW treatment. There were significant differences obviously between the reduction of biofilm cell number after AEW treatment and those after SDW treatment ($p < 0.05$). SDW treatment marginally reduced the population of biofilm cells of *L. monocytogenes* and *V. parahaemolyticus*. In contrast, the *L. monocytogenes* biofilm was more susceptible to AEW treatment than the *V. parahaemolyticus* biofilm. Additionally, the detachment of residual viable cells from a biofilm after AEW treatment was markedly less when compared to biofilms treated with SDW (data were not shown). After AEW treatment, the detached cells which were a potential source of secondary pollution were lower than detecting value.

Extracellular polymeric substance analysis of *V. parahaemolyticus* and *L. monocytogenes* biofilm is shown in **Figures 6A,B**. SDW treatment only result in 3–6% EPS

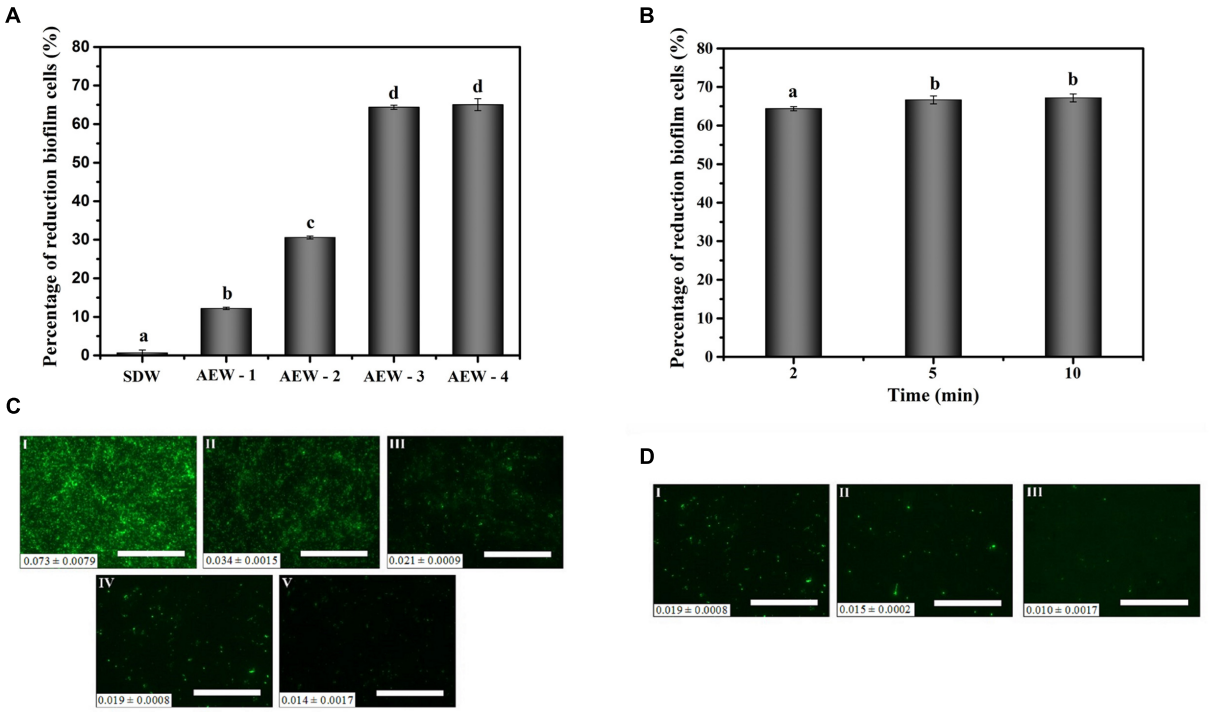

FIGURE 2 | Effect of AEW on *E. coli* cells within biofilms after the SDW, AEW-1, AEW-2, AEW-3, and AEW-4 treatments for different times. Fluorescence density value was presented at the bottom left of each image. The percentages of reduction biofilm cells after the SDW, AEW-1, AEW-2, AEW-3, and AEW-4 treatments for 2 min **(A)**, and the fluorescence images changes of *E. coli* cells within biofilms after the SDW **(C, I)**, AEW-1 **(C, II)**, AEW-2 **(C, III)**, AEW-3 **(C, IV)**, and AEW-4 **(C, V)** treatments for 2 min, respectively. The percentages of reduction biofilm cells after exposed to the AEW-3 treatments for 2, 5, and 10 min **(B)**, and the fluorescence images of *E. coli* cells within biofilm after exposed to the AEW-3 for 2 min **(D, I)**, 5 min **(D, II)**, 10 min **(D, III)**, respectively. The treatment condition of the image **C (IV)** was the same as that for the Image **D (I)**. Scale bar represented 100 μm. Error bars indicated standard deviations of triplicate experiments, and the same letter represented no significant difference ($P \geq 0.05$).

reduction and there was no significant difference between SDW treatment and control. The carbohydrate and protein content of EPS in *V. parahaemolyticus* were reduced by 34 and 44%. Comparatively, there was a reduction of 53% carbohydrate and 75% protein content in EPS of *L. monocytogenes* biofilm. AEW treatment therefore is more effective in reducing protein than carbohydrate content in EPS of *L. monocytogenes* and *V. parahaemolyticus*. Representative Raman spectra of EPS in the spectral fingerprint range of 500–1250 cm^{-1} are presented in **Figures 6C–F**. The Raman bands of EPS in *L. monocytogenes* biofilm at 1090 cm^{-1} (C-O-C glycosidic link) were decreased after AEW treatment, indicating the destruction of the carbohydrate structure (**Figures 6C,D**). Similar trends were observed for carbohydrates at band 565 cm^{-1} (C-O-C glycosidic ring def polysaccharide) and band 1095 cm^{-1} (C-O-C glycosidic link).

The band intensity was also weakened by a big margin after AEW treatment as shown in **Figures 6E,F**. Raman intensity in *V. parahaemolyticus* and *L. monocytogenes* biofilm was also reduced at bands 637–695 cm^{-1} and 1003 cm^{-1} (proteins) after AEW treatment. The Raman bands at 1,003 cm^{-1} could be observed in the spectra of the control and SDW group, but these bands were not present in the spectra of EPS in *L. monocytogenes* biofilm after AEW treatment. The decrease of Raman intensity at band 788 cm^{-1} which corresponds to DNA, arises from the destruction of the ring structure and indicated the degradation of DNA.

The eradication effect of AEW on biofilm formed by *L. monocytogenes* and *V. parahaemolyticus* was further evaluated using SEM. Representative SEM images (**Figures 7A,B**) show that untreated biofilm had nearly uniform and dense mature architecture. *L. monocytogenes* biofilm forms a much stronger structure than *V. parahaemolyticus* biofilm. SDW treatment had no obvious effect in biofilms. In contrast, AEW removed the biofilm cells and destroyed the high ordered structures, resulting in much less dense, and individually formed colonies when compared to the control (**Figures 7A-III,B-III**).

DISCUSSION

Biofilm formation of foodborne pathogens on food processing surfaces is a concern for the food industry (Wang et al., 2016). Controlling pathogen biofilm formation is hindered by EPS which limits the diffusion of sanitizers into the deepest layers of biofilms. In this study, we found that AEW had the ability to disrupt EPS and effectively eradicate foodborne biofilms.

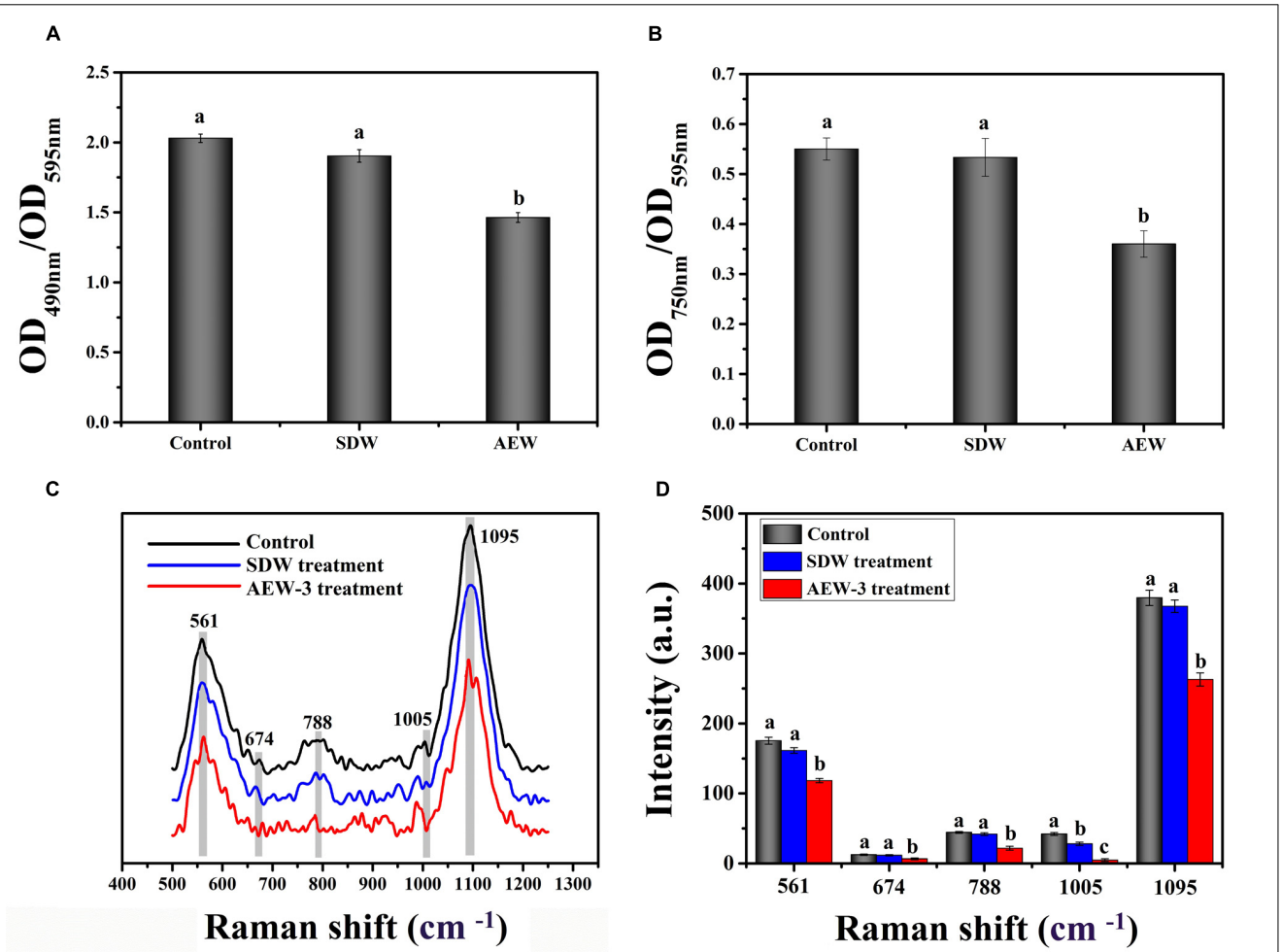

FIGURE 3 | Chemical composition and contents of EPS which represent integrity in *E. coli* biofilms, with untreated, SDW and AEW-3 treatment. **(A)** Total carbohydrates ($OD_{490\ nm}/OD_{595\ nm}$) and **(B)** total protein ($OD_{750\ nm}/OD_{595\ nm}$) in EPS of *E. coli* biofilms. Raman spectrum **(C)** and intensities changes **(D)** of *E. coli* biofilm after untreated, treatment with SDW and AEW-3 for 5 min. Error bars indicated the standard deviations of five measurements and the same letter represented no significant difference ($P \geq 0.05$).

TABLE 2 | Assignment of Raman bands of EPS in biofilms.

Peak position (cm^{-1})	Assignment	Reference
561–582	C-O-C glycosidic ring def polysaccharide; COO^- wag; C-C skeletal	Laucks et al., 2005; Guicheteau et al., 2008; Ivleva et al., 2008
637–695	C-S str and C-C twisting of proteins (tyrosine)	Laucks et al., 2005; Guicheteau et al., 2008; Kahraman et al., 2009
782–788	O-P-O str of DNA	Samek et al., 2014
830–850	Tyr	Schwartz et al., 2009
855–899	C-C str, C-O-C 1,4 glycosidic link	Wagner et al., 2009; Ivleva et al., 2009.
1003, 1005	Ring breath Phe	Laucks et al., 2005; Çulha et al., 2008; Guicheteau et al., 2008; Ivleva et al., 2008; Kahraman et al., 2009
1020–1085	C-C, and C-O str (carbohydrates)	Schwartz et al., 2009
1090–1095	C-C str, C-O-C glycosidic link; ring br, sym	Schenzel and Fischer, 2001; Maquelin et al., 2002; De Gussem et al., 2005; Harz et al., 2005; Neugebauer et al., 2007

def, deformation vibration; str, stretching; Tyr, tyrosine; Phe, phenylalanine; br, breathing; sym, symmetric.

We evaluated the effect of AEW on biofilm using a green fluorescent protein-tagged *E. coli* in preliminary experiments, which allowed non-destructive rapid microscopic visualization of biofilm population without using probes or dyes. There was a direct correlation between population and fluorescence signal of cells (Bron et al., 2006; Montañez-Izquierdo et al., 2012).

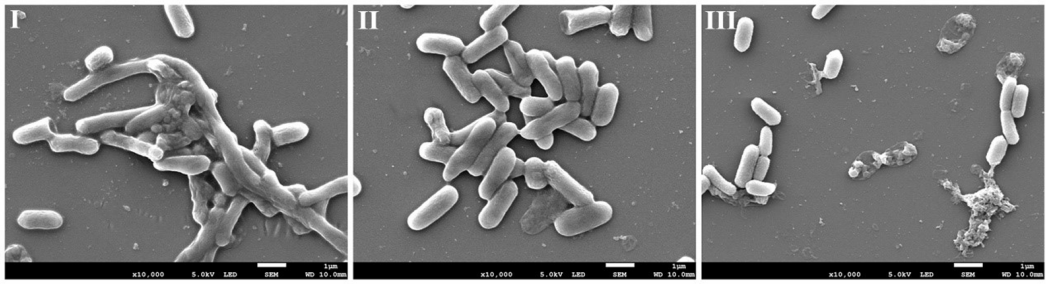

FIGURE 4 | Representative photomicrographs by SEM of biofilm formed by *E. coli* after untreated **(I)**, treated with SDW **(II)**, and AEW-3 for 5 min **(III)**. Scale bar represented 5 μm. Pictures were representative of three independent experiments with three replicates each.

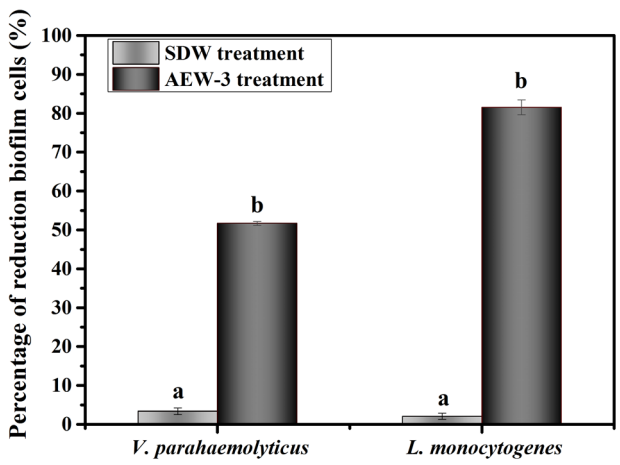

FIGURE 5 | Percentage of reduction biofilm cells of *V. parahaemolyticus* and *L. monocytogenes*.

Multilayers located at different depths and cells aggregates in the biofilms resulted in different fluorescence intensity (Webster et al., 2004). Hence, the eradication effect of AEW on biofilm could be clearly demonstrated with the direct observation of epifluorescence micrographs.

Acidic electrolyzed water electrolyzed at different NaCl concentration was characterized by pH, ACC, and ORP (**Table 1**). Different NaCl concentrations produced different ACC which affected the efficiency of AEW on *E. coli* biofilms (**Table 1**). High ACC causes changes in metabolic compounds within biofilm cells, causing cell death and removal. However, the pH and ORP of AEW were not the main factor contributing to bactericidal ability. A similar finding was reported by both Sun et al. (2012) and Vázquez-Sánchez et al. (2014) who concluded that the available chlorine in AEW might be one of the main factor for the inactivation of *S. aureus* biofilms. In addition, AEW inactivated bacteria due to the oxidative ability of ACC against the cell membrane, various metabolic functions, etc. (Huang et al., 2008). Specifically, AEW causes the degradation of bacterial protective barriers like EPS and increases membrane permeability. Other effects include the leakage of cellular inclusions, and decrease of activity of some key enzymes such as dehydrogenase (Zeng et al., 2010). Also, once the bacterial cells detach from EPS in the biofilm matrix, these cells are more vulnerable to sanitizer agents (Kumar and Anand, 1998).

In our study, the numbers of viable cells in the biofilms of *E. coli*, *V. parahaemolyticus*, and *L. monocytogenes* ranged from 6.77, 6.90, and 7.24 to 2.26, 3.33, and 1.34 log CFU/mL, respectively, after AEW-3 treatment for 5 min. *L. monocytogenes* biofilm was more susceptible to AEW compared to the other two Gram-negative pathogens. Similar results were obtained by Chen et al. (2015), who reported that the effectiveness of the sanitizer to *L. monocytogenes* was more remarkable than other Gram-negative bacteria. Moreover, our results were also supported by Skrivanova et al. (2006), who found that AEW was more effective against planktonic Gram-positive bacteria than planktonic Gram-negative bacteria. One explanation of these phenomena is that the transport of ions across the cell membrane of Gram-positive bacteria is more vulnerable to interference (Skrivanova et al., 2006; Feliciano et al., 2012; Chen et al., 2014). In addition, this apparent discrepancy could partly be explained by the different structural composition of EPS in different biofilms. There were great differences in the proportions of proteins and carbohydrates in EPS in biofilms formed by bacteria. Therefore, AEW efficiency in biofilm removal might vary according to the species of bacteria.

Extracellular polymeric substance makes up about 80% of the biofilm dry mass, primarily consisting of carbohydrates and proteins and plays a major role in mediating biofilms formation (Liu et al., 2007). EPS is responsible for biofilm properties, such as density, porosity, and hydrophobicity. An effective cleaning procedure should break up or dissolve the EPS in the biofilm so that disinfectants gains access to the viable cells (Shen et al., 2016). It is also reported that the main cause of biofilm removal is EPS degradation rather than removal of intracellular components (Christensen et al., 1990). For example, Gao et al. (2016) showed that the radical oxidants from CAT-NP activation of H_2O_2 degraded glucans in EPS via oxidative cleavage. Zhou et al. (2016) also observed that the presence of ozone reduced EPS contents to different extents. However, little is known whether AEW disrupts EPS thus resulting in biofilm eradication. Therefore, we attempted to determine the influence of AEW on biofilm characteristics systematically by investigating

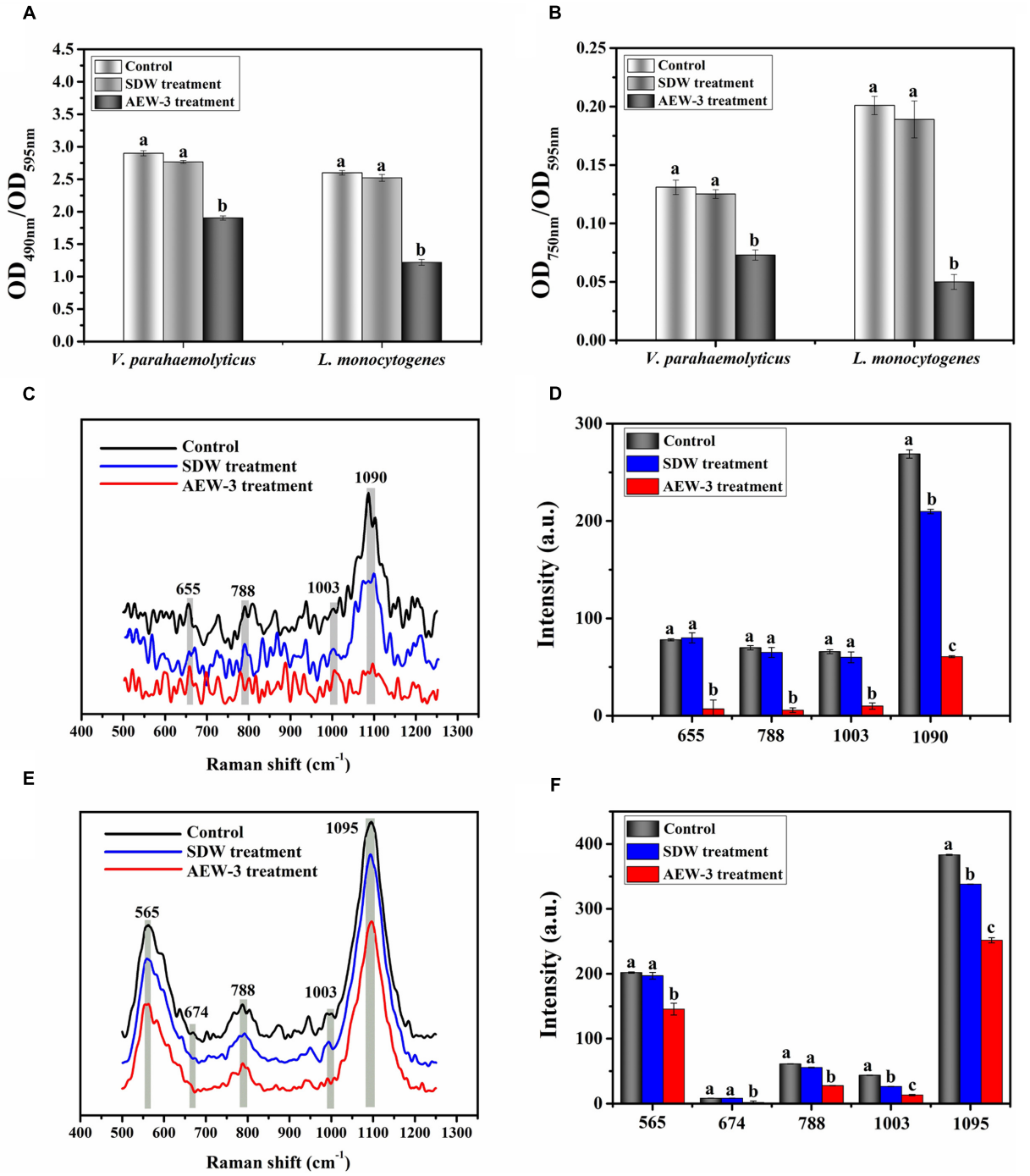

FIGURE 6 | Chemical composition and contents of EPS in *V. parahaemolyticus* and *L. monocytogenes* biofilms with untreated, SDW and AEW-3 treatment. **(A)** Total carbohydrates ($OD_{490\ nm}/OD_{595\ nm}$) and **(B)** total protein ($OD_{750\ nm}/OD_{595\ nm}$) in EPS. Raman spectrum changes of *V. parahaemolyticus* **(C)** and *L. monocytogenes* **(E)** biofilm. Raman intensity changes of *V. parahaemolyticus* **(D)** and *L. monocytogenes* **(F)** biofilm. Error bars indicated the standard deviations of five measurements and the same letter represented no significant difference ($P \geq 0.05$).

EPS content and composition (**Figure 3**). The experimental results show that AEW had a remarkable effect in the disruption of EPS in biofilms. Raman spectroscopic analysis combined with EPS chemical analysis revealed that the band intensities associated with carbohydrates, protein and nucleic acid were significantly decreased after AEW treatment when compared to the control treatment. The changes in the carbohydrate C-O-C group, tyrosine and phenylalanine of proteins were clearly

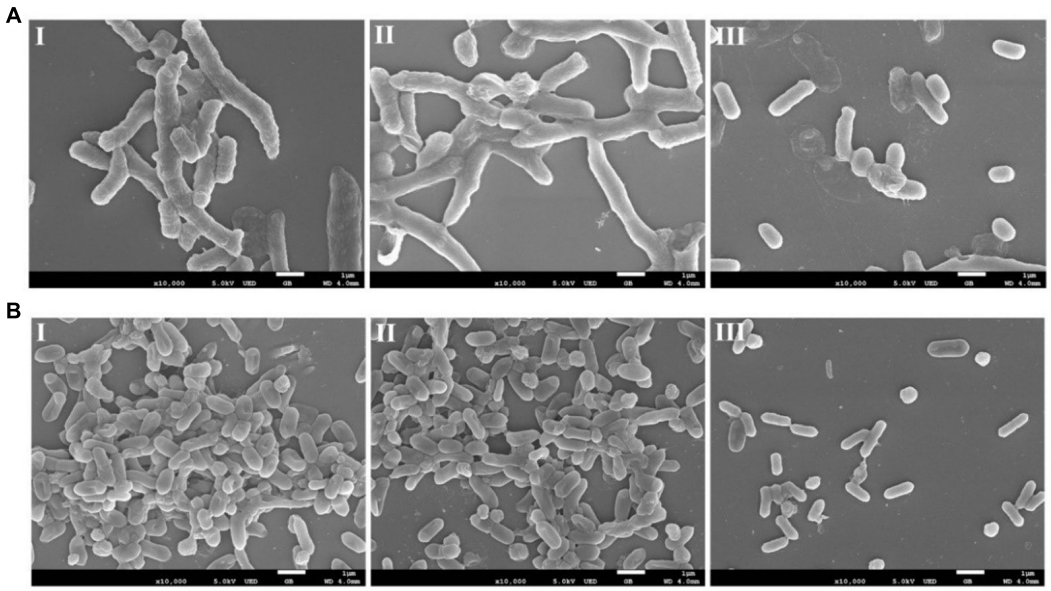

FIGURE 7 | Representative photomicrographs by SEM of biofilm formed by *V. parahaemolyticus* **(A)** and *L. monocytogenes* **(B)** after untreated **(I)**, treated with SDW **(II)**, and AEW-3 for 5 min **(III)**. Scale bar represented 5 μm. Pictures were representative of three independent experiments with three replicates each.

observed. After exposure to AEW, the absence of 1,003 cm^{-1} and 1005 cm^{-1} bands in EPS were attributed to the ring deformation of phenylalanine and aromatic amino acids, indicating that denaturation or conformational changes caused cell death. It was in line with previous reports that the protein in EPS was decreased significantly by antibiotic agents (Jung et al., 2014).

This study shows that EPS disruption could be the basic mechanism of biofilm removal by AEW treatment. The present study hypothesized that the mechanism underlying EPS disruption and biofilms eradication upon exposure to AEW may be associated with ionic interactions. These interactions caused biofilm eradication by changing EPS hydrophobicity and localized charge along the polymer chains. Changes in charge and hydrophobicity would in turn affect the EPS structure. Future research could concentrate on the interaction between EPS and AEW. Some studies have also reported that there are differences in the degree of attachment and biofilm formation by the pathogen as affected by various types of food-contact surfaces, including stainless steel, glass, plastic, and wooden surfaces (Bang et al., 2014). Also, the efficacy of the sanitizer may be affected by surface type, which will be our next research step. In addition, the effect of AEW on mixed-species biofilm models akin to *in vivo* situation is certainly warranted.

Additionally, the residual cells which escapes after exposure to disinfection may further adhere and grow, resulting in a complex matrix (Molobela et al., 2010). These dispersed cells from biofilm showed stronger recalcitrance to disinfection than planktonic cells. Nevertheless, one finding from this study was that biofilm viable cells dispersed by AEW from the biofilm matrix into the ambient environment were under the detection limit, thus AEW treatment may not cause secondary pollution. Therefore, AEW is an excellent alternative to sanitizers and can be applied to control biofilms in food processing facilities as well as protecting foods from cross-contamination.

CONCLUSION

In summary, this study indicated that AEW could effectively eradicate foodborne pathogen biofilms and not caused the secondary pollution. Therefore, AEW is a potent foodborne pathogen biofilms disrupter, which can be used as a reliable and eco-friendly alternative to sanitizer traditionally used in the food industry.

AUTHOR CONTRIBUTIONS

Conceived and supervised the study: YZ, YP, and HL. Designed the experiments: QH and XS. Performed the experiments: QH and XS. Analyzed the data: QH, JF, XS, ZZ, and XW. Revised the paper: ZZ, PM, and YZ. Wrote the paper: QH.

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Differential Survival of Hyper-Aerotolerant *Campylobacter jejuni* under Different Gas Conditions

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Campylobacter jejuni accounts for a significant number of foodborne illnesses around the world. *C. jejuni* is microaerophilic and typically does not survive efficiently in oxygen-rich conditions. We recently reported that hyper-aerotolerant (HAT) *C. jejuni* are highly prevalent in retail poultry meat. To assess the capabilities of HAT *C. jejuni* in foodborne transmission and infection, in this study, we investigated the prevalence of virulence genes in HAT *C. jejuni* and the survival in poultry meat in atmosphere at a refrigeration temperature. When we examined the prevalence of eight virulence genes in 70 *C. jejuni* strains from raw poultry meat, interestingly, the frequencies of detecting virulence genes were significantly higher in HAT *C. jejuni* strains than aerosenstive *C. jejuni* strains. This suggests that HAT *C. jejuni* would potentially be more pathogenic than aerosensitive *C. jejuni*. Under aerobic conditions, aerosensitive *C. jejuni* survived at 4°C in raw poultry meat for 3 days, whereas HAT *C. jejuni* survived in poultry meat for a substantially extended time; there was a five-log CFU reduction over 2 weeks. In addition, we measured the effect of other gas conditions, including N_2 and CO_2, on the viability of HAT *C. jejuni* in comparison with aerosensitive and aerotolerant strains. N_2 marginally affected the viability of *C. jejuni*. However, CO_2 significantly reduced the viability of *C. jejuni* both in culture media and poultry meat. Based on the results, modified atmosphere packaging using CO_2 may help us to control poultry contamination with HAT *C. jejuni*.

Keywords: ***Campylobacter*, aerotolerance, virulence genes, bacterial survival, pathogen inhibition**

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INTRODUCTION

Campylobacter is a leading bacterial cause of human gastroenteritis, annually accounting for approximately 166 million diarrheal cases around the world, particularly in developed countries (Kirk et al., 2015). *Campylobacter* infection in humans develop fever, vomiting, abdominal pains, and diarrhea, and in some cases Guillain–Barré syndrome, an autoimmune disorder characterized by acute and progressive neuromuscular paralysis (Young et al., 2007). Human infection with *C. jejuni* is facilitated by the function of various virulence factors involved in toxin production (e.g., *cdtABC*), cell adhesion (e.g., *cadF*, *peb1A*, and *pldA*) and invasion (e.g., *ciaB*), and colonization of gastrointestinal tracts (Bolton, 2015).

Campylobacter is isolated from a wide range of domestic animals and wildlife (Jokinen et al., 2011). In particular, the gastrointestinal tracts of poultry are colonized by *Campylobacter jejuni*, the major human pathogenic species of *Campylobacter*, at the level of 10^6∼10^8 CFU/g feces or higher (Hermans et al., 2011). Poultry meat is often contaminated with *C. jejuni* during poultry processing, and human campylobacteriosis is most frequently associated with the consumption of contaminated poultry products (Skarp et al., 2016). In addition, cross-contamination in the kitchen is also an important risk factor transferring *Campylobacter* (Chai et al., 2008; Luber, 2009). It has been estimated that a two-log reduction in the number of *Campylobacter* on chicken carcasses may lead to approximately a 30-fold reduction in the number of human campylobacteriosis cases (Rosenquist et al., 2003). To control *Campylobacter* contamination of poultry, various intervention strategies have been examined at the pre- and post-harvest levels, such as bacteriocin and bacteriophages (Hermans et al., 2011; Umaraw et al., 2017).

Unlike other enteric pathogenic bacteria, *C. jejuni* exhibits unique microbiological features. For example, *C. jejuni* is asaccharolytic and has limitations in the utilization of hexose sugars, including glucose, because of the lack of 6-phosphofructokinase in the glycolysis pathway (Parkhill et al., 2000; Velayudhan and Kelly, 2002). To supply carbon sources, *C. jejuni* relies on the utilization of amino acids, organic acids (e.g., lactic acid), and fucose in some strains (Leach et al., 1997; Thomas et al., 2011; Stahl et al., 2012). In addition, *C. jejuni* is microaerophilic and capnophilic and requires both O_2 and CO_2 for growth preferably at 5–10% and 1–10%, respectively (Bolton and Coates, 1983). Despite the fastidious nature of *Campylobacter*, it has not been understood how *Campylobacter* causes such a significant number of human infection cases around the world.

Various tolerance mechanisms have been reported to support the survival of *Campylobacter* under harsh stress conditions, such as heat, cold, acid, and desiccation stresses (Murphy et al., 2006). In addition, *Campylobacter* produces biofilms and switches its physiological state to a viable but nonculturable (VBNC) cell to promote survival under stress conditions. In *C. jejuni*, biofilm formation is stimulated under aerobic conditions, and aeration triggers the formation of VBNC cells (Oh et al., 2015b, 2016), suggesting *C. jejuni* is equipped with multiple survival mechanisms that may support the viability of *C. jejuni* under oxygen-rich conditions. Besides these survival mechanisms, aerotolerance would be the front-line survival mechanism of *C. jejuni* when this microaerophilic pathogen encounters the aerobic environment (Bronowski et al., 2014). Despite our perception about oxygen-sensitivity in *C. jejuni*, interestingly, we recently reported that hyper-aerotolerant (HAT) strains of *C. jejuni* are highly prevalent in retail poultry meat; the HAT strains survive longer than 24 h in vigorous aerobic shaking at 200 rpm. Also, HAT *C. jejuni* often belongs to the multilocus sequence typing (MLST) clonal complexes (CCs) that are frequently implicated in human infection (Oh et al., 2015a), suggesting that HAT *C. jejuni* might be closely related to human infection. To evaluate the virulence potential of HAT *C. jejuni*, in this study, we investigated the prevalence of virulence genes in HAT *C. jejuni* strains. In addition, we measured the survival of HAT *C. jejuni* under different gas conditions, such as N_2 and CO_2, aiming to develop intervention strategies to control HAT *C. jejuni* in poultry meat by using modified atmosphere packaging (MAP) with different gases, since aerotolerance confers tolerance to oxygen, not other gases.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Seventy *C. jejuni* strains that were isolated from poultry were used in this study (Oh et al., 2015a). *C. jejuni* NCTC 11168 is the first genome-sequenced strain of *Campylobacter* and was used as a control in the study (Parkhill et al., 2000). *C. jejuni* 81–176 was used as a positive control for PCR detection of *virB11* (Bacon et al., 2002). In our previous study, we first reported high prevalence of HAT *C. jejuni* that can effectively survive in a vigorous aerobic condition, such as aerobic shaking at 200 rpm (Oh et al., 2015a). Based on the level of aerotolerance, we arbitrarily divided *C. jejuni* into three different groups: (1) aerosensitive *C. jejuni* that loses viability before 12 h by aerobic shaking at 200 rpm, (2) aerotolerant *C. jejuni* that loses viability between 12∼24 h by aerobic shaking at 200 rpm, and (3) HAT *C. jejuni* that survives even after 24 h of aerobic shaking at 200 rpm (Oh et al., 2015a). The 70 *C. jejuni* poultry strains were isolated from retail poultry meat in our previous study and consisted of 20 aerosensitive strains, 25 aerotolerant strains, and 25 HAT strains (Oh et al., 2015a). The *C. jejuni* strains were routinely grown on Mueller–Hinton (MH) agar plates (Difco) at 42°C under microaerobic conditions (85% N_2, 5% O_2 and 10% CO_2).

Determination of *C. jejuni* Survival under Different Gas Conditions

Campylobacter jejuni survival was determined in MH media and chicken meat at 4°C in normal atmospheric conditions and under CO_2 and N_2. Frozen *C. jejuni* strains in 10% glycerol were inoculated on MH agar plates and inoculated plates were incubated at 42°C under microaerobic condition. Overnight cultures of strains of *C. jejuni* grown on MH agar plates were harvested with fresh MH broth and diluted in MH broth to an optical density at 600 nm (OD_{600}) of 0.1. The bacterial suspension was transferred to multiple 96-well plates, and the 96-well plates were incubated at 4°C in air and in an anaerobic jar filled with either CO_2 or N_2. In addition, N_2 gas condition was constructed with 100% nitrogen gas flushing and CO_2 condition was generated with gas pack (>97% CO_2). To prevent desiccation, a container with water was placed nearby the 96-well plates in a refrigerator. Samples were taken at predetermined time for enumeration. In addition, the survival of two strains of *C. jejuni*, which were randomly chosen from each aerotolerance group [HAT strains (#12 and #21), aerotolerant strains (#4 and #29), and aerosensitive strains (#24 and #66)], was determined in raw chicken meat; these strains were selected from different

MLST CCs based on their aerotolerance level. Approximately one-gram of raw chicken meat, including skin and muscle, was prepared with a sterilized razor and placed in a 12-well plate. After applying an aliquot (100 μl) of *C. jejuni* suspension (approximately 8×10^8 CFU/ml) onto each portion of meat and skin mixture, the plate was stored at 4°C under three different gas conditions, including normal atmosphere, CO_2, and N_2. Due to the potential indigenous *C. jejuni* in poultry meat, controls were prepared without addition of *C. jejuni*. The poultry meat samples were transferred to a 50 ml tube containing 2 ml of fresh MH broth. After vortexing for 2 min, the supernatant was collected, serially diluted, and spread onto MH agar plates for enumeration. Each experiment was carried out with duplicate samples, and the experiment was repeated three times.

PCR Detection of Virulence Genes

Overnight cultures on MH agar at 42°C under microaerobic conditions of *C. jejuni* strains were collected in PBS (pH 7.2). Bacterial suspension of overnight culture of *C. jejuni* strains were diluted in PBS to an OD600 of 0.01 (approximately, 8×10^6 CFU/ml) and boiled for 10 min to release gDNA. After centrifugation, the supernatant was used as a template. To evaluate the potential virulence of HAT *C. jejuni* strains, we investigated the prevalence of eight important virulence genes (*cadF*, *cdtB*, *ciaB*, *docA*, *iam*, *peb1A*, *pldA*, and *virB11*), which are associated with toxin production, cell adhesion and invasion, and colonization of gastrointestinal tracts in chickens with PCR with ExTaq polymerase (Takara, Japan). Primers used are listed in **Table 1**. The positive controls for six virulence genes, such as *cadF*, *cdtB*, *ciaB*, *docA*, *iam*, *peb1A* and *pldA,* were amplified from *C. jejuni* NCTC11168, and *virB11* was amplified from *C. jejuni* 81–176. The PCR mixture was amplified with the following conditions: initial denaturation at 96°C for 3 min followed by 35 cycles of denaturation 96°C for 30 s, variable annealing temperature (*cdtB*, *ciaB*, *cadF* and *pldA* at 45°C, *docA*, *peb1* and *virB11* at 50°C, *iam* at 53°C) for 30 s, extension at 72°C for 1 min 20 s and the final extension at 72°C for 7 min. The results were analyzed by electrophoresis with 1% agarose gels and SYBR safe staining dye (Invitrogen).

Statistical Analysis

Two-way ANOVA was performed by using GraphPad Prism 6 (GraphPad Software Inc., United States). Chi-square distribution was used to analyze if the prevalence of virulence genes is dependent on aerotolerance by using SPSS Statistics 21.0 (IBM Predictive Software, United States).

RESULTS

Effect of Aerotolerance on *C. jejuni* Survival in Chicken Meat

To evaluate the impact of hyper-aerotolerance on the survival of *C. jejuni* in poultry meat in this study, raw poultry meat was spiked with two strains of *C. jejuni* from each aerotolerance group (i.e., aerosenstive, aerotolerant, and HAT *C. jejuni* groups) and incubated at 4°C under aerobic conditions. The aerosensitive *C. jejuni* strains lost their viability on poultry meat within 3 days, and the aerotolerant *C. jejuni* strains survived for 7 days (**Figure 1**). Interestingly, HAT *C. jejuni* strains survived in poultry meat for 2 weeks (**Figure 1**). This means that HAT *C. jejuni* strains survived in food in atmospheric conditions approximately four times longer than aerosensitive strains of *C. jejuni*. The results showed that aerotolerance significantly affects the viability of *C. jejuni* in poultry meat under aerobic conditions.

Prevalence of Virulence Genes in HAT *C. jejuni* Strains

In 70 strains of *C. jejuni* from poultry meat, the frequencies of detecting virulence genes were 100, 97.1, 68.6, 81.4, 57.1, 84.3, 64.3, and 11.4% for *cadF*, *cdtB*, *ciaB*, *docA*, *iam*, *peb1*, *pldA*, and

TABLE 1 | Primers used in this study.

Gene	Primer	Sequence (5′-3′)	Size (bp)	Reference
cadF	cadF_F	TTGAAGGTAATTTAGATATG	400	Konkel et al., 1999a
	cadF_R	CTAATACCTAAAGTTGAAAC		
cdtB	cdtB_F	GTTAAAATCCCCTGCTATCAACCA	495	Bang et al., 2001
	cdtB_R	GTTGGCACTTGGAATTTGCAAGGC		
ciaB	ciaB_F	GTTAAAGTTGGCAGT	1163	Konkel et al., 1999a
	ciaB_R	GTTCTTTAAATTTTTCATAATGC		
docA	docA_F	ATAAGGTGCGGTTTTGGC	725	Muller et al., 2006
	docA_R	GTCTTTGCAGTAGATATG		
iam	iamA_F	GCACAAAATATATCATTACAA	518	Konkel et al., 1999a
	iamA_R	TTCACGACTACTATGAGG		
peb1	peb1_F	TAATACGACTCACTATAGGGGAAAATCTTT	775	Biswas et al., 2011
	peb1_R	TTTTCGCTAAAGCATCAATTTCATT		
pldA	pldA_F	AAGCTTATGCGTTTTT	913	Datta et al., 2003
	pldA_R	TATAAGGCTTTCTCCA		
virB11	virB11_F	GAACAGGAAGTGGAAAAACTAGC	708	Bacon et al., 2002
	virB11_R	TTCCGCATTGGGCTATATG		

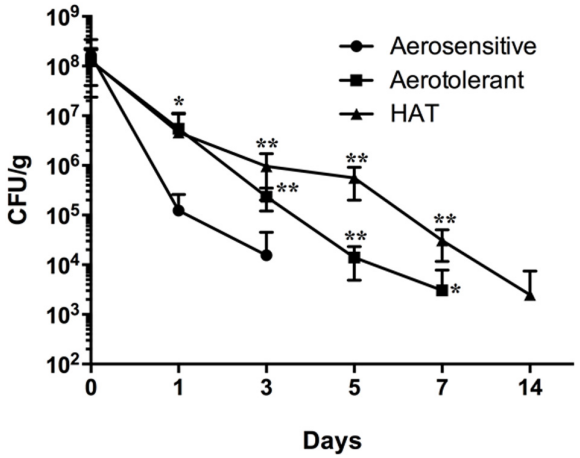

FIGURE 1 | Survival of aerosensitive, aerotolerant, and HAT *C. jejuni* strains in poultry meat at 4°C under aerobic conditions. Two *C. jejuni* strains were randomly selected from each aerotolerance group and used to spike raw poultry meat in duplicate. The results indicate the means and standard deviations of duplicate samples of the two different strains in a single experiment. Three independent experiments were performed, and similar results were obtained in all the experiments. The statistical analysis was performed with two-way ANOVA in comparison with aerosensitive strains. $^*P \leq 0.05$, $^{**}P \leq 0.01$.

virB11, respectively (**Figure 2** and **Table 2**). When we clustered the results based on the aerotolerance level, interestingly, the detection frequencies in HAT *C. jejuni* strains were higher than those in aerosensitive *C. jejuni* strains (**Table 2**), suggesting that HAT *C. jejuni* would potentially be more pathogenic to humans than aerosensitive *C. jejuni*.

Viability of HAT *C. jejuni* Strains in Different Gas Atmospheres

The survival of HAT *C. jejuni* measured under different gaseous conditions. In the food industry, MAP is often employed to extend the microbial shelf-life of meat, and O_2, N_2, and CO_2 are the major gases used for MAP. Thus, we selected N_2 and CO_2 for the viability testing of HAT *C. jejuni* strains. Consistent with their aerotolerance level, there was about approximately a four log reduction in CFU in aerosensitive strains of *C. jejuni*, a three log CFU reduction in aerotolerant strains, and a two log CFU reduction in HAT strains of *C. jejuni* at 4°C in MH broth under the normal atmospheric conditions within 3 days. Incubation in N_2 reduced the survival of *C. jejuni*, and CO_2 further decreased CFU counts in HAT *C. jejuni*, compared with the aerobic conditions (**Figure 3**). The CFU reduction in all the strains were similar between days 3 and 7 (**Figure 3**), and no *C. jejuni* was detected in day 14 (data not shown).

Impact of Different Gas Atmosphere on the Survival of HAT *C. jejuni* in Poultry Meat

The viability of *C. jejuni* strains belonging to different aerotolerance groups was determined in poultry meat stored in different gas atmospheres. In N_2, aerotolerant and HAT *C. jejuni* strains were detected for 14 days, whereas aerosensitive strains survived for 7 days (**Figure 4A**). Compared to aerobic conditions (**Figure 1**), N_2 did not reduce the viability of HAT *C. jejuni* strains in poultry meat. In CO_2, however, HAT strains of *C. jejuni* survived only for a week (**Figure 4B**); this is a significant viability reduction compared to atmospheric conditions where HAT *C. jejuni* strains survived for 2 weeks in poultry meat (**Figure 1**). The results exhibit that HAT *C. jejuni* did not survive well in CO_2, compared to aerobic conditions.

DISCUSSION

Despite the well-known microaerophilic characteristic of *C. jejuni*, our previous study showed that some *C. jejuni* strains are highly tolerant to aerobic stress and these strains are highly prevalent in poultry meat (Oh et al., 2015a). In addition, Rodrigues et al. (2015) recently characterized an unique human isolate of *C. jejuni* strain, named Bf, which can grow aerobically, suggesting that some *C. jejuni* strains are highly resistant to aerobic stress. Increased tolerance to aerobic stress would enable *C. jejuni* to survive during transmission to humans through foods. This would significantly impact the safety of poultry meat because of frequent contamination of poultry meat by *Campylobacter*. In this study, we demonstrated that HAT *C. jejuni* survived in raw poultry meat at 4°C significantly longer than aerosensitive *C. jejuni* (**Figure 1**), confirming the potential threat of HAT *C. jejuni* on the safety of fresh poultry meat.

The *cadF* and *cdt* genes are detected in *C. jejuni* strains from poultry at high frequencies (Rozynek et al., 2005). Similarly, in this study, *cadF* and *cdt* genes were detected in all and most (97.1%) *C. jejuni* strains, respectively (**Table 2**). The *iam* locus has been detected in *C. jejuni* chicken isolates at 54.7% (Rozynek et al., 2005). The *pldA* and *ciaB* genes have been detected from *C. jejuni* poultry isolates at the frequencies of 63.6 and 67.3%, respectively (Melo et al., 2013). Hanning et al. (2010) reported relatively low detection frequencies of *ciaB* (40%) and *pldA* (56%) in *C. jejuni* isolates from poultry carcasses. The *virB11* gene is located in the virulence plasmid pVir, which is often detected in *C. jejuni* strains that cause bloody diarrhea (Bacon et al., 2002; Tracz et al., 2005). The prevalence of *virB11* was 10.7∼17% in human clinical isolates and 9.5∼14% in poultry isolates (Datta et al., 2003; Tracz et al., 2005). When the results were sorted based on the aerotolerance level, the frequencies of detecting virulence genes were significantly higher in HAT *C. jejuni* strains in comparison with aerosenstive *C. jejuni* strains (**Figure 2** and **Table 2**). Interestingly, the most substantial differences in the frequency of detection were observed in the genes associated with invasion, including *ciaB* and *iam* (**Figure 2** and **Table 2**). CiaB shares similarities with SipB (*Salmonella* invasion protein B) from *Salmonella* and IpaB (invasion plasmid antigen B) from *Shigella flexneri* and is translocated to human epithelial cells. Even though a knockout mutation of *ciaB* does not affect *C. jejuni* adhesion to INT407 cells, it significantly

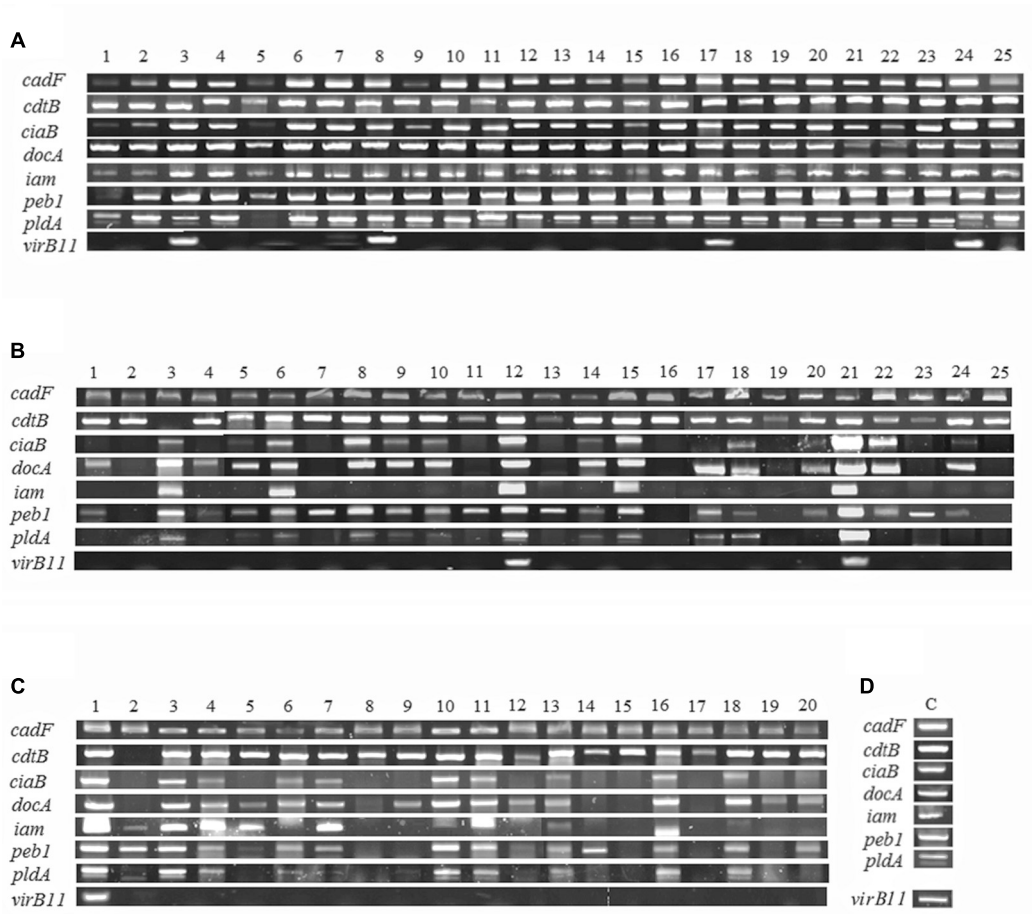

FIGURE 2 | Detection of virulence genes in 70 strains of *C. jejuni* from poultry meat. The results show the prevalence of eight virulence genes in hyper-aerotolerant **(A)**, aerotolerant **(B)**, and aerosensitive **(C)** strains of *C. jejuni*. Positive controls **(D)** were amplified from *C. jejuni* NCTC11168 (*cadF*, *cdtB*, *ciaB*, *docA*, *iam*, *peb1 and pldA*) and 81–176 (*virB11*). Controls were included each batch of PCR testing, and representative results were presented.

TABLE 2 | Prevalence (%) of virulence genes in 70 isolates of *C. jejuni* from poultry meat.

	***cadF*ND**	***cdtB*^{NS}**	***ciaB*******	***docA*****	***iam*******	***peb1****	***pldA*******	***virB11*^{NS}**
HAT *C. jejuni* ($n = 25$)	100	100	100	100	100	100	96	20
Aerotolerant *C. jejuni* ($n = 25$)	100	96	52	68	20	84	48	8
Aerosensitive *C. jejuni* ($n = 20$)	100	95	50	75	50	65	45	5
Total ($n = 70$)	100	97.1	68.6	81.4	57.1	84.3	64.3	11.4

*Statistical significance was performed by chi-square distribution with SPSS ver.21 (IBM). *$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$, NS, Not Significant; ND, Not determined.*

impairs the internalization of *C. jejuni* into INT407 cells (Konkel et al., 1999b). The invasion-associated marker (*iam*) locus was first reported by Carvalho et al. (2001) with random amplified polymorphic DNA techniques (RAPD) and was detected in 85% of invasive strains and 20% of non-invasive strains. The detection frequencies of *pldA* were also significantly different between HAT and aerosensitive *C. jejuni* strains (**Figure 2** and **Table 2**). The *pldA* gene encodes an outer membrane phospholipase A that is involved in hemolysis (Grant et al., 1997). The *pldA* and *ciaB* genes also play a role in *C. jejuni* colonization of chicken intestines (Ziprin et al., 2001). The increased prevalence of the virulence genes in HAT *C. jejuni* strains suggests that HAT *C. jejuni* would be more pathogenic to humans than aerosensitive *C. jejuni*.

The transmission of *C. jejuni* to humans is primarily mediated by contaminated food, mainly poultry meat. Due to the fastidiousness and oxygen sensitivity, *C. jejuni* is not expected to survive efficiently during foodborne transmission in oxygen-rich, atmospheric conditions. However, our results indicate that HAT *C. jejuni* survives longer in poultry meat than aerosensitive strains during transmission to humans in air and would be more capable of causing human infection (**Figure 1**). In this study, we observed that the survival of HAT *C. jejuni* is significantly reduced under CO_2 (**Figures 3**, **4**).

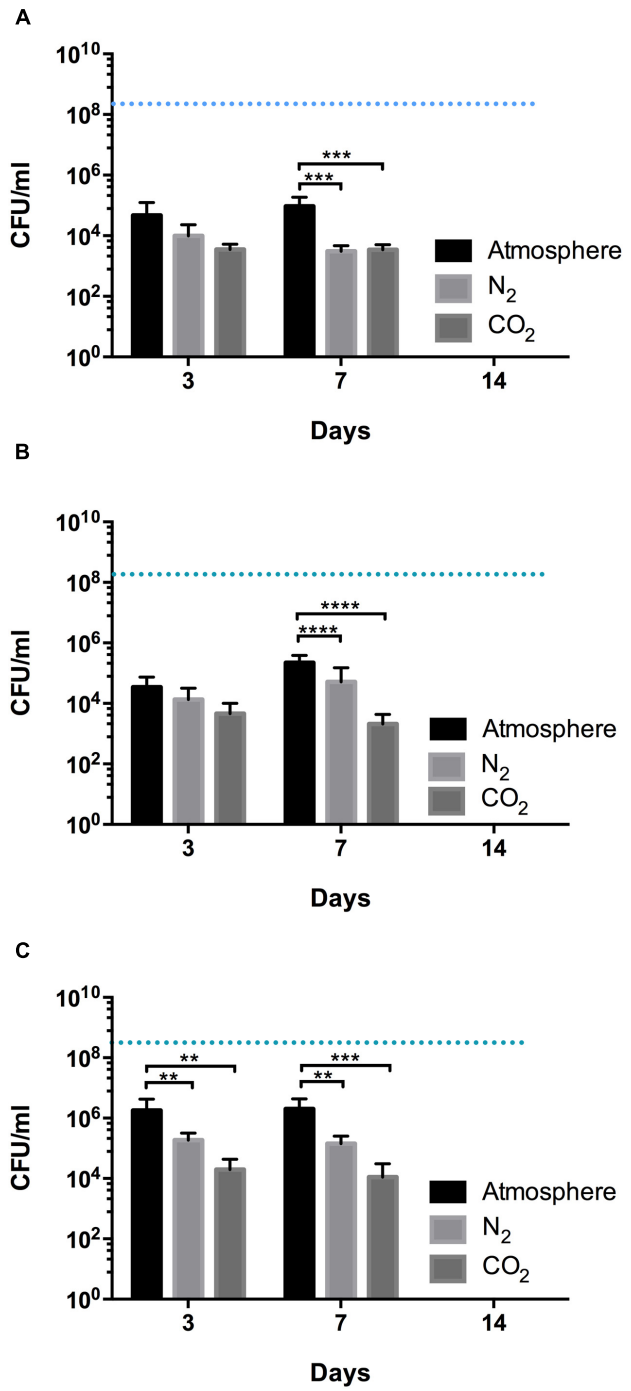

FIGURE 3 | Survival of aerosensitive **(A)**, aerotolerant **(B)**, and HAT **(C)** strains of *C. jejuni* in MH broth under different gas conditions. Incubation was carried out in atmospheric, N_2, and CO_2 conditions. Two strains from each aerotolerance group were randomly selected, and each strain was inoculated in MH broth in triplicate. The initial CFU was adjusted to be approximately 10^8 CFU/ml for all the samples and is indicated with blue dashed lines. The results show the mean and standard deviation of the triplicate samples of two different strains in a single experiment. The experiment was repeated three times, and similar results were obtained in the three independent experiments. Two-way ANOVA testing was carried out for statistical analysis. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$.

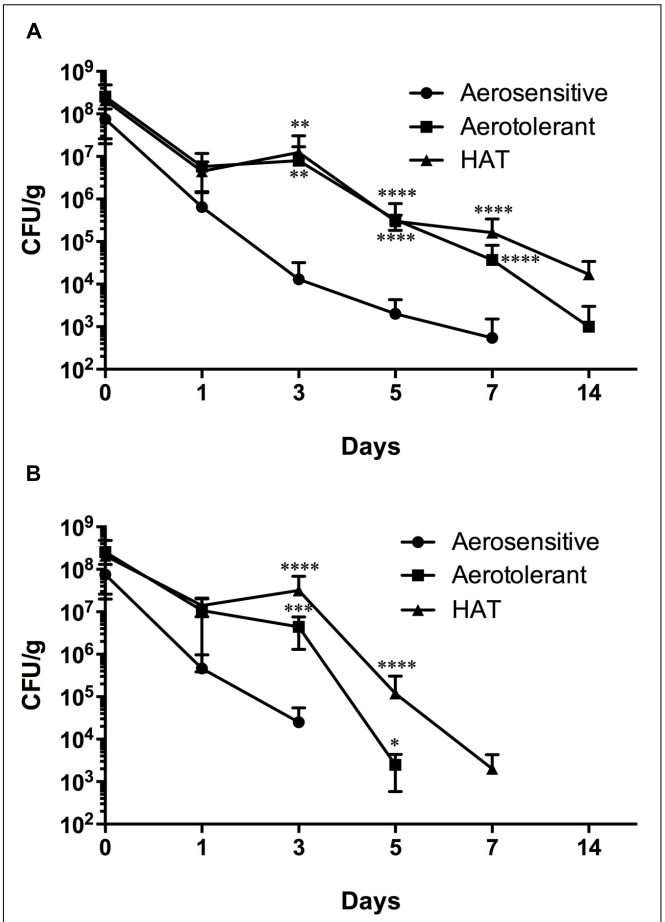

FIGURE 4 | Survival of aerosensitive, aerotolerant, and HAT *C. jejuni* strains at 4°C in poultry meat in N_2 **(A)** and CO_2 **(B)**. Two strains from each aerotolerance group were randomly selected for the experiment. The results indicate the means and standard deviations of duplicate samples of the two different strains in a single experiment. Three independent experiments were performed, and similar results were obtained all the experiments. The statistical analysis was carried out with two-way ANOVA. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$.

This provides important scientific background for developing methods to control HAT *C. jejuni* with MAP. In food industry, CO_2, N_2 and their combinations are generally used for the development of MAP of foods. Compared to aerobic conditions, the survival of HAT *C. jejuni* strains in raw poultry meat was significantly reduced by CO_2 (**Figures 1**, **4B**). Meredith et al. (2014) tested different compositions of the three gases and reported that 40:30:30 of CO_2:O_2:N_2 is the optimum gas mixture both to reduce *Campylobacter* and to extend shelf-life in poultry filets. The threshold CO_2 concentration that critically affects the viability of HAT *C. jejuni* has not been examined, and its determination still awaits future studies for the development of optimal gas mixtures of MAP to control HAT *C. jejuni* in poultry meat.

Our previous study revealed that most HAT *C. jejuni* strains belong to MLST CC 21 (Oh et al., 2015a), the major MLST CC

implicated in human gastroenteritis (Nielsen et al., 2010). It is possible that strains of *C. jejuni* with increased aerotolerance may survive well in foods and are more likely to reach humans, consequently causing human illnesses more frequently than aerosensitive *C. jejuni* strains. At this stage, it remains unknown why HAT *C. jejuni* strains harbor more virulence genes than oxygen-sensitive strains. In this study, we did not provide empirical evidences about the virulence, such as invasion of and adhesion to epithelial cells, and such works will be done in future studies. Nevertheless, this study also showed that MAP using CO_2 may be an interesting approach to control HAT *C. jejuni* in poultry meat.

AUTHOR CONTRIBUTIONS

Design of the project: EO and BJ. Performance of the experiments: EO. Data analysis: EO, LM, LC, and BJ. Writing of the manuscript: EO, LM, LC, and BJ.

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ORIGINAL RESEARCH
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Thymoquinone Inhibits Virulence Related Traits of *Cronobacter sakazakii* ATCC 29544 and Has Anti-biofilm Formation Potential

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The aim of this study was to determine whether thymoquinone, the principal active ingredient in the volatile oil of *Nigella sativa* seeds, could suppress certain virulence traits of *Cronobacter sakazakii* ATCC 29544 which contribute to infection. Sub-inhibitory concentrations of thymoquinone significantly decreased motility, quorum sensing, and endotoxin production of *C. sakazakii* ATCC 29544 and biofilm formation of *C. sakazakii* 7-17. Thymoquinone substantially reduced the adhesion and invasion of *C. sakazakii* ATCC 29544 to HT-29 cells and decreased the number of intracellular bacterial cells within the RAW 264.7 macrophage cells. Thymoquinone also repressed the transcription of sixteen genes involved in the virulence. These findings suggest that thymoquinone could attenuated virulence-related traits of *C. sakazakii* ATCC 29544, and its effects on other *C. sakazakii* strains and *in vivo C. sakazakii* infection need further investigation.

Keywords: *Cronobacter sakazakii*, thymoquinone, sub-inhibitory concentration, HT-29 cells, virulence factors

INTRODUCTION

Cronobacter spp., formerly known as *Enterobacter sakazakii*, is a Gram-negative, non-spore-forming, rod-shaped bacterium (Fang et al., 2012). *Cronobacter* spp. has been isolated from water, sediment, soil and herbs (Iversen and Forsythe, 2004). Powdered infant formula (PIF) and powdered milk have been identified as the most common sources and vehicles of *Cronobacter* spp. transmission (Fang et al., 2012). *Cronobacter* spp. is associated with bacteraemia and sepsis, cerebrospinal and peritoneal fluid accumulation, brain abscesses, cyst formation, necrotizing enterocolitis (NEC), meningitis, and intracerebral infarctions. *Cronobacter* spp. is currently considered to consist of 7 species: *Cronobacter sakazakii*, *C. malonaticus*, *C. universalis*, *C. dublinensis*, *C. muytjensii, C. condiment*, and *C. zurichensis* (Stephan et al., 2014), among which *C. sakazaii* is one of the two group 1 clinically relevant species that form the majority of the clinical isolates.

C. sakazakii possesses an array of virulence factors which aid in tissue adhesion, invasion, and host cell injury (Singh et al., 2015). Previous studies have reported OmpX and OmpA, which are outer membrane proteins (OMPs) of *E. sakazakii*, are potential virulence marker (Himelright et al., 2002; Kim et al., 2010). Various plasmid associated genes such as *Cronobacter* plasminogen activator (*cpa*) and genes responsible for iron acquisition (*eit*CBAD and *iuc*ABD/*iut*A) have been reported in different strains of *Cronobacter* spp. (Stoll et al., 2004; Hunter and Bean, 2013; Singh et al., 2015). Lipopolysaccarides (LPS) is an outer

membrane virulence factor of *E. sakazakii*, which interacts with enterocytes through LPS mediated binding to toll like receptor-4 (TLR4) inducing necrotizing enterocolitis in animals (Hunter et al., 2008). Quorum sensing is a process that enables bacteria to communicate and modify behavior in response to changes in the cell population density. The process involves the production, release, detection and of extracellular signaling molecules called autoinducers (Ng and Bassler, 2009). Phenotypes that are controlled by a quorum sensing system include swarming and the expression of virulence factors such as lytic enzymes, toxins, siderophores, and adhesion molecules (de Kievit and Iglewski, 2000; Miller and Bassler, 2001; Whitehead et al., 2001; De Windt et al., 2003).

With ever-increasing antimicrobial resistant strains, alternative strategies, other than antibiotics, to treat or prevent infections caused by *C. sakazakii* have been explored (Shi et al., 2016a,b). Plant materials have received a great deal of interest as an alternative method to control pathogenic microorganisms. Many studies have demonstrated that components derived from plants (such as essential oils) show antimicrobial activity against a broad spectrum of microorganisms (Cowan, 1999; Turgis et al., 2009).

The black seed (*Nigella sativa*, Ranunculaceae family), also known as Black Caraway Seed and "the Blessed Seed," is an annual herb that grows in countries bordering the Mediterranean Sea, Pakistan and India. The seed has been used as a natural remedy for more than 2,000 years to promote health and treat diseases (Gali-Muhtasib et al., 2006). And the seeds and oil of this plant are demonstrated to have a very low degree of toxicity (Ali and Blunden, 2003). Thymoquinone (TQ), the principal active ingredient in the volatile oil of *N. sativa* seeds (El-Mahmoudy et al., 2002), has been reported to have many significant pharmacological properties, including anti-oxidant, anti-inflammatory, anti-histaminic, analgesic, anti-hypertensive, and anti-mutagenic functions (Ragheb et al., 2009). Investigators have shown that TQ also has significant anti-neoplastic effect on human pancreatic adenocarcinoma, uterine sarcoma, and leukemic cell lines, while it is minimally toxic to normal cells (Worthen et al., 1998). Forouzanfar et al. (2014) have proved that TQ has antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Salmonella enteritidis, Pseudomonas aeruginosa,* and fungi.

Although, the antimicrobial activity of *N. sativa* and its extract has been extensively studied, little information is available in the literature about the anti-virulence potential of thymoquinone on *C. sakazakii*. Therefore, the aim of this study was to investigate the effect of thymoquinone on virulence-related traits of *C. sakazakii* ATCC 29544. In addition, its effect on biofilm formation was also investigated with a strong biofilm producing food-derived *C. sakazakii* isolate 7-17.

MATERIALS AND METHODS

Reagents

The TQ (Tokyo Chemical Industry Co., Tokyo, Japan; CAS: 490-91-5) stock solutions were prepared in 0.1% dimethyl sulfoxide (DMSO) before use as described in a previous study (Shi et al., 2015).

Bacterial Strains and Culture Conditions

C. sakazakii strains ATCC 29544, ATCC 29004, ATCC BAA-894, ATCC 12868 (ATCC, Manassas, USA) were used in this study. Six other *C. sakazakii* strains were taken from our laboratory strain collection, which were originally isolated from various infant formula and infant rice cereal sources in China. Since *C. sakazakii* strain ATCC 29544 showed weak biofilm production in preliminary experiments, the relatively strong biofilm-producing *C. sakazakii* strain 7-17 was selected for use in the biofilm formation study (Li et al., 2016). The QS indicator strain *Chromobacterium violaceum* ATCC 12472 (ATCC, Manassas, USA) was used for QS inhibition assays (Yang et al., 2016). All *C. sakazakii* strains were prepared as described in a previous study (Shi et al., 2015).

Determination of Minimum Inhibitory Concentration (MIC) of TQ

MIC was determined by agar dilution method as described in a previous study (Shi et al., 2016a). Tryptone soya agar (TSA) was aseptically transferred into sterile 24-well plates. TQ was added to the warm medium (45°C) to obtain final concentrations of 0, 200, 400, 600, 1,200, 1,800, 2,400, and 3,600 μmol/L. After hardening, the TSA was spotted with 2 μl of the *C. sakazakii* suspension ($\sim 10^5$ CFU), then the samples were incubated at 37°C for 24 h. The lowest concentration of TQ at which no visible growth of test organisms was observed, was determined as MIC. Ampicillin (100 mg/L) was used as a positive control for the test.

Determination of Sub-Inhibitory Concentration (SIC) of TQ

The SICs of TQ were determined by broth dilution (Li et al., 2014) with some modifications. One hundred and twenty-five microlitres of the tryptone soya broth (TSB) (with 6.0 log CFU of *C. sakazakii*) were inoculated in 96-well microtiter plate. The same volume of TQ solution [TQ dissolved in TSB contain 0.1% DMSO (v/v)] was added to the cultures to obtain final concentrations of 25, 50, 100, 200, 400, 600, 1,200, and 2,400 μmol/L. TSB containing 0.1% DMSO was used as a negative control. The samples were further cultured at 37°C, and cell growth was monitored at 600 nm using a multimode plate reader (Tecan, Infinite™ M200 PRO, Männedorf, Switzerland).

Motility Assay

Swimming and swarming were evaluated in Luria-Bertani (LB) broth containing different agar concentrations as previously described (Li et al., 2014). For analysis of swimming ability, a medium containing 20 ml of LB broth and 0.3% (wt/vol) of agar was used. TQ was added to the warm medium (45°C) to obtain final concentrations of 0, 400 and 600 μmol/L. Then the plates were allowed to dry for 1 h at 25°C before use. Five microliters of each *C. sakazakii* ATCC 29544 culture (∼6.0 log CFU) were inoculated at the center of this semisolid medium and the plates were incubated at 37°C for 7 h. After this, the diameter of the bacterial halo was recorded. The medium without TQ was used as a control.

For analysis of bacterial swarming ability, a medium containing 20 ml of LB broth, 0.5% (wt/vol) agar and 0.5% (wt/vol) glucose was used. Five microliters of *C. sakazakii* culture

(∼6.0 log CFU) were stabbed into the semisolid medium with and without TQ at 0, 400, and 600 μmol/L. The plates were incubated upside down at 37°C for 20 h. The size of the swarm area in the presence or absence of TQ was calculated using AutoCAD.

C. sakazakii ATCC 29544 Endotoxin Assay

ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, U.S.A.) was used according to the method of Amalaradjou et al. (2014). Overnight culture of *C. sakazakii* strains ATCC 29544 was centrifuged (5,000 × g, 10 min, 4°C) and re-suspended in TSB. Then, 50 μl of the cell suspension ($OD_{600\,nm}$ = 0.5) and TQ solution was added in 30 ml TSB to obtain a final concentration of 0, 400, and 600 μmol/L. The bacterial cells were grown at 37°C to its mid-log phase. Non-inoculated TSB was used as a control. Samples were treated following manufacturer instructions and analyzed by a microplate spectrophotometer (Model 680; Bio-Rad, Hercules, CA, U.S.A.).

Quantitative QS Inhibition Assay

The effect of TQ on the QS inhibitory activity was indirectly measured by quantifying the violacein production with the indicator strain *C. violaceum* ATCC 12472 (Taganna et al., 2011). First, the inhibitory concentration of TQ on the growth of *C. violaceum* ATCC 12472 was studied to determine the SIC to be used in further experiments. The two highest concentrations of TQ that did not inhibit *C. violaceum* ATCC 12472 growth after 12 h of incubation were selected as SICs for this study.

A flask incubation assay was used to quantify the QS-inhibitory activity of TQ. An overnight culture of *C. violaceum* ATCC 12472 was diluted to an $OD_{600\,nm}$ of 0.5. Volumes (30 ml) of LB broth containing different concentrations of TQ were placed into separate flasks. Each flask was inoculated with 100 μl of the culture. The flasks were incubated at 30°C for 24 h with 150 rpm. The violacein extraction and quantitation were carried out as previously described by Choo et al. (2006) with minor modifications. Briefly, 5 ml of the *C. sakazakii* culture was centrifuged (5,000 × g, 5 min, 4°C) to precipitate insoluble violacein and the culture supernatant was discarded. Then, 1 ml of DMSO was added to the pellet and the solution was vortexed vigorously for 1 min to completely solubilize violacein. The solution was then centrifuged at 5,000 × g for 10 min to remove the cells. Two hundred microlitres of the violacein-containing supernatants were added to 96-well microtiter plates and the absorbance of violacein-containing supernatants was read with a microplate spectrophotometer (Model 680; Bio-Rad, Hercules, CA, U.S.A.) at a wavelength of 585 nm.

Specific Biofilm Formation (SBF) Inhibition Assay

Biofilm formation assays were performed according to the method described by Naves et al. (2008) with minor modifications. Overnight cultures of *C. sakazakii* strain 7-17 were centrifuged (5,000 × g, 10 min, 4°C) and re-suspended in TSB. Then, 250 μl of the cell suspension ($OD_{600\,nm}$ = 1) were inoculated in sterile 96-well microtiter plates. TQ was added to each of the wells to obtain final concentrations of 0, 400, and 600 μmol/L. Non-inoculated TSB was used as a control. The plates were incubated at 25 or 12°C for 24, 48, and 72 h without agitation. At each time point, the optical densities (ODs) of cell growth were measured at 630 nm using a microplate spectrophotometer (Model 680; Bio-Rad, Hercules, CA, U.S.A.). The suspension was then removed and the wells were rinsed once with 350 μl of distilled water. After being air dried for 30 min, the wells were stained with 250 μl of 1% crystal violet (wt/vol) (Tianjin Kermel Chemical Regent Co., Ltd, Tianjin, China) for 20 min at room temperature. To remove the non-conjugated colorant, the wells were rinsed three times with 350 μl of distilled water. After being air-dried for 30 min, the adhered dye was solubilized in 250 μl of 33% (vol/vol) glacial acetic acid and incubated for 20 min at room temperature before the ODs were read at 570 nm. The SBF was calculated by attaching and stained bacteria ($OD_{570\,nm}$) normalized with cell growth ($OD_{630\,nm}$). The experiment was replicated at least three times.

Cell Culture

The human colonic cell line HT-29 was maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, U.S.A.). Murine macrophage cell line RAW 264.7 cells were grown in RPMI 1640 (Gibco). Both media were supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Hyclone, Logan, UT, U.S.A.), 1% (vol/vol) non-essential amino acids (Gibco) and 1% (vol/vol) double antibiotic solution (100 U/ml penicillin and 100 μg/ml streptomycin; Hyclone). Maintenance of the cell lines and subsequent experiments were carried out at 37°C in a humidified atmosphere containing 5% CO_2.

Cell Viability Assay

HT-29 cells were seeded at a density of 10^5/ml into the wells of 96-well plates and incubated at 37°C with 5% (vol/vol) CO_2 for 24 h. To determine an appropriate non-cytotoxic concentration of TQ for use in this study, cells were grown with different concentrations of TQ and its cytotoxic effect was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Li et al., 2014). After incubation, cultures were removed, 200 μl of 0.5% (wt/vol) MTT dissolved in phosphate-buffered saline (PBS, 0.01 mol/L, pH = 7.4) was added, and the plates were incubated for 4 h. One hundred microlitres of DMSO was then added to each well to dissolve the formazan crystals. Absorbance at 570 nm was measured with a microplate reader microplate spectrophotometer (Bio-Rad). Cell viability was expressed as a percentage of the control (untreated cells).

Adhesion and Invasion of Cells

The effect of TQ on the adhesion and invasion of *C. sakazakii* ATCC 29544 was investigated by using HT-29 cells, according to a previous study (Li et al., 2014). Trypsin-treated cells were seeded in 24-well tissue culture plates and grown in supplemented DMEM (10^5 cells per well) for 24 h. *C. sakazakii* was grown to mid-log phase, centrifuged, and resuspended in cell culture media without double antibiotics. Then, the HT-29 cells were rinsed with PBS and inoculated with 10^8

CFU of *C. sakazakii*, equivalent to a multiplicity of infection (MOI) of 1,000. At the same time, DMEM containing different concentrations of TQ were added to the wells. Plates were incubated at 37°C in a humidified, 5% CO_2 incubator.

For adhesion assay, the infected monolayer cells were rinsed three times in PBS after 1 h of incubation, and lysed with 0.1% Triton X-100 (Amresco, Solon, OH, U.S.A.). The number of viable adherent *C. sakazakii* ATCC 29544 was determined by the serial dilution and plating on TSA plates and incubated at 37°C for 24 h before counting. For the invasion assay, the monolayers were incubated for 1 h following infection, rinsed three times in PBS, and incubated for another 30 min in whole media containing gentamicin (100 μg/ml; Amresco) to kill the extracellular bacteria. Finally, the wells were washed with PBS three times. And the numbers of invaded *C. sakazakii* cells were determined as described in the adhesion assay. The results were expressed as a percentage relative to that of the control group.

Intracellular Survival and Replication of *C. sakazakii* ATCC 29544 in RAW 264.7 Cells

The murine macrophage cell line RAW 264.7 cells were maintained in RPMI 1640 medium with 10% FBS. Twenty-four hours prior to infection, activated cells were seeded in 24-well tissue plates (10^5 cells per well) and cultured at 37°C under 5% CO_2. *C. sakazakii* ATCC 29544 was incubated to its mid-log phase in TSB with various concentrations of TQ (0, 400, and 600 μmol/L). Then the RAW 264.7 cells were washed gently with PBS and infected with 10^7 CFU (100 MOI) of *C. sakazakii* ATCC 29544. The plates were incubated for 45 min at 37°C with 5% CO_2. After incubation, RAW 264.7 cells were re-suspended in RPMI 1640 containing 1% FBS with gentamicin (100 μg/ml) and incubated at 37°C with 5% CO_2 for 30 min.

For intracellular survival assays, the cells were then washed three times with PBS and lysed with 0.1% Triton. After dilution (PBS 1:10), the samples were enumerated on TSA plates. The results were presented as the number of intracellular *C. sakazakii* ATCC 29544 cells after TQ treatment. For replication assays, each well-containing bacterial cells was replenished with RPMI 1640 containing 1% FBS with gentamicin (10 μg/ml) and incubated at 37°C with 5% CO_2 for either 24 or 48 h. Cell washing, lysis and plating procedures were identical to those used in the analysis of bacterial survival. All assays were conducted in triplicate and repeated at least three times on different days.

Quantification of *C. sakazakii* Virulence Gene Transcription Using RT-qPCR

The *C. sakazakii* strain ATCC 29544 was grown in TSB without or with TQ (0, 400, and 600 μmol/L) at 37°C to its mid-log phase. The bacteria were then centrifuged (5,000 × g, 5 min, 4°C) and re-suspended in PBS. The total RNA was extracted with the RNAprep Pure Bacteria Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. To remove all DNA, the purified RNA was treated for 15 min with 30 Units of DNase I. RNA concentrations were measured with a nucleic acid and protein spectrophotometer (Nano-200; Aosheng Instrument Co., Ltd., Hangzhou, China). First-strand cDNA was synthesized from 0.5 μg of each RNA sample in a 10 μl reaction mixture using the PrimeScript™RT reagent kit (TakaRa, Kyoto, Japan) according to manufacturer directions. The primer sequences used for RT-PCR are listed in **Table 1**. RT-PCR was performed in a 25-μl system using SYBR® Premix Ex Taq™ II (TakaRa). The cycling conditions included 1 cycle of 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s, and dissociation steps of 95°C for 15 s and 60°C for 30 s. All samples were analyzed in triplicate and normalized to the endogenous control (ESA_04030) gene. Samples were run on the IQ5 system (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and the transcription of target genes vs. ESA_04030 gene were determined as previously described (Li et al., 2014).

Statistical Analysis

Statistical analyses were performed using SPSS software (version 19.0; SPSS, Inc., Chicago, IL). The data were presented as the mean values ± *SD* and differences between means were tested by Student's *t*-test. Differences are considered significant at $P \leq 0.05$. All experiments were performed at lease in triplicate.

RESULTS

MICs and SICs

The MICs of TQ for 9 *C. sakazakii* strains are presented in **Table 1**. It is shown by agar dilution assay that TQ exhibited antimicrobial activity against all the tested strains, and the MICs ranged from 1,800 to 3,600 μmol/L. Broth dilution assay showed that concentrations below 600 μmol/L exhibited no growth inhibition against *C. sakazakii* ATCC 29544 (**Figure 1**) and were further chosen to study the effects of TQ on *C. sakazakii* virulence. Since TQ was equally effective against our three *C. sakazakii* isolates (ATCC 29544, ATCC 29004, and 7-17), only strain ATCC 29544 was selected for further studies.

Motility

The effect of TQ on *C. sakazakii* motility is shown in **Figure 2**. TQ reduced both swimming and swarming ability of *C. sakazakii* ATCC 29544 (**Figures 2A,B**). The original swimming distance of *C. sakazakii* ATCC 29544 was 7.50 ± 0.15 cm. Addition of TQ at 600 and 400 μmol/L caused swimming distance reductions to 2.76 ± 0.14 cm ($P \leq 0.01$) and 5.75 ± 0.06 cm ($P \leq 0.01$)

TABLE 1 | Minimum inhibitory concentrations of TQ against different strains of *C. sakazakii*.

Strain	Origin	MIC (μmol/L)
ATCC 29544	Child's throat	2,400
ATCC BAA-894	Human clinical specimen	1,800
ATCC 12868	Infant formula	3,600
ATCC 29004	Infant formula	2,400
12-2	Infant rice cereal	3,600
14-15	Infant formula	2,400
18-7	Infant rice cereal	2,400
18-8	infant formula	2,400
18-13	Infant formula	2,400
7-17	Infant formula	2,400

respectively. Swarming motility was also greatly impacted by TQ. The original swarming area of *C. sakazakii* ATCC 29544 was 5.54 ± 0.19 cm^2. TQ at 600 and 400 μmol/L caused swarming area reductions to 0.84 ± 0.22 cm^2 ($P \leq 0.01$) and 1.65 ± 0.23 cm^2 ($P \leq 0.01$) respectively.

TQ Reduces Endotoxin Production in *C. sakazakii*

The original endotoxin concentration of *C. sakazakii* ATCC 29544 was 0.76 ± 0.08 EU/ml (**Figure 3**). After the addition of TQ to the *C. sakazakii*, we observed a clear change in endotoxin concentration. Addition of TQ at 400 μmol/L caused a significant fall ($P \leq 0.05$) in *C. sakazakii* endotoxin concentration to 0.62 ± 0.04 EU/ml. Addition of TQ at 600 μmol/L caused a further decrease in endotoxin concentration ($P \leq 0.01$) to 0.15 ± 0.02 EU/ml.

Anti-QS Activity of TQ

TQ showed no growth inhibition activity against *C. violaceum* at concentrations used in this study (75 and 150 μmol/L) with a broth micro-dilution method (data not shown). As seen in **Figure 4**, anti-QS activity was shown when TQ was used at 75 and 150 μmol/L, as evidenced by a significant decreased production of violacein (about 75.27 and 54.07% of the control, respectively).

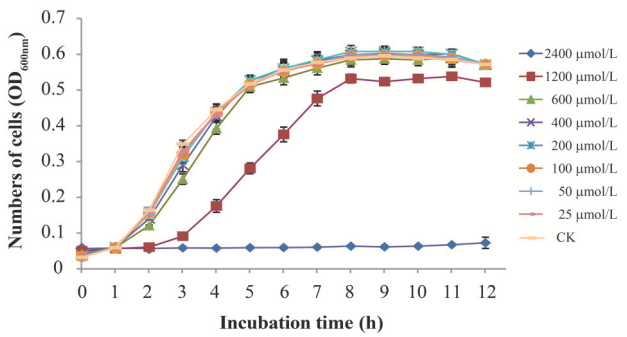

FIGURE 1 | Growth of *C. sakazakii* ATCC 29544 in TSB with various concentrations of TQ. Each value represents the average of three independent measurements.

Anti-biofilm Formation of TQ

The anti-biofilm efficacy of TQ was investigated on *C. sakazakii* strain 7-17 grown at 12 and 25°C for 24, 48, and 72 h on microtiter plates (**Table 2**). Compared with the control, cells treated with TQ showed significantly less biofilm formation. For *C. sakazakii* 7-17, the biofilm formation of cells treated with 600 μmol/L TQ was inhibited by 45.6, 44.4, and 68.3% after 24, 48, and 72 h at 25°C, respectively. And TQ at 600 μmol/L reduced the initial biofilm formation by 31.5, 73.5, and 81.4% of the control level when *C. sakazakii* 7-17 grown at 12°C for 24, 48, and 72 h.

Adhesion to and Invasion of HT-29 Cells

TQ at concentrations of up to 100 μmol/L did not affect cell viability (**Figure 5A**). Therefore, TQ at concentrations of 25, 50, and 100 μmol/L were used in further studies. TQ at 25, 50, and 100 μmol/L inhibited adhesion of *C. sakazakii* to 85.0, 76.3, and 57.5%, respectively compared to the control (**Figure 5B**). TQ was also effective in inhibiting ($P \leq 0.01$) the ability of *C. sakazakii* ATCC 29544 to invade HT-29 cells (**Figure 5C**). The invasiveness of *C. sakazakii* ATCC 29544 treated with 25, 50, and 100 μmol/L TQ was reduced to 50.0, 6.8, and 4.6% of the control, respectively.

Intracellular Survival in RAW 264.7

TQ-untreated *C. sakazakii* ATCC 29544 was able to survive well in macrophage (**Figure 6**). The results showed that TQ at concentrations of 400 and 600 μmol/L were able to significantly ($P \leq 0.01$) decrease intracellular survival of *C. sakazakii* in the macrophages during 48 h when compared to the control.

Virulence-Associated Genes

RT-qPCR demonstrated that TQ significantly decreased the transcription of sixteen virulence-related genes in *C. sakazakii* (**Table 3**). TQ down-regulated the transcription of *fliD*, *flhD*, and *flgJ* genes (critical for flagella regulation) to various degrees. Other down-regulated genes were *ompA* (outer membrane

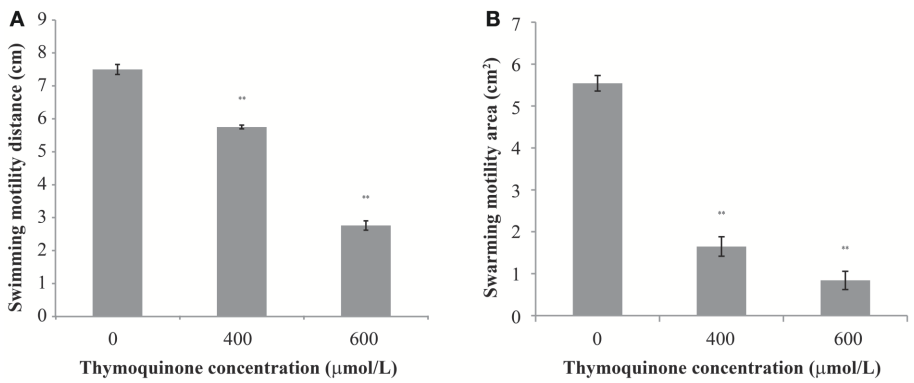

FIGURE 2 | The effect of TQ on swimming motility **(A)** and swarming motility **(B)** of *C. sakazakii* ATCC 29544. Bars represent the standard deviation ($n = 3$). $^{**}P \leq 0.01$ compared to control.

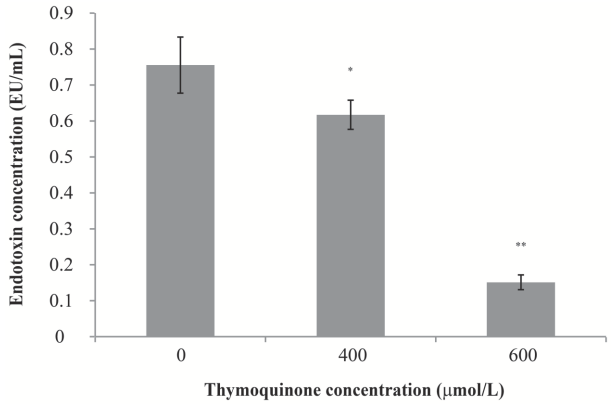

FIGURE 3 | Effect of TQ on endotoxin production by *C. sakazakii* ATCC 29544. Bars represent the standard deviation ($n = 3$). $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ compared to control.

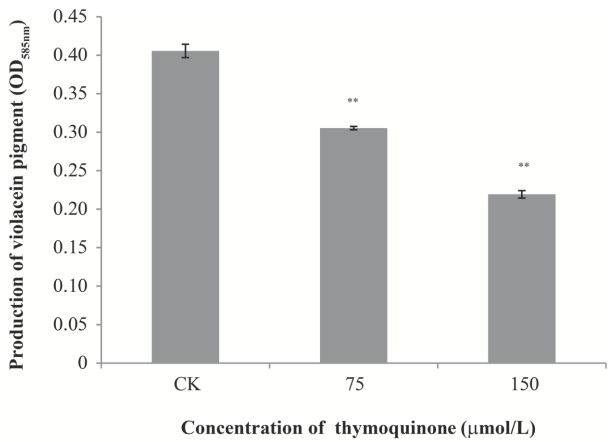

FIGURE 4 | Inhibition of violacein production by *C. violaceum* ATCC 12472 at different concentrations of TQ. Bars represent the standard deviation ($n = 3$). $^{**}P \leq 0.01$ compared to control.

protein A), *ompX* (outer membrane protein X), *uvrY* (adherence and invasion), *motA*, *motB* (flagellar motor protein), *sod* (survival in macrophages), *bcsA* (cellulose synthase catalytic subunit), *bcsG* (cell biosynthesis and biofilm formation), *lpx*, *wzx* (LPS biosynthesis), *luxR* (LuxR family transcriptional regulator), *galE* (colanic acid synthesis), and *kpsT* (K-antigen synthesis) (**Table 3**).

DISCUSSION

The motility is considered as a virulence factor for intestinal and urogenital-tract pathogens (Manson, 1992). To establish infection and incite disease development, bacteria first have to attach and adhere to a surface, either abiotic or biotic. The biofilm of *C. sakazakii* on abiotic surfaces in food and medical sectors constitutes a great public health concerns (Kim et al., 2006). Flagella seem to play a role in the initial phases of biofilm

TABLE 2 | Inhibition of *C. sakazakii* 7-17 biofilm formation by TQ at different concentrations at 25°C and 12°C.

Growth conditions	SBF		
	CK	TQ at 400 μmol/L	TQ at 600 μmol/L
25°C TIME (h)			
24	2.48 ± 0.13	1.42 ± 0.22[b]	1.35 ± 0.18[b]
48	2.25 ± 0.15	1.54 ± 0.10[b]	1.25 ± 0.11[b]
72	2.52 ± 0.20	1.63 ± 0.18[b]	0.80 ± 0.14[b]
12°C TIME (h)			
24	1.08 ± 0.26	0.82 ± 0.09	0.74 ± 0.06
48	2.79 ± 0.21	1.43 ± 0.12[b]	0.74 ± 0.06[b]
72	2.10 ± 0.09	0.34 ± 0.08[b]	0.39 ± 0.07[b]

[b] *$P \leq 0.01$ compared to control.*

development in *C. sakazakii*, in attachment as well as further formation. In addition, motility also plays a role in adhesion to both plant and animal tissues. Hartmann et al. (2010) observed that the absence of flagella greatly reduced the adhesion capacity, suggesting that flagella are important for *Cronobacter* adhesion to biotic surfaces. In this study, we observed that the sub-inhibitory concentration of thymoquinone obviously reduced the swimming and swamming motility area of *C. sakazakii*. To gain further insights into the effect of TQ on *C. sakazakii* motility, three key flagellar structure or biosynthesis genes (Hartmann et al., 2010) were selected to test their transcription. *flhD* encodes the transcriptional activator FlhD. *fliD* encodes the flagellar capping protein, *flgJ* encodes a muramidase gene involved in the flagellar rod assembly protein FlgJ. Interestingly, TQ suppressed *flhD, fliD,* and *flgJ* at transcriptional levels (**Table 3**), the loss of functionality of the flagellum could be the possible mechanism by which TQ suppresses swimming and swarming motility. These findings suggest that TQ can reduce the capacity of *C. sakazakii* to access to a surface actively and further establish effective infection.

It has been reported that some *C. sakazakii* strains are able to form biofilms on glass, silicone, stainless steel, polyvinyl chloride, and enteral feeding tubes in various media (Kim et al., 2006). Biofilms protect the embedded cells against detachment by flow shear and help them resist adverse conditions, such as presence of antimicrobials and biocides (Jung et al., 2013). Because of persistence and high resistance properties of mature biofilm there is an increasing interest to search for substances, which inhibit specific processes in the initial phase of biofilm formation. Szczepanski and Lipski (2014) reported that thyme, oregano, and cinnamon essential oil are able to inhibit the biofilm formation of the genera *Stenotrophomonas, Acinetobacter,* and *Sphingomonas* which were isolated from authentic biofilms in the food industry. Our results showed that TQ inhibited biofilm formation of strong biofilm producer on microtiter plates at 12° and 25°C in 72 h. Cellulose has been identified and characterized as an extracellular matrix component present in the biofilm of *C. sakazakii* (Grimm et al., 2008). The presence of TQ decreased the transcription level of *bcsA*, which encodes the catalytic subunit of the cellulose synthase and *bcsG*, which encodes the conserved hypothetical

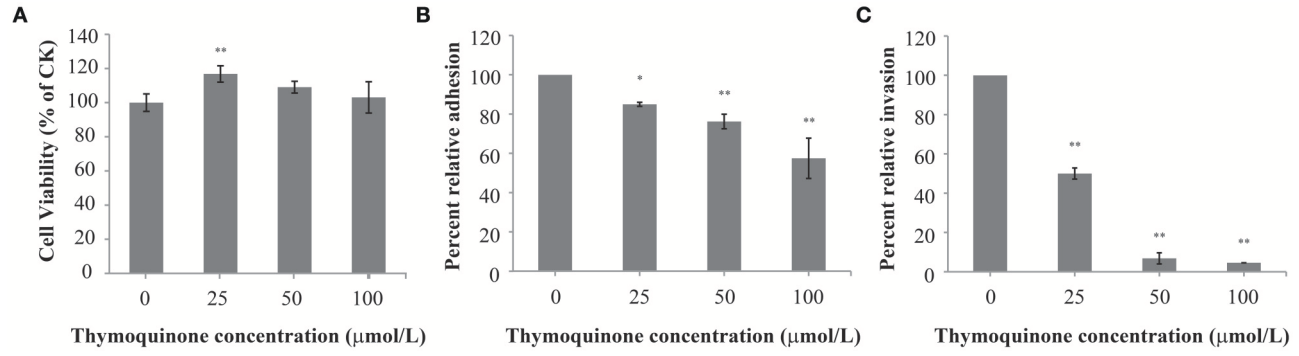

FIGURE 5 | Effect of TQ on HT-29 cells viability **(A)**; Effect of TQ on adhesion **(B)** and invasion **(C)** of HT-29 cells by *C. sakazakii* ATCC 29544. Bars represent the standard deviation ($n = 3$). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ compared to control.

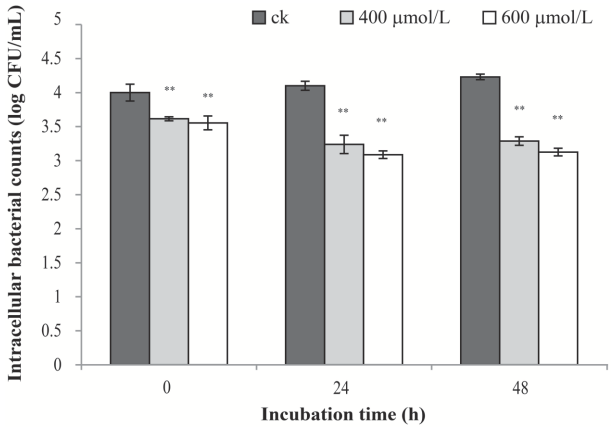

FIGURE 6 | Effect of TQ on bacterial intracellular survival and replication of *C. sakazakii* ATCC 29544 within macrophages. Bars represent the standard deviation ($n = 3$). $^{**}P \leq 0.01$ compared to control.

protein (**Table 3**). Therefore, it is hypothesized that TQ may inhibit *C. sakazakii* biofilm formation by inhibition of cellulose and flagella production.

Bacterial quorum sensing has been reported to control virulence gene expression with the presence of small signal molecules in numerous micro-organisms (Defoirdt et al., 2013). AHL, as the autoinducer signal in QS, accumulates in the medium and activates the transcriptional activator *luxR* to control a variety of physiological processes (Winzer and Williams, 2001). AHL-controlled functions in bacteria include conjugation, secretion of virulence factors, motility, pigment production, biofilm formation, and bioluminescence (Henke and Bassler, 2004). In this study, we used biomarker strain *C. violaceum* ATCC 12472, which can respond exogenous short chain AHL and produce violacein (Lichstein and Sand, 1945). TQ inhibited the production of AHL-regulated violacein pigment in *C. violaceum* possibly through disruption of QS signaling systems. Similarly, Vasavi et al. (2014) reported that inhibition of violacein production by the *Psidium guajava* L. flavonoids in *C. violaceum* indicated possible anti-QS activity. Musthafa et al. (2010) showed *Ananas comosus*, *Musa paradiciaca*, and *Ocimum sanctum* extracts significant reducted violacein production in *C. violaceum* and can be potentially developed as an alternative to antibiotic compounds to prevent AHL-mediated bacterial infection. In addition, RT-PCR assays also confirmed that TQ down-regulated the transcription of the *luxR* gene in *C. sakazakii* in this study.

The binding of pathogens to intestinal epithelial cells is one of the prerequisites for systemic infection. Hunter et al. (2008) found that *C. sakazakii* is able to bind to enterocytes in rat pups at the tips of villi, induces disruption of tight junctions between enterocytes and ultimately causes apoptosis of the cells. Kim and Loessner (2008) demonstrated that *C. sakazakii* exhibited significantly higher invasiveness than other *Enterobacter* species. The adhesion and invasion of *C. sakazakii* play an important role for itself to translocate through the intestinal lumen into the blood circulation and establish a systemic infection with symptoms such as bacteremia and necrotizing enterocolitis. Outer membrane proteins OmpX and OmpA were shown to play critical roles in *C. sakazakii* invasion through both apical side and basolateral side of the host cells, and they are responsible for *C. sakazakii* translocation into the deeper organs (i.e., liver and spleen) (Kim et al., 2010). The *uvrY* gene regulates the ability of *E. coli* to adhere, invade, persist within tissues (Herren et al., 2006). Our results demonstrate that *C. sakazakii* adhesion and invasion of HT29 cells were inhibited by TQ, and TQ significantly downregulated *ompA*, *ompX*, and *uvrY* expression. Likewise, Amalaradjou et al. (2014) reported that trans-cinnamaldehyde was effective in markedly reducing the attachment and invasion of rat intestinal epithelial cells by *C. sakazakii*.

The ability of pathogens to enter, persist and/or grow within macrophages is also important for infection. Almajed and Forsythe (2016) reported that some ST4 and ST1 *C. sakazakii* strains were able to survive for up to 72 h of incubation and multiply significantly within human macrophages cell line U937. In this study, *C. sakazakii* strain ATCC 29544 (ST1) showed the ability to persist within macrophages cell line RAW 264.7 for up to 48 h of incubation. Additionally, our results showed TQ reduced the persistence of *C. sakazakii* with RAW 264.7

TABLE 3 | Differentially expressed virulence-related genes in *C. sakazakii* ATCC 29544 grown in TSB with TQ at different concentrations.

Target gene	Sequence of primers(5′-3′)[c]	Relative gene expression	
		400 μmol/L	600 μmol/L
ESA_04030	F, CCAGGGCTACACACGTGCTA R, TCTCGCGAGGTCGCTTCT	1	1
bcsA	F, CACGATGGTGGCGTTGTTC R, CCTTTGGCGGTGACGTTAA	−1.74 ± 0.3[a]	−4.62 ± 1.49[a]
bcsG	F, ACGACTGGCTGAACAGCTTTTAC R, GCCGGGAAGGTTGTCTGA	−1.32 ± 0.37	−4.74 ± 0.23[b]
flhD	F, CGATGTTTCGCCTGGGAAT R, AGAGTCAGGTCGCCCAGTGT	−1.10 ± 0.06[a]	−2.66 ± 0.16[b]
fliD	F, AAAACCGCAACATGGAATTCA R, CCGCAAACGCGGTATTG	−1.78 ± 0.13[b]	−7.29 ± 0.57[b]
flgJ	F, GACGGCGGGCAAAGG R, GCCGCCCATCTGTTTGAC	−1.12 ± 0.62	−3.17 ± 0.52[b]
motA	F, GGTGTGGGTGCGTTTATCGT R, GCCTTCAGCGTGCCTTTG	−1.06 ± 0.01[a]	−2.83 ± 0.38[b]
motB	F, ACGGCTCGTGGAAAATCG R, CCAGGAAGAAGGCCATCATG	−1.09 ± 0.02[a]	−2.54 ± 0.21[b]
luxR	F, TGTGCGTTCGCCATCCT R, TGGTGTGCAGCGTCAGTTTT	−1.23 ± 0.10[a]	−4.91 ± 1.81[a]
lpxB	F,GCACGACACTTTCCGTAAACTG R,CGCCTGTTCATCGGCATT	−1.05 ± 0.02[b]	−1.69 ± 0.12[b]
ompA	F,GGCCGCATGCCGTATAAA R,GCTGTACGCCCTGAGCTTTG	−2.15 ± 0.01[b]	−2.70 ± 0.37[b]
ompX	F,GTCTTTCAGCACTGGCTTGTGT R,GGTGCCAGCAACAGCAGAA	−1.51 ± 0.16[b]	−4.13 ± 0.68[b]
sod	F,CGAATCTGCCGGTTGAAGA R,CTTGTCCGCCGGAACCT	−1.36 ± 0.14[a]	−1.99 ± 0.08[b]
uvrY	F,GCGAGGACGCCATCAAAT R,ATCCATCAGCACCACATCCA	−1.57 ± 0.25[a]	−2.12 ± 0.48[a]
wzx	F,TGCTTGGGCAGGTACAAAGTG R,CCCTACGGGTGCAGTCACA	−1.39 ± 0.08[b]	−4.28 ± 0.90[b]
galE	F,CTTGAGTATTACGACAACAACG R,GAAACTTTCGACATAAGGGAT	−3.39 ± 0.29[b]	−3.94 ± 0.35[b]
kpsT	F,ATTGGCGGGACGGATAA R,TCGTCCACCAGGTAGTAGTCA	−2.50 ± 0.19[b]	−4.85 ± 0.33[b]

[a]$P \leq 0.05$, [b]$P \leq 0.01$, [c]*F, forward; R, reverse.*

cells. Macrophages and polymorphonuclear cells typically engulf pathogens and also effectively kill nearby organisms by the release of reactive oxygen species. Superoxide dismutases in bacterium provides the capacity to neutralize toxic levels of ROS produced by the host (Lynch and Kuramitsu, 2000). In this study, we found that TQ significantly downregulated transcription of *sod*, which codes for the superoxide dismutases of *C. sakazakii*. Our results indicated that TQ decreased the chances of *C. sakazakii* to use

macrophages as a vehicle for escaping from the immune response and invading the other body organs.

Endotoxin (also known as lipopolysaccharide, LPS), a major amphophilic molecule embedded in the outer envelope of Gram-negative bacteria, is a potent immune activator and essential virulence factor of Gram-negative bacteria. LPS causes release of numerous host proinflammatory cytokines and activates the complement cascade and the coagulation cascade which play an important role in the virulence of Gram-negative pathogens (Das, 2000). Sivamaruthi et al. (2015) reported that *C. sakazakii* LPS is sufficient to affect the *Caenorhabditis elegans* pharyngeal pumping rate, brood size and cause lethality. Townsend et al. (2008) demonstrated the influence of endotoxin on increased translocation of intestinal bacteria in the neonatal rat. Here we proved that TQ inhibited the production of endotoxin in *C. sakazakii* and downregulated the expression of *lpx* and *wzx*, which are essential for the synthesis of LPS in Gram-negative bacteria.

In this study, we demonstrated that SICs of TQ decreased crucial virulence related traits in *C. sakazakii* ATCC 29544 including motility, expression of certain virulence genes, adhesion and invasion to HT-29 cells and intracellular survival of bacterial cells within the RAW 264.7 macrophage cells. In addition, TQ significantly inhibited biofilm formation of *C. sakazakii* 7-17 and interfered with AHL-dependent QS system in *C. violaceum*. The effects of TQ on other *C. sakazakii* strains and *in vivo C. sakazakii* infection model need further investigation before TQ could be potentially developed as an alternative strategy to control the diseases attributed to *C. sakazakii*.

AUTHOR CONTRIBUTIONS

CS, CY, and XX conceived and designed the experiments. YSui, YSun, and DG performed the experiments. YC and TJ analyzed the data. XP and LM contributed reagents, materials, analysis tools. CS and CY wrote the manuscript.

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CORRECTION
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Corrigendum: Thymoquinone Inhibits Virulence Related Traits of *Cronobacter sakazakii* ATCC 29544 and Has Anti-biofilm Formation Potential

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In the first paragraph in the "Introduction" of this article, the number of *Cronobacter* species was incorrectly described as ten. Actually, *Cronobacter pulveris*, *Cronobacter helveticus* and *Cronobacter turicensis* were removed from the genus in 2014.

The original sentence should be corrected as follows: *Cronobacter* spp. is currently considered to consist of 7 species: *Cronobacter sakazakii*, *C. malonaticus*, *C. universalis*, *C. dublinensis*, *C. muytjensii*, *C. condiment*, and *C. zurichensis* (Stephan et al., 2014), among which *C. sakazaii* is one of the two group 1 clinically relevant species that form the majority of the clinical isolates. The authors regret this error.

The original article has been updated.

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Virucidal Activity of Fogged Chlorine Dioxide- and Hydrogen Peroxide-Based Disinfectants against Human Norovirus and Its Surrogate, Feline Calicivirus, on Hard-to-Reach Surfaces

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Human norovirus (NoV) is the leading cause of foodborne illnesses in the United States. Norovirus is shed in high numbers in the feces and vomitous of infected individuals. Contact surfaces contaminated with bodily fluids harboring infectious virus particles serve as vehicles for pathogen transmission. Environmental stability of NoV and its resistance to many conventional disinfectants necessitate effective inactivation strategies to control the spread of virus. We investigated the efficacy of two commercial disinfectants, hydrogen peroxide (7.5%) and a chlorine dioxide (0.2%)-surfactant-based product using a fogging delivery system against human NoV GI.6 and GII.4 Sydney strains as well as the cultivable surrogate, feline calicivirus (FCV) dried on stainless steel coupons. Log_{10} reductions in human NoV and FCV were calculated utilizing RNase RT-qPCR and infectivity (plaque) assay, respectively. An improved antiviral activity of hydrogen peroxide as a function of disinfectant formulation concentration in the atmosphere was observed against both GII.4 and FCV. At 12.4 ml/m^3, hydrogen peroxide achieved a respective 2.5 ± 0.1 and 2.7 ± 0.3 log_{10} reduction in GI.6 and GII.4 NoV genome copies, and a 4.3 ± 0.1 log_{10} reduction in infectious FCV within 5 min. At the same disinfectant formulation concentration, chlorine dioxide-surfactant-based product resulted in a respective 1.7 ± 0.2, 0.6 ± 0.0, and 2.4 ± 0.2 log_{10} reduction in GI.6, GII.4, and FCV within 10 min; however, increasing the disinfectant formulation concentration to 15.9 ml/m^3 negatively impacted its efficacy. Fogging uniformly delivered the disinfectants throughout the room, and effectively decontaminated viruses on hard-to-reach surfaces. Hydrogen peroxide delivered by fog showed promising virucidal activity against FCV by meeting the United States EPA 4-log_{10} reduction criteria for an anti-noroviral disinfectant; however, fogged chlorine dioxide-surfactant-based product did not achieve a 4-log_{10} inactivation. Future investigation aimed at optimizing decontamination practices is warranted.

Keywords: hydrogen peroxide, chlorine dioxide, fogged disinfectant, norovirus inactivation, surface disinfection, public health

INTRODUCTION

Human norovirus (NoV) is the leading etiologic agent of acute gastroenteritis, accounting for 48% of all foodborne outbreaks in the United States (Hall et al., 2014). Human NoV is a non-enveloped virus with a positive-sense RNA genome belonging to the family *Caliciviridae* (Green, 2007). The virus is transmitted either directly through fecal-oral or vomit-oral routes, or indirectly through contact with contaminated surfaces, or through the consumption of contaminated food and water. Once deposited on surfaces, human NoV can remain infectious for several weeks (Escudero et al., 2012; Lopman et al., 2012; Hall et al., 2014). Environmental stability of human NoV is enhanced by resistance to commercial sanitizers and disinfectants, including alcohol-based hand sanitizers and hypochlorite at regulated concentrations (Liu et al., 2010; Tung et al., 2013; Cromeans et al., 2014; Cook et al., 2016). These unique traits of human NoV contribute to the high number of outbreaks observed annually in close quarter environments such as cruise ships, long-term care facilities, and schools, as well as in association with food service (Lopman et al., 2012; Cook et al., 2016). Therefore, innovative methods for inactivation of NoV from these environments where frequent human contact with surfaces is expected are needed to control the spread of the pathogen.

Conventional methods for disinfection of contaminated surfaces are often time-consuming and labor-intensive. Additionally, manual disinfection of surfaces relies on operator compliance to achieve an optimal efficacy. Considering these shortcomings, automated disinfection methods have become increasingly popular. Chlorine dioxide (ClO_2) and hydrogen peroxide (H_2O_2) are two strong oxidizing agents with a broad antimicrobial activity offering a promising potential as contact surface sanitizers (Hoehn et al., 2010; Tuladhar et al., 2012). Gaseous delivery of these disinfectants has shown superior antimicrobial activity over aqueous forms by being more diffusible, penetrable and able to access areas beyond the reach of liquid sanitizers and hard-to-clean sites (Morino et al., 2011; Tuladhar et al., 2012; Yeap et al., 2015).

Despite the widespread use of ClO_2 and H_2O_2 as surface disinfectants, to the best of our knowledge, there is no study in the literature that characterizes the efficacy of these disinfectants against human NoV using a fogging system. We sought to characterize the antiviral activity of two commercially available ClO_2- and H_2O_2-based disinfectants when delivered by a portable fogging device against two epidemiologically important human NoV outbreak strains GI.6 and GII.4 as well as the frequently used cultivable surrogate feline calicivirus (FCV) on stainless steel coupons. In the absence of a practical human NoV cell culture system, we utilized real-time polymerase chain reaction (RT-qPCR) preceded by RNase treatment for the detection and quantification of intact, presumptively infectious virus particles (Knight et al., 2013; Manuel et al., 2015). A standard plaque assay technique was used to determine reduction in infectious titer of FCV particles following exposure to the disinfectants. The experiments for each disinfectant were performed separately with no intention of being a comparative study, although the results of each are described here. This research provides evidence of the efficacy of an antiviral disinfectant delivery system for inactivation of human NoV in enclosed areas.

MATERIALS AND METHODS

Disinfectants

Samples of the two commercial products used in this study were kindly provided courtesy of M. Quinoy (AeroClave™, Winter Park, FL, United States). The disinfectants were (i) H_2O_2 7.5% (inert ingredients 92.5%, United States EPA registration No. 83046-1, AeroClave); and (ii) Vital Oxide® (United States EPA registration No. 82972-1, Vital Solutions, West Palm Beach, FL, United States), a ClO_2-surfactant-based product with United States EPA approval of anti-noroviral efficacy based on infectivity assay against the cultivable surrogate FCV (United States Environmental Protection Agency, 2016b), and claims active ingredients as 0.20% ClO_2, 0.125% alkyl (60% C14, 30% C16, 5% C12, 5% C18) dimethyl benzyl ammonium chloride, 0.125% alkyl (68% C12, 32% C14) dimethyl ethylbenzyl ammonium chloride, and 99.55% as inactive ingredients.

Preparation of Virus Stocks

Fecal specimens collected from outbreaks, and confirmed positive for human NoV GI.6 and GII.4 Sydney strains by sequencing, were kindly provided by S. R. Greene (North Carolina Division of Public Health, Raleigh, NC, United States). Prior to use in experiments, we reconfirmed their identity using genogroup-specific RT-qPCR, as explained below. A 20% suspension (w/v) was prepared in phosphate-buffered saline solution (PBS; pH 7.2), clarified by centrifugation (3,100 × g for 2 min at room temperature), and stored at −80°C until use.

Feline calicivirus strain F9 (FCV) was propagated in Crandell Rees feline kidney (CRFK) cells as previously described (Tung et al., 2013). Briefly, preparation of virus stocks was done by infecting a 90% confluent CRFK monolayer at a multiplicity of infection of 0.6. The cells were incubated at 37°C with 5% CO_2 until >90% of cells displayed cytopathic effects. The cells were lysed by three freeze-thaw cycles at −80°C to release viral particles. Lysates were clarified by centrifugation, passed through a 0.2-μm filter, aliquoted and stored at −80°C until use.

Coupon Preparation and Inoculation

Non-adhesive stainless steel embossing tape (DYMO Co, Berkeley, CA, United States) were utilized as carriers. The tape was cut into 2.5 cm × 5.0 cm pieces, degreased in acetone, and sterilized by autoclaving for 15 min at 121°C. Each coupon was inoculated by placing a 25-μl aliquot of virus stock at the center of each strip (6–7 $\log_{10}$ titer), air-dried in a biosafety hood for 45 min, and immediately used for each experiment. All procedures were carried out in accordance to the United States EPA confirmatory virucidal effectiveness test (United States Environmental Protection Agency, 2016a).

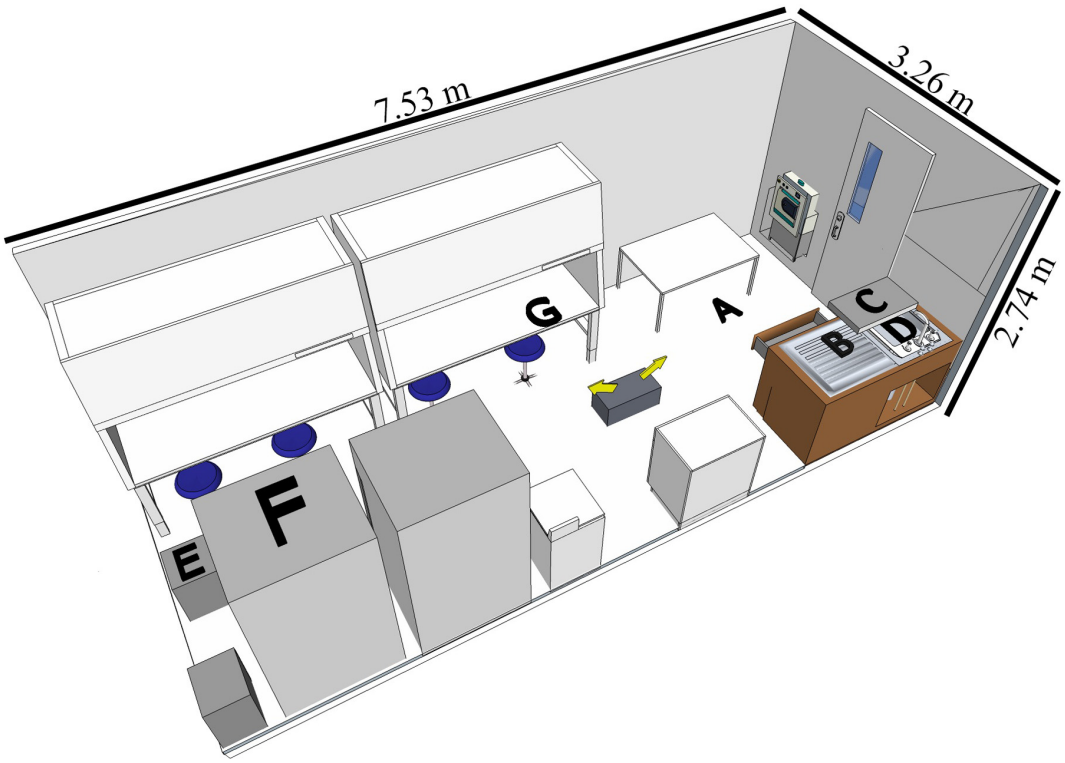

FIGURE 1 | Diagram of BSL-3 laboratory setup with the coupon locations indicated. The fog generator was placed on the ground as indicated by the two arrows showing the directions of fogging. Graphical representation of the laboratory is to scale (room layout created by SketchUp Make 2016, Trimble Navigation Ltd.). Coupon locations as follows: (A) (floor) for both experiments; (B) (counter) and (C) (shelf) for H_2O_2-based product; (D) (sink), (E) (drawer), (F) (top of the refrigerator) and (G) (inside the hood while the door open) for the ClO_2-based disinfectant.

Experimental Conditions and Virus Treatments

A BSL-3 laboratory (7.53 L × 3.26 W × 2.74 H meters, **Figure 1**) in North Carolina Research Campus (Kannapolis, NC, United States) was used for the study. The disinfectants were tested in separate experiments with different assigned locations of the coupons (**Figure 1**) representative of contamination spots on easy-to-reach and hard-to-reach areas for cleaning purposes.

After coupon placement, the room was exited, the air handler unit was turned off, and the fogged disinfectant was delivered into the room (relative humidity 60%, 21°C) using the automated Room Decontamination System 3110 (AeroClave), located at the center of the laboratory floor. The machine was equipped with two nozzles, that according to the manufacturer, was capable of generating fogs at 30 ml/min per nozzle at opposite directions. This allowed us to adjust fog generation time, until desired disinfectant formulation concentrations of 7.1–15.9 ml/m^3 were achieved. The target disinfectant formulation concentration was held for 5 min for H_2O_2 and 10 min for ClO_2-surfactant-based product (according to the manufacturer recommendations), then the ventilation was resumed, and aeration was allowed for 20 min to remove the fogged disinfectant. Exposed carriers were immediately suspended in 3–5 ml PBS in a 15-ml conical tube and vortexed vigorously for 30 s to facilitate virus elution. Eluted viruses were aliquoted and stored frozen at −80°C prior to analysis. For each replicate, positive controls were prepared by placing the inoculated coupons on the bench just outside the BSL-3 laboratory entrance room for the duration of each experiment so that they were not exposed to the disinfectants. Virus $\log_{10}$ inactivation was calculated by subtracting the titer of the disinfectant-treated inoculated coupons from the titer of positive controls.

Human Norovirus RT-qPCR Analysis

Prior to analysis by RT-qPCR, human NoV GI.6 and GII.4 inoculated samples were subjected to an RNase treatment, as previously reported (Manuel et al., 2015). Pretreatment of eluted viral particles with RNase serves as an alternative method to discriminate between infectious and non-infectious viruses through degradation of free floating viral RNA or exposed RNA from partially destructed capsids, preventing them from being amplified during RT-qPCR (Topping et al., 2009; Knight et al., 2013). Briefly, 100-µl of eluate was mixed with 1 U of RNase ONE™ ribonuclease and 1 × reaction buffer (Promega, Madison, WI, United States), and incubated at 37°C for 15 min. Samples were then placed on ice for 5 min to stop the reaction. The viral RNA was immediately extracted with an automated NucliSENS® easyMag® system (BioMérieux, St. Louis, MO, United States) per manufacturer's instructions, eluted

in a proprietary buffer, and stored at −80°C until RT-qPCR analysis.

In all experiments, RT-qPCR amplification targeted the ORF1-ORF2 junction of the human norovirus genome using COG1F/COG1R primers and Ring1(a)/Ring1(b) probes for GI.6 (Kageyama et al., 2003), and JJV2F/COG2R primers and Ring2 probe for GII.4 (Jothikumar et al., 2005). Estimation of genomic copies was performed by comparison with a calibration curve established using RNA transcripts of the ORF1-ORF2 junction of the human norovirus genome (Escudero et al., 2012). The $\log_{10}$-transformed RNA genomic copies were plotted against the threshold cycle (C_t) value (threshold 30) using linear regression to make the calibration curve. Negative amplification control (water) and positive amplification controls (diluted GI and GII RNA transcripts) were incorporated in each RT-qPCR run. All RT-qPCR analyses were performed on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Hercules, CA, United States).

RT-qPCR conditions varied slightly depending on the experiment. For the H_2O_2 experiment, a One-step iScript™ RT Supermix kit (Bio-Rad, Hercules CA, United States) was used in 25-µl master mix composed of 2.5 µl of viral RNA, 200 nM of primers, 200 nM of fluorescently labeled TaqMan probe, 1 × Bio-Rad PCR reaction buffer (Bio-Rad), and 0.5 µl Bio-Rad iScript RT mix. The reaction mixture was subjected to a one-step thermal cycling profile under the following amplification conditions: (i) reverse transcription for 10 min at 50°C, (ii) initial denaturation for 5 min at 95°C, and (iii) 45 cycles of 15 s at 95°C and 30 s at 55°C.

For the experiments on ClO_2-surfactant-based product, a SuperScript™ III One-Step RT-PCR with Platinum® Taq High Fidelity DNA polymerase (Invitrogen, Carlsbad, CA, United States) was used. The reaction volume of 25 µl was composed of 2.5 µl of RNA template, 1 × reaction mix, 0.5 µl SuperScript® III RT/Platinum® Taq mix, 0.25 U RNasin® Plus ribonuclease inhibitor (Promega), 200 nM of each primer and 200 nM of GII probe or 120 nM of each of the two GI probes. The RNA was reverse-transcribed at 50°C for 15 min, the Platinum Taq polymerase was activated at 95°C for 2 min, then followed by thermal cycling for 15 s at 95°C, 30 s at 54°C, and 30 s at 72°C for a total of 45 cycles.

Feline Calicivirus (FCV) Infectivity Assay

Infectious titers of FCV were determined using the United States EPA standard plaque assay technique (United States Environmental Protection Agency, 2016a). Briefly, CRFK cell monolayers at 80–90% confluency were infected with 450 µl of 10-fold serially diluted eluates. After overlay and incubation for 2–3 days at 37°C in 5% CO_2, cells were fixed in 3.7% formaldehyde, and plaques visualized by staining with 0.1% (w/v) crystal violet solution. The cells were rinsed with water and plates with 5–50 plaque-forming units (PFU) were used to determine infectious virus titer. Neutralizer control (using PBS) and cell viability controls were included in accordance with the United States EPA method (United States Environmental Protection Agency, 2016a).

Statistical Analysis

Each experiment tested a single disinfectant formulation concentration, and was replicated three times with duplicate measurements. All data are reported as mean ± standard error. Statistical analysis was done by one-way ANOVA followed by Tukey's HSD for pair-wise comparisons of means using RStudio (Version 0.99.903, RStudio Inc., Boston, MA, nited States). A p-value of smaller than 0.05 was considered statistically significant.

RESULTS

The purpose of this research was to evaluate the applicability of both H_2O_2 (7.5%) and ClO_2 (0.2%)-surfactant-based disinfectants against human NoV and the cultivable surrogate virus, FCV, on stainless steel contact surfaces. We employed RNase RT-qPCR on human NoV to select for intact virus particles, and plaque assay to assess infectivity of FCV. Highest quantifiable degree of virus inactivation, based on virus stock concentration and assay detection limits, was 2.1–2.8 and 3.2–3.7 $\log_{10}$ genomic copies for NoV GI.6 and GII.4 Sydney, respectively, and 4.2–5.1 $\log_{10}$ PFU for FCV.

Hydrogen Peroxide (H_2O_2)

No trend was observed for human NoV GI.6 reduction as a function of H_2O_2-based disinfectant formulation concentration ($p > 0.05$, **Figure 2A** and Supplementary Data Sheet 1). However, increasing the concentration from 7.1 to 12.4 ml/m^3 enhanced viral genomic copy number reduction for GII.4 from 1.5 ± 0.3 to 2.7 ± 0.3 $\log_{10}$ copies, respectively ($p < 0.05$, **Figure 2B**). For GII.4 samples, the inoculated coupons placed on the shelf (site C, **Figure 1B**) and exposed to 7.1 and 8.8 ml/m^3 H_2O_2 had significantly lower overall reductions (0.7 $\log_{10}$) as compared to the coupons placed on a counter top (site B, 2.0 $\log_{10}$, $p < 0.05$) or floor (site A, 2.2 $\log_{10}$, $p < 0.05$). No significant location effect for carriers was observed for GI.6 samples ($p > 0.05$). Norovirus GII.4 displayed a significantly lower reduction in RNA genomic copies as compared to GI.6 when carriers were placed on either the shelf or counter and exposed to lower disinfectant formulation concentrations of H_2O_2 ($p < 0.05$, **Figures 2A,B**).

Virucidal activity of H_2O_2 against FCV was enhanced as the disinfectant formulation concentration of fogged disinfectant increased in the atmosphere, reaching the maximum reduction of 4.3 $\log_{10}$ in infectious FCV particles ($p < 0.05$, **Figure 2C**). At all locations, at least 10.6 ml/m^3 H_2O_2 was required to ensure a 4-$\log_{10}$ reduction for infectious FCV. No location effect was observed for FCV inactivation in any of the samples tested at any disinfectant formulation concentration ($p > 0.05$).

ClO_2-Surfactant-Based Product

This product was used at the recommended disinfectant formulation concentration of 12.4 ml/m^3 and a higher concentration of 15.9 ml/m^3 to assess any enhanced virucidal activity. Human NoV GI.6 and GII.4 strains showed 1.7 ± 0.2 and 0.6 ± 0.1 $\log_{10}$ reductions in genome copy number, respectively, following application at 12.4 ml/m^3 (**Figures 3A,B**

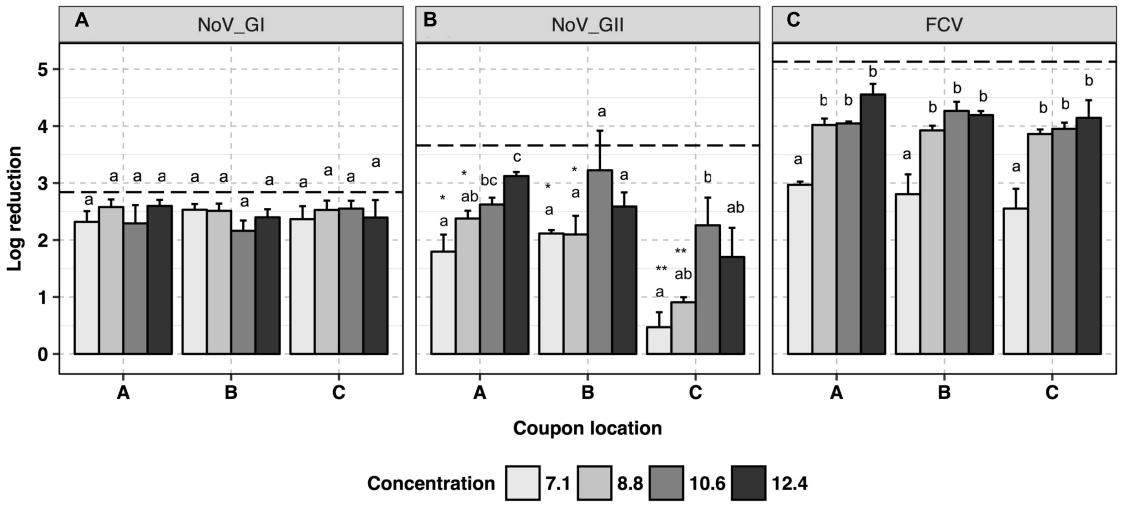

FIGURE 2 | Efficacy of H_2O_2 fogging on GI.6 and GII.4 Sydney human NoV and feline calicivirus (FCV) at various disinfectant formulation concentrations (ml/m^3). For each location within each section (virus strain): (1) uppercase letters denote coupons location as shown in **Figure 1**; (2) lowercase letters indicate statistically significant differences in each location across disinfectant formulation concentrations; (3) asterisks denote significant differences for each disinfectant formulation concentration across locations (only observed for human NoV GII.4 on the samples located on location C, shelf). Results are expressed as $\log_{10}$ reduction in genomic copies by RNase RT-qPCR for human NoV, and plaque-forming units (PFU) for FCV. Error bars represent standard error of the mean. All experiments were performed in triplicate. Long-dashed lines represent the highest quantifiable degree of virus $\log_{10}$ inactivation.

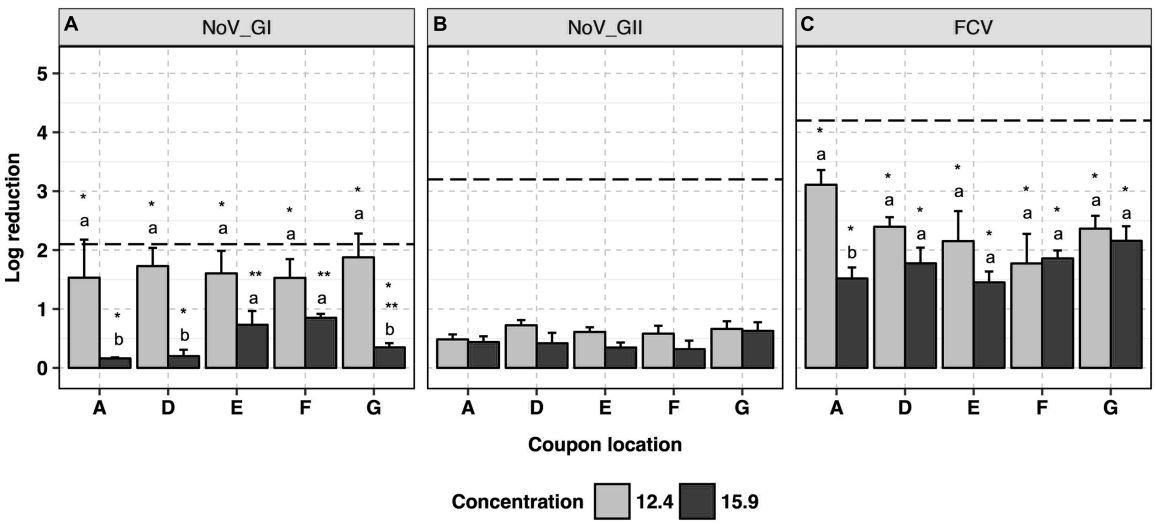

FIGURE 3 | Efficacy of ClO_2-surfactant-based product fogging on GI.6 and GII.4 Sydney human NoV and FCV at various disinfectant formulation concentrations (ml/m^3). For each virus: (1) uppercase letters denote coupons location as shown in **Figure 1**; (2) lowercase letters indicate statistically significant differences in each location across disinfectant formulation concentrations; (3) asterisks denote significant differences for each concentration across locations [statistical analysis was not carried out for human NoV GII Sydney due to the negligible reduction (<1 $\log_{10}$) observed in those samples]. Results are expressed as $\log_{10}$ reduction in genomic copies by RNase RT-qPCR for human NoV, and PFU for FCV. Error bars represent standard error of the mean. All experiments were performed in triplicate. Long-dashed lines represent the highest quantifiable degree of virus $\log_{10}$ inactivation.

and Supplementary Data Sheet 1) with no impact of coupon location on the efficacy of the disinfectant ($p > 0.05$). The antiviral efficacy decreased ($p < 0.05$) when the product was applied at the higher disinfectant formulation concentration (15.9 ml/m^3), wherein less than 1.0 $\log_{10}$ reduction in genome copies was achieved in the majority of the samples; this was consistent across the replicates. At 15.9 ml/m^3, marginal differences in GI.6 $\log_{10}$ inactivation were observed as a function of the location of the coupons, and were not always statistically significant ($p > 0.05$).

The average $\log_{10}$ reduction in infectious FCV titer as the result of exposure to ClO_2-surfactant-based product was

2.4 ± 0.2 at 12.4 ml/m^3 versus 1.8 ± 0.1 at 15.9 ml/m^3 (**Figure 3C**). Log_{10} reduction in infectious FCV was, respectively, 0.7 and 1.7 log_{10} higher than GI.6 and GII.4 log_{10} genome copy number reductions when 12.4 ml/m^3 was applied ($p < 0.05$). Similar to the data for human NoV, the product was uniformly efficacious against FCV across coupon locations with a reduced efficacy at higher disinfectant formulation concentration. Slight condensation on the ground around the fog generator were observed when ClO_2-surfactant-based product was applied at 15.9 ml/m^3.

DISCUSSION

Fomites (porous and non-porous surfaces) can be contaminated with human NoV through direct contact with the feces or vomitus shed by infected individuals, and serve as vehicles to spread the virus. Findings have demonstrated the stability of human NoV on hard surfaces and their resistance to common disinfectants, contributing to the NoV persistence in the environment and rapid transmissibility (Escudero et al., 2012; Tung et al., 2013; Liu et al., 2015; Pringle et al., 2015).

We investigated the antiviral activity of two disinfectants against human NoV GI.6 and GII.4 and FCV, the cultivable surrogate, using a fogging device. Human NoV GII.4 fecal suspensions dried on stainless steel coupon have been shown to be stable for at least 240 min when exposed to air under standard laboratory conditions (Manuel et al., 2015). Therefore, the observed virus reduction in our experiment could be representative of virus inactivation as the result of the exposure to the disinfectants. Our experimental design includes four unique features: (i) incorporation of an RNase treatment prior to RT-qPCR for human NoV to provide data more representative of viral infectivity by targeting intact viruses (Knight et al., 2013); (ii) use of two different human NoV genogroups to evaluate potential genotype-associated differences in response to the disinfection; (iii) use of a cultivable surrogate to evaluate virus infectivity in parallel, and as recommended by the United States EPA for registration of anti-norovirus product claims (United States Environmental Protection Agency, 2016a); and (iv) consideration of the impact of contamination location on fogging efficacy. The experiments on each disinfectant were performed separately and this is not intended to be a comparative study. In addition, it should be noted that the virucidal performance observed here is a function of the method of application, formulation concentration in the atmosphere and the contact times under which this study was conducted.

In general, both disinfectants were effective against human NoV and FCV. We observed a higher reduction in human NoV GI.6 viral genomic copies at low formulation concentrations of the disinfectants when compared to GII.4 located in the same area (**Figures 2**, **3**). This suggests that GII.4 could be more resistant than GI.6 to the disinfectants, in agreement with a previous report of human NoV susceptibility to alcohols, chlorine, and high hydrostatic pressure (Cromeans et al., 2014). Given that the majority of human illnesses are caused by GII.4 epidemic strains (Pringle et al., 2015), it is tempting to link the enhanced environmental resistance of these strains to their widespread prevalence, although additional studies are needed to test this hypothesis. Although not the focus of our research, slight variation in the resistance of human NoV strains of a certain genogroup to disinfectants may exist, as a recent study reported a lower resistance of GII.4 Sydney to alcohol as compared with GII.4 New Orleans strain (Park et al., 2016).

To our knowledge, this study represents the only one of its kind to investigate the impact of fogged H_2O_2 and a ClO_2-surfactant-based product on both outbreak-associated human NoV strains, GI.6 and GII.4, and the cultivable surrogate FCV. We found that fogged H_2O_2 could inactivate GI.6 and GII.4 human NoV by 2.4 and 1.4 log_{10} at the lowest applied disinfectant formulation concentration (7.1 ml/m^3). In a similar study, Tuladhar et al. (2012) examined the inactivation of human NoV GII.4 positive stool under exposure to fogged H_2O_2 and observed less than 1.0 log_{10} reduction in genomic copies; however, the authors did not perform RNase treatment prior the RT-qPCR and hence their results may underestimate reduction in viral genomic copies and product efficacy.

In the case of ClO_2-surfactant-based product, using monoclonal antibody-conjugated immunomagnetic beads to select for infectious viruses, Liu et al. (2015) demonstrated no reduction for human NoV GI and GII in suspension assays. Even though a superior antiviral activity for chemical disinfectants is usually expected when antiviral activity is examined in suspension as compared with surface assays (Morino et al., 2011), we found that ClO_2-surfactant-based product, when delivered as a fog at 12.4 ml/m^3 provided 1.7 and 0.6 log_{10} reduction in GI.6 and GII.4 Sydney genomic copies when inocula were dried on stainless steel coupons. As stated above, the differences in these results may be a function of methodological approaches to estimating surviving infectious human NoV.

Both H_2O_2 and ClO_2 are strong oxidizing agents that can destroy both proteins and nucleic acids (McDonnell and Russell, 1999; Yeap et al., 2015). Given the structure of enteric viruses, it would be logical to assume that the initial hit to the virus occurs at the capsid, after the destruction of which follows attack of viral RNA. Our data supports the notion that both disinfectants attack the virus capsid; however, given our experimental design, no conclusion on the impact of the disinfectants on viral genomes could be drawn. A recent study published by our group revealed that copper appears to inactivate human NoV by attacking both viral genome and capsid (Manuel et al., 2015). Since the antimicrobial mechanism of action of copper is similar to that of H_2O_2 (i.e., generation of reactive free radicals), it is reasonable to speculate that H_2O_2 fogging is likely to also impact the viral genome of human NoV, although this requires further confirmatory studies. Gaseous ClO_2 has been shown to inactivate murine norovirus, a cultivable human NoV surrogate, through degradation of both viral capsid protein and genome (Yeap et al., 2015). In the case of sodium hypochlorite, however, no significant degradation of the viral genome at 1,000 ppm was observed for human NoV GI, GII and the surrogates, except for FCV (Cromeans et al., 2014). Surface assays showed that sodium hypochlorite, even at high concentrations (e.g., 5,000 ppm), does not seem to have a significant role in degradation of

the viral genome after 4 min contact with a NoV GII.4 fecal suspension inoculated on steel coupons (Park and Sobsey, 2011).

Evaluating the virucidal efficacy of a disinfectant solely based upon reduction in viral genomic reduction may underestimate the efficacy of disinfectants (Yeargin et al., 2015); therefore, testing the infectious titer of a non-enveloped cultivable surrogate provides further evidence on the antiviral activity of a disinfectant (Tung et al., 2013; Cromeans et al., 2014). Among the human NoV cultivable surrogates, FCV is the approved surrogate established by the United States EPA to assess the anti-noroviral efficacy of disinfectants (United States Environmental Protection Agency, 2016a). The 4.3 $\log_{10}$ reduction in infectious titer we reported for FCV during exposure to H_2O_2 fog is in accordance with a previous study (Bentley et al., 2012). After exposure to fogged ClO_2-surfactant-based product, we observed a comparatively lower reduction of FCV titer (2.4 $\log_{10}$) at 12.4 ml/m^3. The efficacy of disinfection depends on a number of factors including mode of application, disinfectant concentration, contact time, organic load of the inoculum, and virus type. Since there have not been previous fogging studies with FCV exposed to chlorine dioxide by fog, the only comparisons that can be made are hard surface studies with ClO_2 gas. Morino et al. (2011) demonstrated less than a 3 $\log_{10}$ reduction in FCV titer even after a 4-h contact time with 0.05 ppm ClO_2 gas. Other surrogates such as murine norovirus are likely to be more resistant than FCV to oxidizing disinfectants such as sodium hypochlorite (Cromeans et al., 2014), meaning this disinfectant may be even less efficacious than reported in our study.

To test the impact of contamination location on the efficacy of the disinfection system, we placed the coupons at different elevations and distances to the fogger (**Figure 1**). The only location effect occurred in H_2O_2 experiment when carriers containing GII.4 Sydney virus were placed on the shelf (site C, 1.6 m height) and subjected to the two lowest disinfectant formulation concentrations of 7.1 and 8.8 ml/m^3 (**Figures 1**, **2B**). Interestingly, a previous study failed to observe any location effect when carriers containing poliovirus were placed on top of a 2.0 m high closet and subjected to H_2O_2 fogging using a similar delivery mechanism and fogging parameters as compared to our study (Tuladhar et al., 2012). In the case of ClO_2-surfactant-based product, no location effect was observed for any of the viruses, even when the product was fogged at the recommended disinfectant formulation concentration of 12.4 ml/m^3.

When used at a disinfectant formulation concentration of 15.9 ml/m^3, the efficacy of fogged ClO_2-surfactant-based product achieved a 0.4 $\log_{10}$ reduction in both GI and GII and 1.8 $\log_{10}$ reduction in FCV. The negative impact of increased ClO_2-surfactant-based product concentration on virus inactivation was observed both in human NoV RNA genome copies as well as FCV PFU across replicates. Though not explored experimentally, we believe that over-saturation of the fog at disinfectant formulation concentrations higher than that recommended (12.4 ml/m^3) resulted in condensation of the disinfectant and more rapid removal from the atmosphere. In a similar study, a 3.0 $\log_{10}$ reduction in murine norovirus was reported after 2 min exposure to 2.5 ppm ClO_2 gas; and when 2.0 ppm ClO_2 gas was applied, a similar virus inactivation could be obtained only after extending the contact time to 5 min (Yeap et al., 2015). Therefore, we speculated that the efficacy of the ClO_2-surfactant-based product tested in this study might be enhanced if applied at the recommended disinfectant formulation concentration of 12.4 ml/m^3 but for a longer contact time.

Our observation of adequate dispersion of both products when used at appropriate disinfectant formulation concentrations illustrates a significant advantage to fogging in that it can uniformly provide exposure to disinfectant throughout the room, covering even hard-to-reach surfaces. Further, since fogging is semi-automated, covers a wide area, and should inactivate airborne virus (not evaluated in our study), it should allow higher sanitation efficacy, increased compliance, and better worker productivity. A disadvantage of these systems is their high cost relative to conventional surface sanitation methods and that they can only be used in semi-enclosed spaces which must be evacuated for 30–60 min before reentry. They are also designed to be used on pre-cleaned surfaces. While we did not perform experiments using additional simulated organic (soil) load, previous studies using oxidizing disinfectants including H_2O_2, ClO_2 or sodium hypochlorite against a variety of human norovirus surrogates (murine norovirus and FCV) have shown that organic load significantly reduces virucidal efficacy (Urakami et al., 2007; Ayyildiz et al., 2009; Morino et al., 2011). Despite the reasonable virucidal efficacy achieved by applying the 7.5% H_2O_2 disinfectant by fogging, corrosivity concerns after long-term use remain (Rutala et al., 2008). Unfortunately, at lower concentrations H_2O_2 may lose its anti-noroviral activity (Kingsley et al., 2014).

Overall, this study represents the only one of its kind to investigate the impact of fogging on multiple human NoV strains (GI.6 and GII.4) along with the cultivable surrogate FCV under the same set of experimental conditions. Major findings from this study include evidence that exposure to H_2O_2 fogging results in an approximate 2.5 and 2.7 $\log_{10}$ reduction in RNA genomic copies for human norovirus GI.6 and GII.4 Sydney (respectively), and a 4.3 $\log_{10}$ reduction in infectious FCV at 12.4 ml/m^3. On the other hand, the ClO_2-surfactant-based product was mildly efficacious against the tested viruses, achieved only a 1.7 and 0.6 $\log_{10}$ reduction in RNA genomic copies for human NoV GI.6 and GII.4 (respectively), and 2.4 $\log_{10}$ reduction in PFU for FCV when used at the recommended disinfectant formulation concentration of 12.4 ml/m^3. Both experiments indicated that GII strains of human norovirus may be more resistant to the fogged disinfectants than GI strains. In conclusion, fogging systems, especially when used with hydrogen peroxide disinfectant, show promise application for NoV disinfection of enclosed areas, allowing the disinfectant to saturate the air and reach hard-to-disinfect surfaces. It is important to note that due to differences in experimental design and methods, this work does not allow for the direct comparison of the efficacy of the tested disinfectants delivered as a fog. Future studies should focus on improving the surface decontamination by optimizing the application technologies and disinfection parameters.

AUTHOR CONTRIBUTIONS

NM, CM, and EM designed and performed the experiments, and contributed to the preparation of the manuscript. JK and LW provided the BL-3 facility and technical insights on utilization of facility. L-AJ led the research.

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SUPPLEMENTARY MATERIAL

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A Multiplex RT-PCR Assay for *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. Detection in Raw Milk with Pre-enrichment

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This study firstly developed a multiplex real-time PCR (RT-PCR) technique combined with a pre-enrichment step to simultaneously detect *Staphylococcus aureus* (*S. aureus*), *Listeria monocytogenes* (*L. monocytogenes*) and *Salmonella* spp. in raw milk and the dairy farm environment (feces, soil, feed, water) in one reaction. Brain heart infusion (BHI) broth was selected for the enrichment step to increase the density of the target bacteria by using an incubation of 4 h before multiplex RT-PCR. The results showed that the detection limit of the multiplex real-time assay was approximately 10^2 CFU/mL for pure cultures and artificially contaminated milk without enrichment, while 12, 14, and 10 CFU/25 mL, respectively, for *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. after pre-enrichment. The newly developed multiplex RT-PCR assay was applied to 46 dairy farm environmental samples and raw milk samples covering a wide variety of sample types. The results demonstrated that the multiplex RT-PCR assay coupled with the BHI enrichment broth was suitable for the simultaneous screening of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. in the pasture environment and in raw milk. The multiplex RT-PCR assay clearly and successfully shortened the total detection time and reduced labor compared to conventional culture-based methods for testing natural samples.

Keywords: multiplex real-time PCR, enrichment step, *S. aureus*, *L. monocytogenes*, *Salmonella* spp., dairy farm environment, raw milk

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INTRODUCTION

Waterborne and foodborne pathogens are ubiquitous in the environment. The threat to human health posed by foodborne pathogens has attracted public attention, and the incidence of illness or death caused by major known pathogens has increased worldwide (European Food Safety Authority/European Centre for Disease Control [EFSA/ECDC], 2014, 2015a,b). Infections or outbreaks caused by major foodborne pathogens can be the result of consuming contaminated foods, including beef, milk products, fresh vegetables, and contaminated water (Burgess et al., 2016). In addition, food itself is a complex system, as well as a complicated environment, which can supply the enough nutrition for the bacteria.

Milk is a very popular food around the world, supplying nutrients essential for human health. The total annual worldwide production of milk has reached 695 million tons and created a value of 117 billion in EU annual sales for the top 20 dairy companies (Papademas and Bintsis, 2010). Recently, consumption of raw unpasteurized milk has been increasingly welcomed as having enhanced nutritional qualities, taste, and benefits (Oliver et al., 2009). However, dairy farms and milk products are nutrient-rich reservoirs for microbes, especially for waterborne and foodborne pathogens; these farms and products supply a transmission pathway for bacteria when they come into contact with contaminated sources. Pathogens, including *S. aureus*, *L. monocytogenes,* and *Salmonella* spp., can cause mastitis and can be directly excreted into milk (Oliver et al., 2005). A survey showed that the prevalence of *S. aureus* was 25.3% amongst 51,963 raw milk samples in California (Heidinger et al., 2009). *L. monocytogenes* is also ubiquitous in farm environments and is found in contaminated animal feces, low-quality feed, unsanitary dairy farms, and even in milk (Sanaa et al., 1993; Fox et al., 2009; Latorre et al., 2010; Hunt et al., 2017). *Salmonella* spp. have been the most commonly reported foodborne pathogens isolated from bulk tanks, ranging from 0 to 11% (Oliver et al., 2009). These three pathogens can be transmitted through feces, soil, water, and feed, resulting in cross-contamination. Monitoring the dairy farm environment is a key point for controlling major pathogens and pathogenic diseases. Therefore, in order to minimize the risk of infection for consumers, the development of a rapid, accurate, and internationally accepted assay for the detection of these major pathogens in the dairy farm environment and milk products has become increasingly important for the food industry and for public health.

Classical culture-based approaches offer standardized procedures for the detection of these three foodborne pathogens (e.g., ISO standards), including culture enrichment, selective plating, and biochemical confirmation. These procedures are labor-intensive, complicated, and time-consuming (Kim et al., 2012; Garrido et al., 2013; Ma et al., 2014; Zhang et al., 2015). With the development of biotechnology, molecular assays such as PCR have increasingly gained attention for their ability to rapidly detect foodborne and waterborne microbiological pathogens in food and animal feed (Kagkli et al., 2011). Automated RT-PCR eliminates the need for an electrophoresis step after amplification (Elizaquível et al., 2011; Ma et al., 2014) and has the added advantage of reducing the risk of cross contamination. Multiplex PCR systems have also been applied to detect pathogenic bacteria. Many previous PCR and qPCR methods have been developed for the detection of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. and have been applied in simplex or multiplex formats (Elizaquível and Aznar, 2008; Rantsiou et al., 2008; Chua and Bhagwat, 2009; Suo et al., 2010; Garrido et al., 2013; Yang et al., 2013; Ma et al., 2014; Xiao et al., 2014). However, the limitations and reliability of PCR-based detection methods partly depend on the number of target bacterial cells, especially the copy numbers of the target molecules present in the sample (Garrido et al., 2013). Low contamination levels in food samples make the detection of target pathogens difficult and time consuming. Thus, considering the actual conditions, an enrichment step is necessary to improve the efficiency of the procedure. The important advantages of enrichment are that it increases the target pathogen concentration in the sample and physiologically resuscitates stressed or injured cells, which reduces the potential risk of sublethal bacteria.

In some countries, there is 'zero tolerance' for pathogenic bacteria. Due to assay detection limits, some pathogens cannot be detected, causing false negative results. Therefore, pre-enrichment is highly necessary for analyzing the environment and for tests in food industry. Real-time PCR coupled with an enrichment step has been applied for the detection of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. in various food samples, such as pine nuts, meats, fish, and eggs (McGuinness et al., 2009; Chen and Deutscher, 2010; Garrido et al., 2013; Wang et al., 2015). Two-step enrichment was carried out on selective media but was inconclusive regarding the necessity of selective cultivation (Taskila et al., 2012). One-step cultivation has been accepted and applied for the enrichment of foodborne and waterborne pathogens. And one-step enrichment was chosen instead of two-step enrichment for use in the multiplex PCR system to shorten the total detection time (Maks and Fu, 2013; Zheng et al., 2013). Currently, there are no investigations that have studied suitable broths to simultaneously enrich *S. aureus*, *L. monocytogenes*, and *Salmonella* spp.

In this study, a multiplex RT-PCR assay for the simultaneous detection of *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. was developed. Our protocol is unique in that we increased the enrichment step before DNA extraction and screened media to find one suitable for these three pathogens to get the detection limit in a short time. The medium selected was evaluated for its ability to achieve high bacterial density of these three pathogens over a short period of time when cultured either individually or together. The multiplex RT-PCR assay with the enrichment step was characterized by its low limit of detection and reduced assay time compared with non-enrichment and conventional methods. To the best of our knowledge, this is the first study to report the use of multiplex RT-PCR with an enrichment step for the simultaneous detection of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. in milk and dairy farm samples.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The bacterial strains used to test the specificity of the multiple RT-PCR in this study are listed in **Table 1**. *S. aureus* (ATCC 25923), *L. monocytogenes* (ATCC 19115) and *Salmonella typhimurium* (ATCC 14028) were obtained from the American Type Culture Collection (ATCC) and used to establish the multiplex RT-PCR. *S. aureus* strains were grown in tryptic soy broth (TSB; Beijing Land Bridge Technology Company Ltd, Beijing, China) supplemented with 10.0% NaCl and incubated at 37°C for 18 h. The other strains were cultured in TSB at 37°C for 18 to 20 h. Following the incubation, 8 mL of the enriched culture were pooled into sterile centrifuge tubes and centrifuged at

TABLE 1 | Specificity of the multiplex real-time PCR primers for different bacterial strains.

Bacterial stains	Source[a]	Multiplex real-time PCR results		
		nuc	*hlyA*	*orgC*
Staphylococcus aureus	ATCC 25923	+	−	−
	CICC 21648	+	−	−
	CICC 10786	+	−	−
	CMCC 41002	+	−	−
	CMCC 26003	+	−	−
	CGMCC 1.89	+	−	−
	CGMCC 1.128	+	−	−
Listeria monocytogenes	ATCC 19115	−	+	−
	ATCC 19114	−	+	−
	ATCC 19112	−	+	−
	ATCC 19116	−	+	−
	ATCC 19118	−	+	−
	CMCC 54002	−	+	−
Salmonella spp.				
Salmonella typhimurium	ATCC 14028	−	−	+
Salmonella typhimurium	CMCC 50362	−	−	+
Salmonella enterica	CMCC 50041	−	−	+
Salmonella Paratyphi B	CMCC 50094	−	−	+
Other stains				
Campylobacter jejuni	ATCC 33560	−	−	−
Vibrio parahaemolyticus	ATCC 33847	−	−	−
Vibrio parahaemolyticus	ATCC 17802	−	−	−
Vibrio vulnificus	ATCC 27562	−	−	−
Escherichia coli O157:H7	ATCC 43889	−	−	−
Escherichia coli O157:H7	CICC 10907	−	−	−
Escherichia coli	CMCC 44568	−	−	−
Enterococcus faecalis	CGMCC 1.2135	−	−	−
Listeria welshimeri	ATCC 43548	−	−	−
Listeria welshimeri	ATCC 43550	−	−	−
Listeria innocua	ATCC 33091	−	−	−
Bacillus subtilis	CGMCC 1.4255	−	−	−
Bacillus cereus	CMCC 70331	−	−	−
Shigella boydii	CGMCC 1.10618	−	−	−
Shigella flexneri	CGMCC 1.10599	−	−	−

[a]*ATCC, American Type Culture Collection, United States; CMCC, China Medical Culture Collection, China; CICC, China Center of Industrial Culture Collection, China; CGMCC, China General Microbiological Culture Collection Center, China.*

3000 rpm, 25°C for 10 min. The cell pellets were suspended in sterile peptone water (PW; 0.85% NaCl, 0.1% peptone) to obtain concentrations ranging from 10^1 to 10^8 CFU/mL.

Preparation of Artificially Contaminated Milk Samples

Ultra High Temperature (UHT) treated milk was purchased from a local supermarket (Shanghai, China). It was confirmed negative for *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. by culturing in Baird-Parker agar (BP; Beijing Land Bridge Technology Company Ltd, Beijing, China), PALCAM agar (Beijing Land Bridge Technology Company Ltd, Beijing, China), and bismuth sulfite agar (BS; Beijing Land Bridge Technology Company Ltd, Beijing, China), respectively. An overnight culture of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. (∼10^8 CFU/mL) was added to 1 mL of milk to final concentrations of 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 CFU/mL.

Enrichment Optimization by Growth Kinetics

Five different commercial enrichment broths were evaluated to enrich samples for RT-PCR analysis: alkaline peptone water (APW; Beijing Land Bridge Technology Company Ltd, Beijing, China), BHI broth (Beijing Land Bridge Technology Company Ltd, Beijing, China), Luria-Bertani broth (LB; Beijing Land Bridge Technology Company Ltd, Beijing, China), TSB broth (Beijing Land Bridge Technology Company Ltd, Beijing, China), and PW. The effects of different enrichment broths on *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. growth were determined from turbidity growth curves using an automatic Bioscreen C (Labsystems, Helsinki, Finland). Twenty-microlitre volumes of bacterial suspension samples at the appropriate concentrations were inoculated in a 100-well honeycomb plate containing 180 μL of the corresponding medium in each well, rendering a final viable count of 10–100 CFU, following ISO 11133-2:2003 recommendations regarding the productivity of the inocula (ISO 11133-2, 2003). In addition, 200 μL of the corresponding medium was added to three of the wells as blank controls. The honeycomb plate was placed in the Bioscreen C reader an incubated at 37°C for 48 h. The OD600 was measured at 30-min intervals, and the honeycomb plates were shaken at medium intensity for 20 s before every measurement (Xuan et al., 2017). The modified Gompertz model (Zwietering et al., 1990) was employed to fit the growth data using Origin pro 8.6 (Origin Lab Corp., Northampton, MA, United States). The equation of the modified Gompertz model is as follows:

$$Y = y_0 + (y_{\max} - y_0) \times \exp\{-\exp[\frac{\mu_{\max}}{y_0} \times (\lambda - t) + 1]\}$$

There are three growth parameters in this model, namely, maximum growth rate ($U_{\max}$), which is an intrinsic parameter in a constant environment and describes the different multiplication rate; time to detection (TD), which is similar to the function of the lag phase time and describes the duration of time that the bacteria react to the enrichment broth and the time to reach the detection limit of turbidity; and the maximum population density (MPD), which indicates the highest level the bacteria can reach under given environmental conditions (Zheng et al., 2015).

DNA Extraction

Bacterial DNA was extracted using a TIANamp Bacteria DNA Kit (Tiangen Biotech Beijing Co., Ltd., China) according to the manufacturer's instructions. This method was modified according to the previous studies (Ye et al., 2013; Zhang et al., 2015), where the incubation time in lysozyme was increased to 1 h, and the incubation time in proteinase K was increased to 2 h.

Primers and Probes Used for Multiplex Real-Time PCR

Primers and probes targeting *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. were used as previously described with minor modifications (Day et al., 2009; Li et al., 2015; Omiccioli et al., 2009). TaqMan primers and probes were synthesized by Invitrogen Corp (Shanghai, China). The sequences of the primers and probes for the multiplex real-time PCR experiments are provided in **Table 2**.

Multiplex Real-Time PCR

The multiplex real-time PCR reaction was carried out in a final volume of 20 μL with the following components: 2 μL of 10 × PCR Buffer (Invitrogen, United States), 1.2 μL of 50 mM $MgSO_4$ (Invitrogen, United States), 0.5 μL of 10 mM dNTP mix (Invitrogen, United States), 0.2 μL of Taq DNA polymerase (5 U/mL) (Invitrogen, United States), 1 μL of template DNA per reaction tube, 0.5 μL of 10 mM primers and 0.2 μL of 10 mM probe for each strain.

A 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, United States) was used. The cycling protocol consisted of 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Analysis of the results was performed using 7500 Software version 2.0.6.

Standard Curves and Amplification Efficiency

To create RT-PCR standards and determine the amplification efficiency, pure cultures and artificially contaminated samples seeded with 10-fold diluted suspensions of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. were prepared as described in Sections "Bacterial Strains and Culture Conditions and Preparation of Artificially Contaminated Milk Samples," respectively. A standard curve was obtained by using genomic DNA extracted from serial dilutions of the pure culture and the artificially contaminated samples. Each reaction was amplified in triplicate. Negative (no template) controls were included in each RT-PCR run. A direct plate counting procedure was conducted for the quantification of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. in pure cultures and samples. The cultures were incubated for 24 h at 37°C on BP agar for *S. aureus*, PALCAM agar for *L. monocytogenes*, and BS agar for *Salmonella* spp.

The different bacterial concentrations (log10 CFU/mL) were plotted against the corresponding *C*t-values and had a linear relationship. The amplification efficiencies (E) were determined by using the slope of the curve and applying the equation: $E = 10^{-1/slope} - 1$ (Kawasaki et al., 2010).

Evaluation of the Limit of Detection (LOD) and Enrichment Time by Multiplex Real-Time PCR

The LOD was evaluated before and after the process of enrichment. To determine the LOD without the enrichment step, nine samples were prepared as follows: 3 mL of the suspensions of 10^9 CFU/mL *S. aureus*, *L. monocytogenes,* and *Salmonella* were inoculated in 27 mL UHT milk to make the initial dilution. Then, 10-fold dilutions were generated to achieve the final contaminations of the three pathogens in UHT milk, which were 10^1, 10^2, or 10^3 CFU/mL, as determined by plate counts. Then, DNA extraction was performed on 1 mL sample.

The enrichment optimization experiments were conducted using 10^1 CFU values of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. in 25 mL of UHT milk to determine the LOD after enrichment. All samples were initially confirmed as negative for *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. by plate count before the inoculation. Twenty-five milliliters of low contaminated samples (10^1 CFU/25 mL) were added to 225 mL of selected enrichment broth and grown at 37°C. The cell growth was monitored by collecting 1 mL of the sample for DNA extraction at hourly intervals during a 5-h incubation, which was described in Section "DNA Extraction."

Multiplex RT-PCR was conducted as described in Section "Multiplex Real-Time PCR," and the results were gathered from both the non-enrichment tube and enrichment broth and statistically compared to evaluate the effect of the culturing time on the enrichment step.

Detection of *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. in Raw Milk and Dairy Farm Samples

A total of 46 milk and environmental samples were tested in this study, including fifteen normal raw milk samples, seven mastitis milk samples, six soil samples, six feed samples, six fecal samples, and six water samples from dairy farms in three areas of China. The sampling time for all samples did not exceed 5 h, and the

TABLE 2 | Primers and probes used in the multiplex real-time PCR.

Gene	Target bacteria	Primers/Probes	Product sizes(bp)	Reference
nuc	*S. aureus*	CACCTGAAACAAAGCATCCTAAA CGCTAAGCCACGTCCATATT FAM-TGGTCCTGAAGCAAGTGCATTTACGA-BHQ1	149	Li et al., 2015
hlyA	*L. monocytogenes*	ACTTCGGCGCAATCAGTGA TTGCAACTGCTCTTTAGTAACAGCTT ROX-TGAACCTACAAGACCTTCCAGATTTTTCGGC-BHQ1	137	Omiccioli et al., 2009
orgC	*Salmonella* spp.	CTTTATGATGCATTCTACCAACGACTG CCGAATCACCACTGTTAGGA VIC-CGCTTCCTGAGTCAGCCTCTTCTGAAACG- BHQ1	121	Day et al., 2009

samples were kept in the cold before extraction. The samples were analyzed by multiplex RT-PCR with enrichment and using standard culture methods for *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. (ISO 11290-02, 1998; ISO 6579, 2002).

We added 25 mL/g of samples into 225 mL of selected enrichment broths weighed into a sterile plastic bag and enriched for a certain number of hours at 37°C in preparation for DNA extraction. The enrichment time was determined by the results of the previous step [see Evaluation of the Limit of Detection (LOD) and Enrichment Time by Multiplex Real-Time PCR]. One milliliter of supernatant was transferred to a new tube and centrifuged at 12,000 rpm for 10 min for DNA extraction, and then multiplex RT-PCR was carried out. For the standard culture method, 25 mL/g samples were added to the enrichment broths weighed into a sterile plastic bag and incubation for 24 h at 37°C. After enrichment, 100 μL was transferred to selective agar (BP agar for *S. aureus*, PALCAM agar for *L. monocytogenes* and BS agar for *Salmonella* spp.) and incubation for 48 h at 37°C. The typical colonies were picked to make the biochemical confirmation where the presumptive *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. colonies were validated using the API STAPH test (BioMérieux), the API LISTERIA test (BioMérieux) and API 20E test (BioMérieux), respectively.

Statistical Analysis

Statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, United States).

RESULTS

Specificity of Primers and Probes for Multiplex Real-Time PCR

The specificity and sensitivity of the designed primers and probes (**Table 1**) were tested individually and in combination using various bacterial strains available for this study. As shown in **Table 1**, none of the target bacteria showed a negative signal; all produced a specific band corresponding to the amplicons. By contrast, non-target bacteria did not produce an amplified signal. Such results indicated that the multiplex RT-PCR was specific to *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. and did not amplify products from other species.

Enrichment Optimization Based on Growth Kinetics and Growth Parameters

The average growth curves of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. in the five enrichment broths were obtained by fitting to the modified Gomperz model and were shown in **Figures 1A–C**. The coefficients of determination (R^2-values) for the fitted growth curves (APW, BHI, LB, and TSB) were greater than 0.98 (data not shown). These three pathogens grew vigorously in APW, BHI, LB, and TSB broths. However, as shown in **Figures 1A–C**, the growth of *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. in PW was insufficient to develop a full growth curve; therefore, growth could not be fitted to the model. PW could maintain the survival of the bacteria but could not provide the necessary nutrients for bacterial growth.

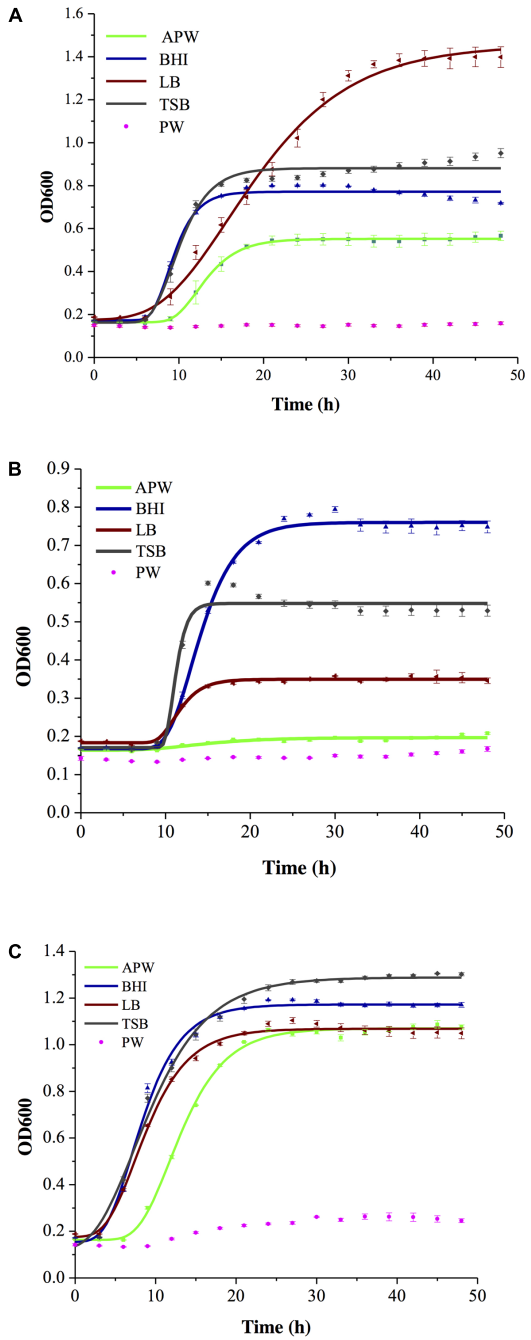

FIGURE 1 | Growth curves of *S. aureus* **(A)**, *L. monocytogenes* **(B)**, and *Salmonella* spp. **(C)** in five enrichment broths.

The growth parameters of these three pathogens in the five enrichment broths were also calculated using the modified Gompertz model, and they are shown in **Table 3**. *S. aureus* and *Salmonella* spp. had the highest growth rate in BHI broth. This increased growth rate allowed these target pathogens to grow

TABLE 3 | The growth parameters of three pathogens in five enrichment broths.

		APW	BHI	LB	TSB	PW
S. aureus	U_{max}	$0.062 \pm 0.004^{*c}$	0.093 ± 0.022^{a}	0.022 ± 0.004^{d}	0.089 ± 0.048^{b}	NF
	TD	9.445 ± 0.221^{b}	6.830 ± 0.541^{d}	7.904 ± 0.925^{c}	14.41 ± 0.366^{a}	NF
	MPD	0.552 ± 0.004^{c}	0.773 ± 0.016^{b}	1.455 ± 0.027^{a}	0.880 ± 0.611^{b}	NF
L. monocytogenes	U_{max}	0.032 ± 0.015^{d}	0.058 ± 0.006^{c}	0.100 ± 0.013^{b}	0.195 ± 0.024^{a}	NF
	TD	7.617 ± 0.350^{a}	10.189 ± 0.344^{c}	9.113 ± 0.387^{b}	9.988 ± 0.153^{c}	NF
	MPD	0.195 ± 0.148^{d}	0.760 ± 0.010^{a}	0.349 ± 0.003^{c}	0.547 ± 0.005^{b}	NF
Salmonella spp.	U_{max}	0.042 ± 0.004^{b}	0.046 ± 0.010^{a}	0.048 ± 0.007^{a}	0.024 ± 0.009^{c}	NF
	TD	7.850 ± 0.303^{a}	3.697 ± 0.495^{b}	3.799 ± 0.400^{b}	2.297 ± 0.768^{c}	NF
	MPD	1.069 ± 0.128^{c}	1.163 ± 0.027^{b}	1.068 ± 0.019^{c}	1.287 ± 0.044^{a}	NF

U_{max}, maximum rate of growth; TD, time to detection, the time to reach the detection limit of turbidity and is measured in hours; MPD, the highest level the bacteria can reach under given environmental conditions; NF, none fit. Values are the mean of triplicate measurements ± standard deviation; values with different lowercase letters in the same row showed a significant difference at $P < 0.05$.

rapidly and reach a detectable level, shortening the detection time. The highest MPDs achieved by *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. were in LB, BHI, TSB, respectively, and the lowest were in APW, APW and LB, respectively. The aim of the enrichment step was to facilitate the bacterial density reaching the same concentrations of the limit of detection as without enrichment in a short time. Therefore, U_{max} is the most important factor in the selection of media. The results obtained indicate that BHI broth is the best of these five commercial broths for the enrichment of *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. before multiplex real-time PCR.

Standard Curve and Amplification Efficiency

Three replicates were analyzed for the efficiency evaluation of the multiplex real-time PCR method in pure culture and in contaminated milk. In our standard curves, the correlations (R^2) for pure culture and contaminated milk were greater than 0.99, indicating high linearity (**Figure 2**). The slopes of the linear regression curves for the pure cultures were −3.06 for *S. aureus*, −3.50 for *L. monocytogenes* and −3.02 for *Salmonella* spp. The amplification efficiencies for *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. were 112, 95, and 114%, respectively, in pure culture. In artificially contaminated milk, the slopes were −3.19 for *S. aureus*, −3.47 for *L. monocytogenes*, and −3.35 for *Salmonella* spp., and the amplification efficiencies for *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. were 106, 94, and 98%, respectively.

Evaluation of the LOD in Artificially Contaminated Milk Samples Using Multiplex Real-Time PCR with and without Enrichment

For evaluation of the LOD in artificially contaminated milk samples using multiplex RT-PCR with and without enrichment, two groups of 18 samples were analyzed following the procedure described in Section "Evaluation of the Limit of Detection (LOD) and Enrichment Time by Multiplex Real-Time PCR." The LOD results are listed in **Table 4**. The first group of samples, which had no enrichment, gave a LOD for *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. of 10^2 CFU/mL for each pathogen, with *C*t-values between 27 and 31. For the second group, when incubated for 1, 2, or 3 h, the *C*t-values were higher than 35 and showed the negative results. However, when incubated for 4 h of the enrichment step, the multiplex RT-PCR system could detect these three pathogens successfully, and gave a LOD of 12 CFU in 25 mL for *S. aureus*, 14 CFU in 25 mL for *L. monocytogenes*, and 10 CFU in 25 mL for *Salmonella* spp., with *C*t-values lower than 30.

Detection of Three Major Bacteria in Samples Based on Multiplex Real-Time PCR with and without Enrichment and Using Standard Culture Methods

In total, 46 samples collected from three areas of China were analyzed for *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. infection using both the multiplex real-time PCR method (with enrichment) and the culture method. The results obtained from the multiplex real-time PCR method and standard culture method are listed in **Table 5**. In the case of the 46 environmental samples of *S. aureus*, 11 (23.9%) were positive by the multiplex real-time PCR method and were found in raw milk, mastitis milk, and animal feed, however, 10 (21.7%) were positive based on standard culture method. From the 46 samples analyzed for *L. monocytogenes*, 4 (8.7%) were positive by the both multiplex real-time PCR method and standard culture method and were found in raw milk, mastitis milk, feces, and animal feed. There were no *Salmonella* spp. positive samples among the 46 natural samples by using both RT-PCR method and standard culture method. Compared to the RT-PCR method, the culture method was time-consuming and labor-intensive, taking 1 week for the whole process. In addition, when screened on selective medium, many species of bacteria appeared on one plate, causing difficulty in picking single colonies because of the non-specific selectivity of the medium.

DISCUSSION

Detection of multiple pathogens in the same system is currently needed for the dairy industry to reduce the cost of detecting each

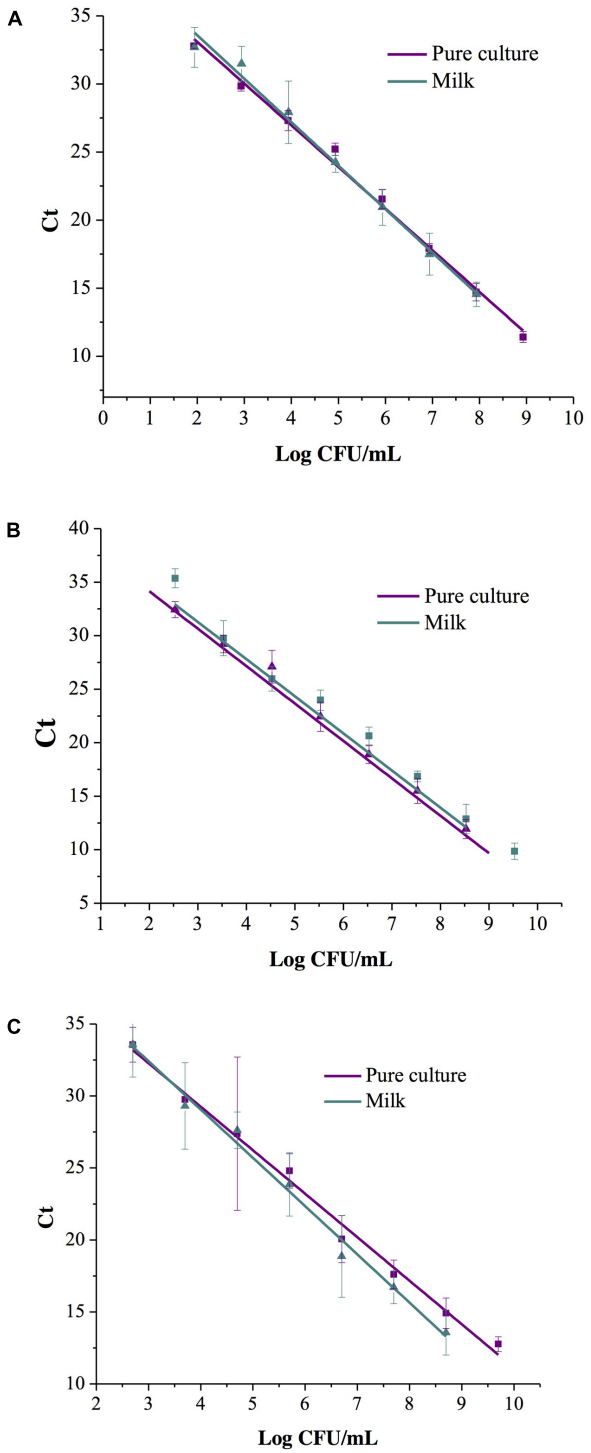

FIGURE 2 | Standard curves of the multiplex real-time PCR for three pathogens of *S. aureus* **(A)**, *L. monocytogenes* **(B)**, and *Salmonella* spp. **(C)** in pure culture and artificially contaminated milk.

TABLE 4 | Evaluation of the LOD for simultaneous detection of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. with and without enrichment step.

	LOD		
	S. aureus	***L. monocytogenes***	***Salmonella* spp.**
Without enrichment	10^2 CFU/mL	10^2 CFU/mL	10^2 CFU/mL
With enrichment	12 CFU/25mL	14 CFU/25mL	10 CFU/25mL

pathogen (Kim and Bhunia, 2008). Multiplex PCR can be used for the detection of multiple pathogens in various industries, including the simultaneous detection of *Cronobacter sakazakii*, *S. aureus*, and *Bacillus cereus*, as well as the simultaneous detection of *Vibrio parahaemolyticus*, *L. monocytogenes*, and *Salmonella* spp. (Wolffs et al., 2007; Yang et al., 2013; Li et al., 2015; Zhang et al., 2015; Alves et al., 2016; Li F. et al., 2016). This was the first study to investigate the simultaneous growth of *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. in commercial enrichment broth. We developed a rapid and efficient multiplex RT-PCR assay for the simultaneous detection of *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. in milk and dairy farm environment with a pre-enrichment step. Our multiplex real-time PCR showed excellent specificity for the simultaneous detection of three target pathogens when placing these three probes and primers in one single reaction system, and they showed strong strain specificity and exclusivity (**Table 1**). This specificity was confirmed by the detection of seventeen target strains and fifteen non-target strains. The results for the pure cultures and artificially contaminated milk showed highly efficient detection (**Figure 2**).

In the present study, the limit of detection of this multiplex real-time PCR was 10^2 CFU/mL in artificially contaminated milk without enrichment. Previous studies have identified the LOD of these three pathogens in food samples without enrichment. Forghani et al. (2016) developed a multiplex RT-PCR for the simultaneous detection of *Bacillus cereus*, *L. monocytogenes*, and *S. aureus* in milk, rice, and lettuce with an LOD value of 3.7×10^3 CFU/g. Xiao et al. (2014) obtained an LOD value of 3.5×10^2 CFU/mL for *Salmonella* and *L. monocytogenes* and of 3.5×10^3 CFU/mL for *S. aureus* in food by high-resolution melting real-time PCR. Compared to previous studies, the LOD values obtained from our method are one order of magnitude lower. The reasons for the lower LOD we got were because the specific probes and suitable reaction conditions used in our reaction system. However, there is some uncertainty about the limit of detection due to the lack of pre-enrichment before multiplex RT-PCR.

Pathogens in environmental samples and the food matrix may exist in very low numbers and may sometimes be accompanied by competitor organisms. This results in difficulty in the detection of pathogens using either molecular-based or culture-based methods without a pre-enrichment step (Baylis et al., 2000; Margot et al., 2015). Most of the bacteria in marketed foods are usually in an injured/stressed state, and some are in a viable but non-culturable (VBNC) state due to heat, radiation, or low temperature treatments. *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. have been demonstrated to possess the ability to enter the VBNC state when under extreme conditions

TABLE 5 | Results obtained for milk and dairy farm environment samples with multiplex real-time PCR (with enrichment step) and standard culture method.

Sample type	P/T[a]		*S. aureus* (PCR/culture)[b]			*L. monocytogenes* (PCR/culture)			*Salmonella* spp. (PCR/culture)		
	PCR	Culture	+/+	+/−	−/+	+/+	+/−	−/+	+/+	+/−	−/+
Raw milk	5/15	5/15	4	0	0	1	0	0	0	0	0
Mastitis milk	4/7	4/7	3	0	0	1	0	0	0	0	0
feces	3/6	3/6	2	0	0	1	0	0	0	0	0
Soil	0/6	0/6	0	0	0	0	0	0	0	0	0
Feed	3/6	2/6	1	1	0	1	0	0	0	0	0
Water	0/6	0/6	0	0	0	0	0	0	0	0	0

[a]*P/T, Positive sample number/Total sample number.* [b]*+/+, positive for both real-time PCR and standard culture method; +/−, positive for real-time PCR method and negative for standard culture method; −/+, negative for real-time PCR method and positive for standard culture method.*

(low temperature, tap water) (Ramamurthy et al., 2014; Li J. et al., 2016). At present, most of the multiplex or simplex PCR studies do not involved in the pre-enrichment step and there are no clear conclusions about the suitable broths and enrichment time for the pre-enrichment step. In this study, one of the major advantages was to enrich the three target bacteria in one broth and to obtain a lower LOD. The aim of the pre-enrichment step was to resuscitate the stressed target bacteria in a natural sample. BHI has been used to resuscitate VBNC *L. monocytogenes* (Lindbäck et al., 2010), which helped the cells recover from stressful conditions. In addition, BHI broth has been evaluated as an enrichment medium for *Salmonella* spp. in sprout-related studies (Tu et al., 2003; Kisluk and Yaron, 2012). As shown in **Table 3**, the growth rate of *S. aureus* and *Salmonella* spp. in BHI broth was faster than in the other enrichment broths. Therefore, BHI was the most suitable broth for the enrichment of the three targeted pathogens in raw milk and in dairy farm environmental samples using a short enrichment time. The results for artificially contaminated milk samples showed that the detection limits for all three targeted pathogens were 12 CFU/25 mL for *S. aureus*, 14 CFU/25 mL for *L. monocytogenes*, and 10 CFU/25 mL for *Salmonella* spp. (**Table 4**).

Previous studies have reported selective broths for enrichment of one or two among *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. using one or two steps (Dailey et al., 2014; Khueankhancharoen et al., 2016). The use of mTA10 broth with 100 mM buffer and secondary enrichment to detect *Salmonella* spp. and *Listeria monocytogenes* has been reported by Garrido et al. (2013). The enrichment process was relatively complicated and time consuming. Rappaport-Vassiliadis soy broth (RVS) has also been used as enrichment media for the selection of *Salmonella* spp. (Van Schothorst and Renaud, 1985). Selective medium is more specific than non-selective medium to prevent the growth of non-target bacteria and to reduce the impact of competitor microorganisms on the growth of target bacteria (Kim and Bhunia, 2008; Taskila et al., 2012). Clearly, selective broth is more suitable for one or two species of bacteria when the targeted cells are at high concentration (Chen et al., 1993). However, we are committed to the simultaneous detection of three bacteria, and therefore, a single selective medium is not suitable for the pre-enrichment process. The development of enrichment medium for the simultaneous isolation of several pathogenic bacteria in one system is a major trend. The use of non-selective medium and the control of the enrichment time can effectively increase the concentration of the target bacteria. Compared to the other non-selective broths, *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. had the highest growth rate in BHI broth. We controlled for the enrichment time, 4 h, which avoided the overgrowth of the background flora.

Microbial transfer in farm soil, animal feed, feces, water, and herds may result in the contamination of milk products. Doyle et al. (2016) demonstrated that the dairy farm environment in which the herds were kept in was the primary driver of the composition of the milk microbiota. While it is clear that our multiplex RT-PCR method could accelerate the analysis of milk and dairy farm environmental samples (soil, water, feed, and feces) for *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. contamination, its success is linked to the ability of these three target bacteria to compete with resident micro-flora. In this study, a total of 46 samples of raw milk, mastitis milk, feces, soil, animal feed, and water from three areas of China were detected using multiplex RT-PCR with 4-h incubation in BHI broth. In these natural samples, 15 samples yielded positive results (**Table 5**). *S. aureus* was detected in four samples of raw milk, three samples of mastitis milk, two samples of feces and two samples of feed. *L. monocytogenes* was detected in one sample each of raw milk, mastitis milk, feces and feed. Lastly, *Salmonella* spp. could not be detected in milk or dairy farm environmental samples. Our results suggest that the sample contents and the microflora background do not affect the PCR amplification. We recommend 4 h of pre-enrichment time in BHI broth before the multiplex real-time PCR for simultaneous detection of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. in milk and dairy farm environmental samples.

A drawback of the present study is that the method could not accurately quantify the viable bacteria in the environment and food samples. As a result, the DNA of some dead bacteria was also included in the information of the total bacterial DNA. However, when the samples were enriched, the numbers of viable bacteria were increased in the sample, which can reduce the risk of false positive results due to the DNA of dead bacteria.

Although propidium monoazide (PMA) coupled with multiplex RT-PCR is a useful tool for the quantification of viable bacteria (Bae and Wuertz, 2012; Yang et al., 2013), it still a drawback to the limit of detection. Li et al. (2015) combined DNA and PMA to quantify the viable *Legionella pneumophila*, *S. typhimurium*, and *S. aureus* in tap and river water, and this method could detect 10^1 CFU/mL viable bacteria. Therefore, the pre-enrichment step combined with the multiplex PCR method is more suitable for the detection of pathogenic bacteria in the food environment and in complicated food matrixes, especially for the detection of bacteria in a few numbers.

CONCLUSION

This study presents a tool for the simultaneous detection of *S. aureus*, *L. monocytogenes,* and *Salmonella* spp. The pasture environment and animal feed can be assessed to reduce the risk of microbial cross-contamination in the aquaculture environment, especially the bacteria in the aquaculture chain, including soil, water, feed, and feces. Multiplex RT-PCR coupled with the pre-enrichment step can be used to recognize the potential infection risk of sublethal and VBNC bacteria by allowing them to recover from stressful conditions.

AUTHOR CONTRIBUTIONS

TD and YS drafted the manuscript. All authors listed, have edited the manuscript, and made substantial and direct contribution to the work. All authors gave approval for publication of the manuscript.

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