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Antibiotic Resistance in Foodborne Pathogens- a Cause for Concern?

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Antimicrobial resistance in foodborne pathogens- A cause for concern?

Short Title: Antibiotic and Disinfectant Resistance in Food-borne Pathogens

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Abstract

The widespread use of antibiotics in food animal production systems has resulted in the emergence of antibiotic resistant zoonotic bacteria that can be transmitted to humans through the food chain. Infection with antibiotic resistant bacteria negatively impacts on public health, due to an increased incidence of treatment failure and severity of disease. Development of resistant bacteria in food animals can result from chromosomal mutations but is more commonly associated with the horizontal transfer of resistance determinants borne on mobile genetic elements. Food may represent a dynamic environment for the continuing transfer of antibiotic resistance determinants between bacteria. Current food preservation systems that use a combination of environmental stresses to reduce growth of bacteria, may serve to escalate development and dissemination of antibiotic resistance among food related pathogens. The increasing reliance on biocides for pathogen control in food production and processing heightens the risk of selection of biocide-resistant strains. Of particular concern is the potential for sublethal exposure to biocides to select for bacteria with enhanced multi-drug efflux pump activity capable of providing both resistance to biocides and cross-resistance to multiple antibiotics. Although present evidence suggests that biocide resistance is associated with a ~~fitness~~ physiological costs, the possibility of the development of adaptive mutations conferring increased fitness cannot be out-ruled. Strategies aimed at inhibiting efflux pumps and eliminating plasmids could help to restore therapeutic efficacy to antibiotics and reduce the spread of antibiotic resistant food borne pathogens through the food chain.

1. Introduction

Over the last two decades there has been an increase in the number of antibiotic resistant bacteria isolated from humans and animals. The overuse and misuse of antibiotics in both human and veterinary medicine has contributed to this global pandemic of antibiotic resistant bacteria [1]. In contrast to human medicine, antibiotics are used therapeutically, prophylactically and sub-therapeutically as growth promoters in food animals [2]. This has led to the development of resistance to antibiotics in foodborne pathogens which can ultimately be transmitted to humans *via* the food chain [3,4]. Although most cases of foodborne disease result in self-limiting diarrhoea, antibiotic therapy is warranted in cases of persistent enteritis, bacteraemia and in immunocomprised individuals. Infection with antibiotic resistant bacteria can complicate initial treatment and result in prolonged duration of illness, an enhanced risk of mortality or invasive illness and increased healthcare costs. The Centers for Disease Control and Prevention estimate that foodborne diseases are responsible for about 76 million illnesses, resulting in 325,000 hospitalisations and 5,000 deaths in the United States each year [5].

Antibiotic resistance typically occurs as a result of target gene mutation, drug inactivation and decreased accumulation resulting from decreased permeability and /or increased efflux [6]. The horizontal transfer of resistance determinants on mobile genetic elements such as plasmids, transposons and integrons, promotes the rapid spread of antimicrobial resistance genes between different species and genera of bacteria and the development of a multi-drug resistance phenotype. More recently, considerable attention has

focussed on the role of multi-drug efflux pumps in mediating multi-resistance [7].

In response to the concern about the growing impact of antibiotic resistance in clinical practice, European Union (EU) regulations have banned the use of antibiotic growth promoters in animal feed [8]. Consumer demands for safe food has resulted in an increase in biosecurity measures in the food production industry, including the use of biocides to control and reduce microbial communities associated with food spoilage and disease [9]. Additionally, the increased public awareness of hygiene has resulted in a deluge of consumer healthcare and cleaning products touting antimicrobial uses [10]. Increased exposure to biocides has contributed to the emergence of pathogens showing decreased suceptibility to them. Resistance to biocides may be due to target gene mutations [11, 12], however, it is more commonly associated with increased efflux pump activity [13,14,15]. As antibiotics are substrates for these pumps, ~~also~~ this also raises the concern that biocides can select for multi-drug resistance in clinically important bacteria in the absence of antibiotic selective pressure [16].

This review examines the contribution of stresses encountered in the food processing environment to the emergence and spread of antibiotic resistance. Consideration is given to the use of biocides by the food industry and the emergence of resistance to these agents. Mechanisms associated with biocide resistance and the potential for biocides to select for antibiotic

resistant bacterial are explored. Finally, the ~~fitness~~ physiological cost of biocide resistance is ~~examined~~explored.

2. Horizontal transfer of antibiotic resistance

Although chromosomal mutations can be responsible for the development of antibiotic resistance [17], mutation occurs at a relatively low frequency of one per 1 billion cell divisions [18]. Consequently, transferable resistance poses a greater threat as it can achieve much larger dimensions owing to widespread rapid dissemination among bacteria of different taxonomic and ecological groups [18]. Genetic exchange can occur by conjugation (plasmid-mediated), transduction (bacteriophage-mediated) or transformation (which may involve plasmids and naked DNA). Conjugation is the most frequently recognised mechanism for horizontal gene transfer. Plasmids may contain particular genetic structures including composite and/or complex transposons, known as "jumping genes" along with the more recently described integron structures (Figure 1), which can increase the rate of dissemination of resistance genes between bacteria. Integrons can capture antibiotic resistance encoding genes *via* site-specific recombination [19, 20]. These integrons possess a conserved structure on the proximal end (known as the 5'-CS) containing an integrase gene (*intI*), a recombination site (*attI*) and a promoter (P_{ant}), along with a conserved distal region (3'-CS) containing a *qac~~Δ~~EΔ1* [conferring resistance to quaternary ammonium compound(s) (QAC's)] and a *sul1* determinant (conferring resistance to sulphonamide). These CS regions flank a variable central locus, into which gene cassettes are recombined, composed of one or

more open reading frames (ORF) encoding antibiotic resistance gene(s) [21]. More then 60 different gene cassettes have been identified, with some integrons possessing multiple gene cassettes arranged in a classical 'head-to-tail' orientation [22]. As these resistance determinants are under the control of a single strong upstream promoter (located towards the 3' end of *intI*), all recombined gene cassettes are co-expressed. Therefore, selective pressure imposed by the use of a particular antibiotic, can co-select for another resistance determinant located within an adjacent gene cassette [23]. Additionally, exposing integron-containing bacteria to sub-inhibitory levels of QACs may co-select for antibiotic resistance [24].

2.1 Antibiotic resistance transfer in food and the effect of food preservation stresses

Gene transfer has been shown to occur in a variety of complex media including the gut of various animals [25], the human colon [26], cultured human cells [27], bovine rumen fluid [28], sewage [29], surface water [30] and calf faeces [31]. Several studies to-date have successfully demonstrated laboratory-based gene transfer by conjugation with food-borne bacterial strains in broth (liquid mating) [32, 33, 34] or by filter (solid surface) mating [35, 36]. However, there is limited data describing gene transfer in the *in situ* food matrix [37].

Walsh *et al.* (2008) reported the transfer of an ampicillin resistance marker *via* a R-plasmid from *S.* Typhimurium DT104 to a susceptible recipient *E. coli* K12 in broth, milk and ground meat, at 25 and 37^{o}C within 24 h. A higher rate of

transfer (10^{-2} cfu/g transconjugants per recipient) was reported in ground meat at 48 h [38]. Similarly, Van der Auwera *et al.* ~~(2007)~~ reported plasmid transfer (at 10^{-1} cfu ml/g transconjugants per recipient) for *Bacillus thuringiensis* in broth, milk and milk pudding [39]. Cocconelli *et al.* ~~(2003)~~, reported the transfer of a vancomycin resistance gene *via* a conjugative R-plasmid in enterococcal strains during cheese and sausage fermentation [37]. These authors reported a 2-3 log (cfu/g) increase in the transfer rate of plasmids in meat, and suggested that factors including plasma in the meat matrix could play an important role in increasing the rate of plasmid transfer [40].

Recent evidence suggests that plasmid transfer may also be more rapid between bacteria in minimally processed foods, held under sub-lethal food preservation stresses such as high/low temperature, osmotic and pH stress. Using experimental broth/filter mating conditions, McMahon et al., ~~(2007)~~ reported significantly increased rates of antibiotic resistance plasmid transfer between *E*. coli strains and *E*. coli and *S*. Typhimurium strains held under the typical environmental food preservation stresses used by the food industry [41]. However, it was not determined whether this was due to increased donor plasmid transfer or more efficient plasmid capture by the recipient. On a positive note, biocides have been show to reduce the rates of plasmid transfer (by both conjugation and transduction) [68]

3. Biocides and their use in food production

Biocides refer to a broad category of agents including sanitizers, disinfectants and food preservatives [24] (see Figure 2-which provides a general summary of this classification). For the purposes of this review we will confine our discussion to the first two listed categories. Biocides which include disinfectants and sanitizers can be further differentiated based on the organism(s) they target and directions for use [42]. A disinfectant must completely eliminate all the organisms against which it is directed, whilst a sanitizer need not eliminate all of the organisms that it is targeted against. The efficacy of biocides and the types of organisms that they inhibit vary considerably, and is dependent on the compositional concentration and synergism among the components [43]. Compared to antibiotics, the mode of action of biocides is relatively non-specific. They damage cytoplasmic membranes and can react unspecifically with functional groups of proteins or nucleic acids [44].

Biocides are used as part of the biosecurity measures in livestock production to prevent outbreaks and spread of disease and to decontaminate animal housings. In the food processing environment they are used to prevent product contamination with pathogens. Commonly used biocides for environmental and surface cleaning include, quaternary ammonium compounds (QAC's), oxidising compounds, acid anionics, hypochlorite and chlorine dioxide. Triclosan and chlorhexidine are used extensively in handcare products [45]. Unusually unlike the approval process for antibiotics, a risk assessment of the development of biocide resistance is not considered during the approval of biocides for food industry use.

3.1. Mechanisms associated with decreased susceptibility to biocides

It was considered highly unlikely that resistance to biocides would ever occur, as most biocides are complexes of antimicrobial agents that act in unison to inactivate multiple cellular targets [46,47]. However, reduced susceptibility to biocides was first reported a century ago [48] and our current high dependance on biocides, has resulted in some reports of reduced bacterial susceptibility [49-53]. While reduced susceptibility to biocides does not necessarily correlate with product failure, the implication of ineffective pathogen control may be damaging to the food industry. Factors reducing the effectiveness of biocides include the presence of organic material and biofilm growth. Inadequate disinfection procedures in livestock production facilities and food processing plants may contribute to the selection of biocide resistant isolates as a result of exposure to sublethal biocide concentrations. Staphylococci showing decreased susceptibility to QACs have been isolated from food processing plants (54, 55). Langsrud and Sundheim ~~(1997)~~ reported that more than 30% of *Pseudomonas* spp. isolated from poultry carcasses could grow in the presence of benzalklonium chloride at concentrations used in the poultry plant (56). Resistance to QACs has been demonstrated in *Listeria* spp. isolated from poultry products, red meat and cheese [57]. In contrast a recent report showed that biocide resistance was not a contributing factor to the persistence of strains of *L. monocytogenes* and *E. coli* in the products and environment of five chilled food production facilities (58). Although little is known about the effects of low concentrations of biocides on bacterial biofilms, of potential significant to the food industry is

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the results of in-*vitro* studies showing that incomplete elimination of a biofilm might lead to increased resistance after biofilm growth possibly due to the selection of highly resistant clones. [59, 60]

Mechanisms of aquired antibiotic resistance have been widely studied and clearly elucidated [19,20]. Comparatively, mechanisms of acquired biocide resistance have been poorly evaluated. Biocide resistance can result from mutation or from acquisition of resistance determinants on plasmids [54]. Resistance resulting from biocide inactivation is rare but has been documentated for organomercurials [61,62]. Resistance can also result from changes in cell permeability but is more often associated with enhanced biocide efflux [61].

A number of efflux transporters capable of effluxing a single substrate, or a wide variety of structurally unrelated agents including antibiotics and biocides, have been identified in bacteria. The contribution of these efflux pumps to antibiotic resistance is well documented and has been the subject of numerous reviews [63-66]. Bacterial efflux systems are classified into five families: the major facilitator (MF) superfamily, the ATP (adenosine triphosphate)-binding cassette (ABC), the resistance nodulation-division (RND) family, the small multi-drug resistance (SMR) family (a member of the much larger drug/metabolite transporter (DMT) superfamily), and the multi-drug and toxic compound extrusion (MATE) family [67]. Of these, MF transporters are the most prevalent in Gram-positive bacteria [Figure 3 (a)] and the RND transporters are the most common in Gram-negative bacteria [~~(see~~ Figure 3 (b)]~~)~~. RND efflux pumps are organised as tripartite structures consisting of an inner membrane pump, an outer membrane protein and a

periplasmic linker protein. Multi-drug efflux pumps such as RND pumps, are generally chromosomally encoded, and their over-expression can result from mutations in local or global regulators. This contrasts with drug-specific efflux pumps, which are more usually encoded by mobile genetic elements [67] and are single component structures [68].

Here we will review the mechanisms of resistance associated with some of the commonly used biocides, namely; triclosan, quaternary ammonium compounds (QAC's) and chlorhexidine [47,66] (see Figure 4- for chemical structure).

Triclosan

Triclosan works specifically on enoyl-acyl reductase, an enzyme which is essential for fatty acid synthesis. Modification, repression or deletion of the specific cellular target *fabI* (encoding enoyl-acyl reductase) results in reduced bacterial susceptibility to triclosan [11,12,68]. Triclosan has been reported to target the FabI enzyme of a number of bacteria including spoilage organisms such as *P. aeruginosa* and food pathogens such as *E. coli, Salmonella* and *Campylobacter.* Characterisation of triclosan resistant mutants, revealed a single-amino-acid change in *fabI* in the codon for glycine 93 in *E. coli* and glycine 95 in *P.aeruginosa* (68). Additionally, many of the RND family pumps associated with resistance to clinically important antibiotics are able to accomodate triclosan. These include the AcrAB-TolC pump of *E. coli* [15] and *Salmonella* [69], the CmeABC and CmeDEF of *C. jejuni* [70], and several of the Mex pumps in *P. aeruginosa* [71].

Quaternary ammonium compounds

Quaternary ammonium compounds (QAC e.g. benzalkonium chloride) act by physical disruption and partial solubilisation of the cell and membrane. A variety of plasmid and chromosomally encoded efflux determinants of QAC resistance have been described in both Gram-negative and positive bacteria [68]. Transporters capable of accomodating QACs in Gram-negative bacteria are generally chromosomally encoded and include a number of MATE (PmpM in *P.aeruginosa*), RND (AcrAB-TolC, AcrEF-TolC and YhiUV-TolC pumps of *E. coli*), [64] and SMR family (EmrE in *E. coli*) [72] multi-drug transporters. The SMR transporters, QacE and QacEΔ1, QacF and QacG found in Gram-negative bacteria are plasmid-encoded [73,64]. Interestingly, as stated earlier the *qacAE1* is contained on the conserved distal region (3'-CS) of a class 1 integron structure (Figure 1c). The chromosomal efflux determinants of QAC resistance in Gram-positive bacteria include the MF family NorA multi-drug transporter (usually associated with fluoroquinolone resistance), the MF family MdeA and the MATE family MepA in *S. aureus* [74, 75, 76). In contrast to Gram-negative bacteria, the main mechanism of QAC resistance in Gram-positive bacteria is plasmid-borne efflux, due to the SMR family transporters QacC/D and QacEΔ1, QacG, QacH and QacJ and the MF family QacA/B transporter [64].

Chlorhexidine

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Bactericidal concentrations of chlorhexidine result in a denaturation of cytoplasmic proteins and coagulation of the cell contents. The specific

mechanism(s) associated with chlorhexidine resistance still remain to be elucidated. However, chlorhexidine resistance has been associated with *cepA,* encoding a putative efflux mechanism in *K. pneumoniae* [77]. Indirect evidence for the role of RND family exporters in chlorhexidine resistance has been provided. Benzalkonium chloride and triclosan adapted *E. coli* displayed a multi-drug-resistance phenotype including reduced susceptibility to chlorhexidine, consistent with increased expression of an RND multi-drug transporter [78]. Chlorhexidine has been shown to induce expression of the MexCD-OprJ efflux pump in *P. aeruginosa*, although a role in chlorhexidine resistance was not examined [79]. QacA/B in Gram-positive bacteria reduces susceptibility to chlorhexidine [64, 79]

3.2 Links between biocide and antibiotic resistance

The link between biocide and antibiotic resistance has mainly been studied in the laboratory by selecting bacteria with decreased susceptibility to biocides. Numerous studies have clearly demonstrated that selection for biocide resistance can result in cross-resistance to antibiotics [80-83]. Furthermore, several studies have unequivocally demonstrated that selection for multiple antibiotic resistance results from increased expression of multi-drug transporters capable of accommodating both biocides and antibiotics [9, 15, 16, 71, 84, 85, 86]. Triclosan has been shown to select for multiple antibiotic resistant *P. aeruginosa* overpressing the MexCD and MexJK efflux systems [71, 87], *E. coli* [15] and *Salmonella* [9] overexpressing the AcrAB multi-drug efflux pump and more recently *Stenthrophomonas maltophilia* overproducing the SmeDEF multi-drug efflux pump [88]. Similarily, QAC can select for resistant *S. aureus* isolates showing cross resistance to fluoroquinolones as a

result of increased *norA* expression [89] and a modest cross resistance to several antibiotics as a result of overproduction of MdeA [90]. Exposure to sub-lethal levels of a QAC disinfectant containing formaldehyde and glutaraldehyde has also been shown to select for multiple antibiotic resistance in *Salmonella* associated with overexpression of AcrAB-tolC [9].

In vivo, the relationship between biocide use and antibiotic resistance is less clear, with no conclusive evidence being provided to date to suggest that the observed laboratory phenomena have relevance to the real world [63]. A recent study reported a lack of correlation between biocide and antibiotic resistance in bacteria isolated from homes that used or did not use biocide containing products [91]. Similarly, no correlation was evident between biocide and antibiotic resistance in a large number of clinical *S. aureus* and *P. aeruginosa* isolates studied over a 10 year period [92]. In contrast, a comparison of clinical and industrial isolates of *P. aeruginosa* revealed that antibiotic/biocide correlations occured with clinical strains only. Studies on Gram-negative organisms found in urinary tract infections revealed significant correlations between biocide resistance and multiple antibiotic resistance [93, 94]

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4. Physiological impacts associated with biocide resistance-is there a link?

Studies have shown that when cells encounter a stress, such as the selective pressure imposed by an antibiotic or a biocide, the cell alters its physiological status enabling it to survive [80,95]. The precise detail of how different

bacterial cells respond and what occurs at the functional level is currently incomplete. Nevertheless, it is possible to generalize about some of the molecular responses (as shown in Figure 5). In the absence of externally applied stresses, a bacterial cell would be expected to maintain its normal physiological state (Figure 5 [a]), assuming that appropriate nutrients and other factors were plentiful. Imposing a particular stress (Figure 5 [b] - antibiotic or biocide) causes the cell to transduce this signal into the ~~cell~~cytolasm. At a molecular level, one or several genes will respond [24,96]. In some cases expression is switched on (eg: as typified by the chaperones *groEL* and others) [97 98], whilst in others gene expression is switched off. These effects are probably transient and normal status is reinstated once the stress has disappeared. In contrast, when a different stress is imposed (Figure 5 [c] –acid) the cell reacts accordingly and yetanother set of genes display differential regulation patterns. Some of these genes may be common and react similarly as in the former case. However, at a functional level, the resulting phenotype may be very different.

Exposure of bacteria originating in animals treated with antibiotics may provide for a pre-adaptation in some important zoonotic bacteria (including *Salmonella* and *Campylobacter*). The capacity of food-borne bacteria to survive stresses encountered along the food chain is an important factor aiding their transmission from animals to humans. Subsequent stressing of these organisms further along the food chain may serve to increase their ability to resist any new stresses imposed (combinations of effects outlined in Figures 5[b] and [c]).

The nature of the relationship between antibiotic/biocide resistance and associated physiological impacts, is only now beginning to be explored. Early descriptions are at best conflicting. Both antibiotics and biocides can induce the expression of efflux pumps, but this is likely to be just one of many responses at the functional level and no clear description has emerged. Several studies have shown that low level biocide resistance exhibited by cells results in slower growth rates compared to their isogenic parents [99-100]. Similarly, when *S.* Typhimurium was selected after exposure to aldehyde, oxidizing and tar-acid based disinfectants, these mutants displayed reduced growth rates, reduced colony size and were less invasive when co-cultured with Caco-2 cells, compared to their isogenic non-selected parent strains [102, 103]. Karatzas, *et al.*, ~~(2007)~~ also reported that when *S.* Typhimurium was selected on a number of commercially applied disinfectants (including triclosan), mutants recovered had reduced growth rates reduced invasiveness, as determined using Caco-2 cells [9]. These observations conflicted with those reported by Webber *et al.* ~~2008~~, showing increased fitness in *Salmonella* following selection in triclosan [104].

In *E. coli* triclosan has been reported to down-regulate some virulence promoters of outer membrane protein X and p-fimbriae, however these effects appear to be transient [105]. Starvation and exposure of *E. coli* O157:H7 to sodium hypochlorate (such stresses are likely to be encountered by bacteria in the food production environment) influenced the virulence potential of this organism. Virulence factors including *stx* (encoding verocytoxins) and

attachment components were up-regulated [106]. These contributed to the survival of this human pathogen.

Conclusions

The advent of multi-drug resistant (MDR) pathogens has created a public health issue. Recent reports highlight the importance of food as an avenue for the dissemination of antibiotic resistant genes to humans, thereby reducing the efficacy of our current arsenal of drugs. To effectively combat this problem, a comprehensive understanding of the mechanisms involved are essential. The isolation of antibiotic resistant pathogens from retail food products, underpins the fact that a potential reservoir of antibiotic resistant bacteria may exist within the food chain. It is speculative whether or not, depending on the genotypes of these organisms, the efficacy of biocides may ~~be~~ also be compromised.

It is unclear, at present whether a link between antibiotic and biocide resistance exists *in-vivo*. Although laboratory data appears to support such a link, there is a lack of convincing evidence from natural sources, despite the involvement of some common mechanisms including efflux pumps. However, all studies agree the prudent use of antimicrobials (antibiotics and biocides) is important.

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–In the absence of any new antibiotics, the importance of plasmid-mediated resistance transfer and efflux pumps, in the development and maintanance of antibiotic resistance cannot be underestimated. Effective strategies aimed at plasmid curing and inhibition of efflux pump activity would be legitimate targets to pursue in the expectation that novel inhibitors could be developed. Neither compound type has been licensed for use in the treatment of bacterial infections in human and veterinary medicine. In gaining a better understanding of these mechanisms, it may be possible in the future to develop a rationally-based inhibitor(s), based on the kowledge of structurally vulnerable targets.

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Figure legends

Figure 1: Structure of composite (a), complex (b) transosons and integrons associated with antibiotic resistance in bacteria.

Figure 2: A schematic representation of the current categorisation of antimicrobials

Figure 3: Schematic diagram of the main efflux determinants of biocide resistance in (a) Gram-positive and (b) Gram-negative bacteria

Figure 4: Chemical structure of benzalkonium chloride (QAC's), chlorhexidine and triclosan

Figure 5: A general schematic illustrating the responses at a molecular level in a cell under normal (a), antibiotc/biocide stress (b) and acid stress (c) conditions.

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