Prevalence and antimicrobial resistance profiles of *Vibrio* spp. and *Enterococcus* spp. in retail shrimp in Northern California

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Abstract

Shrimp is one of the most consumed seafood products globally. Antimicrobial drugs play an integral role in disease mitigation in shrimp and other aquaculture settings to meet production demand, but their prevalent use in these industries raises public health concerns about the emergence and spread of antimicrobial resistant microorganisms. *Vibrio* spp., as the most common causative agents of seafood-borne infections in humans and illness in shrimp, and *Enterococcus* spp., as an indicator organism, are focal bacteria of interest for the monitoring of antimicrobial resistance (AMR) in seafood.

In this study, 400 samples of retail shrimp meat were collected from randomly selected grocery stores in the Greater Sacramento, California, area between September 2019 and June 2020, and were tested for *Vibrio* spp. and *Enterococcus* spp. The prevalence of *Vibrio* and *Enterococcus* in these samples were 60.25% (241/400) and 89.75% (359/400), respectively. Subsamples of confirmed *Vibrio* and *Enterococcus* isolates (n = 110 each) were subjected to antimicrobial susceptibility testing (AST) using the NARMS Gram-negative and Gram-positive drug panels, respectively. Results from AST indicated that *Vibrio* isolates had high phenotypic resistance to ampicillin (52/110, 47.27%) and cefoxitin (39/110, 35.45%). *Enterococcus* were most frequently resistant to lincomycin (106/110, 96.36%), quinupristin-dalfopristin (96/110, 87.27%), ciprofloxacin (93/110, 84.55%), linezolid (86/110, 78.18%), erythromycin (58/110, 52.73%), and chloramphenicol (43/110, 39.09%). Multidrug resistance (resistance to ≥3 drug classes) was observed in 8.18% of *Vibrio* isolates (9/110) and 93.64% of *Enterococcus* isolates (103/110). No significant differences in the prevalence of AMR ($P > 0.05$) were found between isolates from farm raised and wild caught shrimp ($P = 1.0$), nor between isolates from shrimp of domestic and imported origin ($P = 1.0$) in *Vibrio* isolates. Similarly, *Enterococcus* isolates from

wild caught shrimp samples were no different from those raised in farms (P = 0.377), and those of domestic origin were not statistically different from those that were imported (P = 0.321).

Whole genome sequencing (WGS) of a subset of *Vibrio* isolates (n = 42) speciated isolates as primarily *V. metschnikovii* (24/42; 57.14%) and *V. parahaemolyticus* (12/42; 28.57%), and detected 27 unique antimicrobial resistance genes (ARGs) across these isolates, most commonly *qnrVC6* (19.05%, 8/42), dfr_{A31} (11.90%, 5/42), dfr_{A6} (9.5%, 4/42), *qnrVC1* (9.5%, 4/42). Additionally, WGS predicted phenotypic resistance in *Vibrio* isolates with an overall sensitivity of 11.54% and specificity of 96.05%.

Keywords: Resistance, *Vibrio*, *Enterococcus*, Shrimp, Antimicrobial, Antibiotic, Whole Genome Sequencing, California

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Chapter 1: LITERATURE REVIEW

Introduction

Overview of the shrimp industry

Over three billion people around the world rely on seafoods as primary sources of dietary protein, and many more include them regularly in their diets (Lund, 2013). In 2020, a total of 178 million metric tons of seafood were produced by fisheries and aquaculture globally, constituting nearly a quarter of the world's meat supply (Edwards et al., 2019; FAO, 2022a). The same year, the seafood industry was valued at 401 billion USD (FAO, 2022a). Shrimp constitutes a significant portion of this production volume and economic value, and in 2020, global production of shrimp exceeded 7 million metric tons live weight, nearly as much as all offshore finfish aquaculture combined (FAO, 2022a). By 2022 farmed shrimp was worth 24.7 billion USD and constituted nearly 10% of all seafood produced by mass, extending a longstanding trend of accelerating growth (FAO, 2022a; NFI Media, 2022).

Much of the shrimp industry's growth owes to a multidecadal trend of increased shrimp consumption in countries with developing economies. Economic growth in some countries, primarily in Asia, has enabled more consumers to access delicacy foods like shrimp, while a simultaneous technology-driven rise in production in the 21st century has reduced market prices (FAO, 2022a; Kumar & Engle, 2016). Historically, shrimp consumption has been highest in high income markets such as the United States (U.S.), European Union (E.U.), and Japan (FAO, 2022a). Shrimp is the single most-consumed seafood in the U.S., and per capita shrimp consumption in the U.S. rose to nearly five pounds in 2020 (NFI Media, 2022; National Marine Fisheries Service, 2022). Still, nearly 90% of Americans eat less shrimp than is recommended, reflecting the oft-

recommended potential for humans to increase their seafood consumption (Troell et al., 2019; USDA & USDHHS, 2020; Steenson & Creedon, 2022; Thomsen et al., 2022), and in 2022, China usurped the U.S.'s top shrimp importer ranking despite the country also having world-leading domestic shrimp production (FAO, 2023).

One of the reasons for shrimp's global popularity is its nutritional quality. The most consumed shrimp species are rich in essential amino acids and polyunsaturated fatty acids (Liu et al., 2021), while also containing fewer unhealthy fats than red meat or poultry (USDA & USDHHS, 2020). Further, because shrimp occupy a low trophic level in aquatic food webs, they don't bioaccumulate as much methylmercury as many other popular seafoods like salmon and tuna (Smith & Guentzel, 2010; USDA & USDHHS, 2020). The palatability and healthy nutrient profile of shrimp help explain the increase in demand observed in recent decades, and production methods have evolved to keep pace.

Shrimp production

Wild shrimp capture fisheries predominated production in the early days of the shrimp industry, and shrimp are still extracted from benthic marine and estuarine habitats around the world in great quantities. However, they are often fished with unsustainable practices and without sufficient regard for the long-term stability of shrimp stocks (Alam et al., 2022; Asche et al., 2022). Fishers utilize trawling techniques to catch shrimp, which have been shown repeatedly to be environmentally, economically, and socially unsustainable (Thrush et al., 1998; Foster & Vincent, 2010; Zaima, 2014; Texeira et al., 2019). Trawling results in bycatch of non-target species, and affects the long-term sustainability of wild shrimp populations, their benthic habitats, and human fishing communities (Dellapenna et al., 2006). While there are ongoing efforts to negate the

negative impacts of trawling, the practice remains commonplace and destructive (Rodrigues Filho et al., 2020; Larsen et al., 2021).

Yields of shrimp and other seafoods from capture fisheries have also failed to keep pace with rapid increases in global demand over the past few decades. Annual production of all seafood animals from fisheries fluctuated between 79.0 and 89.4 million metric tons between 1990 and 2020. This is true despite 93% of fisheries being fished at or above maximum sustainable levels in 2019, which suggests there is little upward mobility in yields from capture fisheries (Kumar & Engle, 2016; FAO, 2020; FAO, 2022a). Yet, global per capita and gross seafood production consistently increased over the same twenty-year period (FAO, 2020).

Seafood demand that cannot be fulfilled by capture fisheries is satiated by aquaculture. Shrimp aquaculture is the fastest growing animal food sector in the world, and already produces triple the shrimp of capture fisheries (Kumar et al., 2016; FAO, 2022c; Golder et al., 2022). Shrimp farming techniques can vary significantly in terms of scale, intensity, and inputs. Small scale, extensive shrimp farms are most common, but their production capacity is limited (Thornber et al., 2020). These farms most often feature a flow-through design in which water is redirected from a nearby source like a brackish estuary to passively fill and replenish man-made pond impoundments, and wastewater outflow drains back into the same water body (Boyd et al., 2022). However, the trajectory of shrimp farming through time has been towards higher intensity operations (Kumar & Engle, 2016; Thornber et al, 2020).

Technological advancements which allow for more effective aeration, accelerated selection of favorable genetic traits, optimization of diets, and more effective disease prevention and treatment have facilitated higher stocking densities and yields in shrimp aquaculture (Kumar & Engle, 2016). Today, the majority of farmed shrimp are produced from large, intensive operations

(Thornber et al., 2020; FAO, 2022a). Most shrimp aquaculture takes place in East and Southeast Asia (namely China, India, Indonesia, Vietnam, Thailand, and the Philippines), and South and Central America (especially Ecuador and Mexico) (FAO, 2022a). While the geographies of shrimp consumption are shifting as mentioned previously, many of the largest import markets remain located in North America and Europe, necessitating long-distance distribution from processing sites.

There are a wide variety of shrimp products available to consumers, for which processing can greatly differ. Shrimp can be sold packaged or in bulk; raw, blanched, or fully cooked in ready-to-eat products; shell-on or peeled; head-on or headless; deveined or veined; whole, butterflied or chopped; plain or coated in breading or sauce; etc. One constant among all shrimp products, though, is that if they are not sold live, the entirety of processing and distribution post-slaughter must be chilled (Hannan et al., 2022). Shrimp meat is highly susceptible to the growth of spoilage and pathogenic microbes which can affect meat quality and pose public health risks, and chilling and freezing are necessary to slow their growth throughout the slaughter-gate-to-consumer-gate life cycle (Hannan et al., 2022). This is especially true considering the long-distance shipping that's often required from top production sites to import markets. Fan et al. (2020) found that the sensory quality of whiteleg shrimp meat kept refrigerated at 4°C declined noticeably after just five days. Freezing at very low temperatures improves the shelf life of shrimp meat but can be inaccessible for small scale operations and processors in impoverished regions (Hannan et al., 2022; Khan et al., 2022). Even when this infrastructure is available, however, the risks of microbial spoilage and spreading of pathogens to consumers are of significant concern.

Risk of infectious disease in farmed shrimp

While more intensive shrimp farms are capable of greater production capacity, they are also faced with different sources of increased risk. One of the primary risks in any shrimp farming is infectious disease, as shrimp are highly susceptible to pathogens. One reason for this is that shrimp, like all invertebrates, lack an adaptive immune system (Smith et al., 2003; Hauton & Smith, 2007; Ali et al., 2018). Operations with high stocking densities are at especially high risk when faced with disease spread, which means that when an outbreak affects high-output farms it can cause disruptions on an incredible scale. In some cases, disease outbreaks have destroyed entire countries' shrimp industries. Taiwan, for example, was the leading shrimp producing country in the mid-1980s, but multiple disease outbreaks collapsed its shrimp industry and production never fully recovered (Kumar & Engle, 2016). More recently, an outbreak of Early Mortality Syndrome on shrimp farms in Thailand in the early 2010s decimated what was also once world-leading shrimp production (Sanguanrut et al., 2018; FAO, 2022a). Early Mortality Syndrome had also caused production declines in China a few years earlier, and its spread to Thailand was blamed on poor biosecurity (Flegel, 2019).

This disease risk also has a serious impact on which shrimp species are cultured. A wide variety of shrimp species are fished from capture fisheries for human consumption, but shrimp aquaculture is dominated by only a few. Whiteleg shrimp (*Penaeus vannamei*) became the predominant species in shrimp aquaculture due to the development of specific pathogen free, specific pathogen resistant, and specific pathogen tolerant broodstock, even though other species like the black tiger shrimp (*Penaeus monodon*) had been preferred previously (Barman et al., 2012; Thornber et al., 2020). In 2020, whiteleg shrimp constituted more than half of all global crustacean production, though other species including the black tiger shrimp, giant river prawn

(*Macrobrachium rosenbergii*), and oriental river prawn (*Macrobrachium nipponense*) are also cultured in large volumes (FAO, 2022a).

Disease Management and Use of Antibiotics in Shrimp Aquaculture

Although significant preventative efforts are undertaken at the production, processing, and regulatory levels, shrimp biology and the culture techniques employed in the aquaculture operations that account for the vast majority of global shrimp production could result in high risk of catastrophic disease outbreaks on farms. Disease prevention and treatment are thus of significant concern in shrimp aquaculture, and there are many methods used by farmers to mitigate losses from outbreaks. Organizations like Best Aquaculture Practices and ASEAN's Good Aquaculture Practices for Food Fish offer certifications for seafood products that are produced following their environmental and social stewardship standards and play an important role in disease prevention and treatment on farms. However, compliance with the standards among farmers can be inconsistent (Tlusty & Tausig, 2014).

Non-antibiotic methods of disease prevention and treatment in shrimp aquaculture

Some disease prevention efforts at shrimp farms fall under the umbrella of biosecurity. This includes measures employed to prevent animal exposure to external sources of infectious disease or spread of diseases from infected farm animals to the wild environment (Pruder, 2004). Examples of biosecurity measures include chemical disinfection of farm workers and equipment, assessments and quarantining of new animal shipments, water tests, precautionary bacterial culturing, health monitoring, and recordkeeping (Delphino et al., 2022). Biosecurity practices are not foolproof, however, and some farms may be unable or choose not to utilize some precautions;

it has been observed that shrimp farm scale positively correlates with adherence to biosecurity protocols (Delphino et al., 2022).

Vaccination has also gained favor in finfish aquaculture in the 21st century (Gudding & Van Muiswinkel, 2013). However, traditional vaccination is not an option in shrimp as a consequence of their lack of an adaptive immune system (Sommerset et al., 2005; Gudding & Van Muiswinkel, 2013; Thornber et al., 2020). While new, DNA-based vaccination methods have shown some promise in shrimp, their efficacy is limited and their function is not well understood (Chang et al., 2018). For example, Rout et al. (2007) and Rajesh Kumar et al. (2008) injected an antigen-encoding plasmid to black tiger shrimp and observed significant but short-lived protection from white spot syndrome virus. Li et al. (2010) had similar results with whiteleg shrimp.

Another preventative step taken by some shrimp farmers is the application of probiotics to inoculate ponds and shrimp microbiomes with beneficial microbes that can exclude pathogens and aid in nutrient uptake (Garriques & Arevalo, 1995). However, mislabeling of probiotic products has been known to result in the introduction of unintended and antimicrobial resistant bacteria into farms (Noor Uddin et al., 2015; Uma & Rebecca, 2018). In a 2015 evaluation of the bacterial composition of probiotic products used on Vietnamese shrimp farms, Noor Uddin et al. (2015) found that all of their samples contained bacterial species that were not listed on packaging, and that many harbored antimicrobial resistance genes (ARGs).

Antibiotics used in shrimp aquaculture

Traditional disease prevention in shrimp aquaculture involves prophylactic and metaphylactic applications of antibiotics. with antibiotics at subtheraputic concentrations. Prophylaxis, the use of antibiotics at subtheraputic concentrations, has historically been popular in

the culture of many terrestrial and aquatic species both for disease prevention and growth promotion (Holmström et al., 2003; Sarmah et al., 2005; Cabello, 2006). While this practice was once commonplace in shrimp culture, it has declined throughout the 21st century with the exception of hatchery settings (Holmström et al., 2003; Zhang et al., 2011; Smith, 2012; Thornber et al., 2020).

Metaphylactic application of antimicrobial drugs is still the most common response when disease outbreaks do occur in shrimp farming, however (Thornber et al., 2020). Because infectious disease-related risk is so high, and shrimp is such a valuable commodity, farmers are incentivized to apply antimicrobials in great quantities; an incredible 2.7% of all antimicrobials used worldwide are applied in shrimp farms (Schar et al., 2020; Thornber et al., 2020). Most commonly, and most directly, these drugs are administered to animals as feed additives (Thornber et al., 2020).

Farmers have reported using a wide variety of antimicrobial drugs when treating diseases in shrimp, though specific drugs and frequency of use vary geographically and farm-to-farm (Rico et al., 2013; Li et al., 2016; Lulijwa et al., 2020). Rico and colleagues' 2012 review of antimicrobial usage in Asian aquaculture reported upwards of 30 unique antimicrobial compounds applied in shrimp farms (Rico et al., 2012). The most common drugs used in global shrimp aquaculture, though, are the antibiotics oxytetracycline, enrofloxacin, ciprofloxacin, florfenicol, sarafloxacin, and 1st, 2nd, 3rd, and 4th generation quinolones (Bermúdez-Almada & Espinosa-Plascencia, 2012).

Regulations differ between countries regarding which antimicrobials can be used in aquaculture within their borders. The U.S. Food and Drug Administration, for example, has stringent restrictions on the antimicrobial agents approved for aquaculture; only oxytetracycline, sulphadimethoxine-ormetoprim, florfenicol, and sulphamerazine are approved and used with

regularity (Lulijwa et al., 2020; FDA, 2023). All the top shrimp producing countries also have written regulations to this effect, though, generally, holding farmers to them has proven a challenge (Collignon et al., 2018). Farmers are often tasked with self-reporting their antimicrobial usage in written surveys and may be incentivized to misrepresent use to produce higher yields and access regulated export markets (Shamsuzzaman & Biswas, 2012; Pham et al., 2015; Lulijwa et al., 2020; Thornber et al., 2020). The U.S. and E.U., for example, both have stringent limits on antimicrobial residue levels on incoming seafood, and regularly reject shipments which violate them (Willette & Cheng, 2018; Thornber et al., 2020).

Risks Associated with Antimicrobial Resistance in Bacteria Originating from Shrimp

Antimicrobial drugs are humanity's primary tool for treating dangerous bacterial infections in both veterinary and human clinical contexts. However, exposure to antimicrobial drugs tends to select for antimicrobial resistance (AMR) in bacteria (CDC, 2019). This is especially true when these drugs are misused, but this risk is present even when drugs are used as directed (CDC, 2019). Antimicrobial resistance has repeatedly been identified as one of the most significant global health challenges of the current era (Wise et al., 1998; CDC, 2022). In 2016, it was estimated that without efforts to stop the spread of AMR, drug resistant infections could cost a staggering 10 million lives annually and a cumulative 100 trillion USD by 2050 (O'Neill, 2016).

Shrimp farming is an environment particularly conducive to the development and spread of AMR in bacteria. Aquaculture at large is generally considered an important reservoir for antimicrobial resistant bacteria (ARB) (Marti et al., 2014), but other factors make shrimp of unique concern in this regard. As previously discussed in this review, the biology and immunology of shrimp, coupled with market pressures that lead farmers to stock shrimp at high densities,

incentivize frequent use of a variety of antimicrobial drugs. Further, the flow-through design of many shrimp farms allows unmetabolized drugs suspended in farm effluent to be spread into estuarine and marine habitats. As a result of anthropogenic non-point sources and agriculture use, antimicrobial drugs have been detected in wild aquatic ecosystems, and even low concentrations have been shown to select for AMR in environmental and shrimp-associated bacteria (Gullberg et al., 2011; Wistrand-Yuen et al., 2018). Moreover, many drugs currently and historically employed in shrimp and other aquaculture are also used in human medicine (Alderman & Hastings, 2003; Bermúdez-Almada & Espinosa-Plascencia, 2012; WHO, 2019). Some shrimp farms, most commonly in low- and middle-income regions like southeast Asia, also employ integrated farming practices wherein ponds are fertilized with human and livestock waste which could harbor antimicrobial agents, resistant bacteria, or pathogenic bacteria (Suzuki & Hoa, 2012; Kim et al., 2013). Shrimp farming also takes place primarily in rural areas of low- and middle-income countries, where regulation of antimicrobial drug use is sometimes lacking (Collignon et al., 2018).

Even if a resistant organism is nonpathogenic, ARGs can be spread to pathogens by horizontal gene transfer (Lulijwa et al., 2020). Often, through the phenomenon of co-selection, multiple ARGs are transferred simultaneously in a single horizontal gene transfer event – even including ARGs which confer resistance to drugs the bacteria have not been directly exposed to (Lulijwa et al., 2020). Some ubiquitous, commensal bacteria, such as *Enterococcus* spp., are especially adept at transferring ARGs in this manner and have been regarded as potentially dangerous genetic reservoirs for AMR (Byappanahalli et al., 2012; Di Cesare et al., 2013).

Ultimately, pathogenic bacteria are the organisms that actualize the dangers of AMR. In shrimp, the main bacterial pathogens are *Vibrio* spp. which primarily infect immunosuppressed animals (Johnson, 1989; Karunasagar et al., 2004). Namely, *V. parahaemolyticus* and *V. harveyi*

have caused mass deaths in farmed shrimp (Johnson, 1989; El-Far et al., 2015). Some *Vibrio* spp. are also significant human pathogens; *V. parahaemolyticus* and *V. vulnificus* cause more seafood-related infections and deaths in the U.S., respectively, than any other bacteria (Elmahdi et al., 2016). The U.S. Centers for Disease Control and Prevention report that 45,000 Americans are infected with *V. parahaemolyticus* each year, and while only around 100-200 cases of *V. vulnificus* occur each year in the U.S., roughly 20% of infections are fatal (CDC, 2020; Brumfield et al., 2021). *V. cholerae* has also infamously been the causative agent of multiple human pandemics. While *V. cholerae* infections are still relatively common and dangerous, especially in low and middle income countries, the frequency and severity of outbreaks have trended down globally as hygiene, medicine, and water treatment technologies have advanced (Colwell, 1996). Other prominent bacterial pathogens associated with shrimp and other seafoods are pathogenic strains of *E. coli*, *Salmonella*, *Campylobacter*, and *Listeria monocytogenes*, though unlike *Vibrio* spp., these are secondary contaminants that only associate with shrimp meat after harvest (Stephen et al., 2023).

Active surveillance for the emergence and spread of resistant bacteria, changing patterns in AMR, and for areas or populations that may require intervention is a crucial first step in preventing the spread of ARB (Grundmann, 2014; Lee et al., 2022; Public Health Agency of Canada, 2022; Tate et al., 2022). Commercially available are numerous and can vary greatly in geographic origin, production, and processing, so surveillance from a single market or area can provide insight into demographic factors concerning the variation of AMR in shrimp-associated bacteria. It was recently suggested in a study conducted through the National Antimicrobial Resistance Monitoring System that *Vibrio* spp. and *Enterococcus* spp. would be good candidates

for tracking AMR because of their high prevalence relative to other organisms and because of their pathogenicity and role as ARG reservoirs, respectively (Tate et al., 2022).

Conclusion

The popularity and profitability of shrimp have caused shrimp aquaculture to become the fastest growing animal food sector in the world. However, farming techniques that have facilitated this growth also make outbreaks of infectious disease a significant concern. As a result, antimicrobial drugs are used extensively in shrimp farming, which can select for antimicrobial resistance in shrimp-associated bacteria. Those resistance characteristics threaten human and animal health when they are present in pathogens, thus good aquaculture practices and prudent use of antimicrobials are of grave importance. One of the most important parts of human efforts to quell the spread of antimicrobial resistance in shrimp and other foodstuffs is active surveillance.

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Chapter 2: PREVALENCE AND ANTIMICROBIAL RESISTANCE PROFILES OF VIBRIO SPP. AND ENTEROCOCCUS SPP. IN RETAIL SHRIMP IN NORTHERN CALIFORNIA

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Introduction

Shrimp is America's most popular seafood and is vaunted by the USDA as a healthy protein due to its nutrient density, lower unhealthy fat content than red meat or poultry, and lower levels of methylmercury many other seafoods (NFI Media, 2022; USDA & USDHHS, 2020). Annual per-capita consumption of shrimp in the U.S. approached five pounds in 2020, even though nearly 90% of Americans eat less than the recommended quantity of shrimp and other seafoods (USDA & USDHHS, 2020; National Marine Fisheries Service, 2022). To meet domestic demand, the United States is one of the most significant importers in a global shrimp industry that in 2018 was valued at 24.6 billion USD and was still found to be growing at an accelerating rate (FAO, 2020; NFI Media, 2022).

Shrimp aquaculture, which already outproduces wild shrimp fisheries three times over, is the fastest growing animal food sector in the world (Kumar et al., 2016; FAO, 2022; Golder et al., 2022). The majority of shrimp production occurs in countries in Asia (primarily China, Thailand,

Indonesia, and India) and South and Central America (especially Ecuador) (FAO, 2022). Farm raised shrimp are highly susceptible to infectious disease due to high stocking densities and their lack of an adaptive immune system (Smith et al., 2003; Hauton & Smith, 2007). Outbreaks can endanger entire harvests without quick and aggressive treatment. Vaccination, which has gained traction in finfish aquaculture, is not an option for shrimp (Sommerset et al., 2005; Gudding & Van Muiswinkel, 2013). Consequentially, bacterial infectious diseases in farmed shrimp are almost always treated with antimicrobial agents. Since the scale of the industry and the risks posed to by those diseases are so great, an enormous volume of antimicrobial drugs are used in shrimp production. Indeed, 2.7% of all global antimicrobial usage of any type is attributable to shrimp aquaculture (Schar et al., 2020; Thornber et al., 2020).

The most common form of antimicrobial use in shrimp farming is feed-mediated metaphylaxis after the detection of an infection (Thornber et al., 2020). Prophylactic use of antimicrobials in shrimp aquaculture was once commonplace and is an ongoing practice, especially in hatchery settings, but has generally declined through the 21st century (Holmström et al., 2003; Zhang et al., 2011; Smith, 2012; Thornber et al., 2020). While antimicrobials are the first line treatment against pathogens in food production and clinical contexts, their use can select for resistance in bacteria and lead to untreatable infections in human and animals.

Inappropriate antimicrobial use practices can increase the selective pressure in bacteria and result in the development of antimicrobial resistance (CDC, 2019). Shrimp-associated pathogenic bacteria are thus at high risk of developing antimicrobial resistance, which reduces the ability to treat infections that compromise animal welfare, human health, and industry. Moreover, since ARGs can spread via horizontal gene transfer, even non-pathogenic bacteria, or those which are pathogenic for a different species, that develop resistance can spread resistance to bacteria which

are pathogenic for a species of interest like shrimp or humans (Lulijwa et al., 2020). Monitoring the prevalence and patterns of resistance in foodborne bacteria is therefore critical to evaluate food safety and public health risks.

Considering the scale of the shrimp industry and the seriousness of the public health risks posed by the spread of antimicrobial resistance, it is imperative to track resistance patterns and ARGs in shrimp meat. Recently, a pilot study by the National Antimicrobial Resistance Monitoring System (NARMS) detected *Vibrio* and *Enterococcus* as the most prevalent Gram-negative and Gram-positive bacteria in retail seafood samples, respectively, and highlighted them as good candidates for tracking AMR (Tate et al., 2022). *Vibrio* spp. are cosmopolitan and are normal flora in the coastal and estuarine habitats of wild shrimp, but are also the most common seafood-borne pathogens in humans (Raissy et al., 2012; Costa et al., 2015a) and the main pathogens of shrimp (Johnson, 1989; El-Far et al., 2015). *Enterococcus* spp. are primarily commensal bacteria in the environment and animal gut microbiomes, but are often employed as indicator organisms for the monitoring of antimicrobial resistance since they have the capacity to readily acquire ARGs conferring resistance to nearly every drug in use and transfer them to other bacteria, including pathogens (Byappanahalli et al., 2012). The present assessment aims to expand knowledge in this area and establish a baseline for future assessment by characterizing the resistance found in *Vibrio* and *Enterococcus* isolates sourced from shrimp samples collected from grocery stores in the greater Sacramento area, California.

Materials and Methods

Sample Collection

A total of 400 shrimp samples, either prepackaged or in half-pound packages from bulk seafood counters, were collected from grocery stores in the Greater Sacramento, California, area in four seasonal periods between September 2019 and June 2020. A list of 100 grocery stores were randomly selected among those located in Sacramento zip codes according to Google Maps. During each sampling event, stores were randomly selected from this pool. Along with the samples themselves, metadata including production type (farm raised or wild caught), country of origin, species and size of shrimp, store handling method, sold forms (fresh, frozen, or previously frozen), and time of collection was collected during sampling. Samples were kept on ice during transport, refrigerated upon receipt at the laboratory, and processed within 72 hours of collection.

Sample Processing, Bacterial Isolation, and Confirmation

Samples were processed using the NARMS seafood pilot laboratory protocol (https://www.fda.gov/media/149957/download). Briefly, two aliquots of 25 g from each shrimp sample were placed into two sterile stomacher bags, one containing 225 mL of alkaline peptone water (APW) and another with buffered peptone water (BPW). Samples were homogenized in a Neutec Masticator Paddle Blender (Neutec Group, Inc., Farmingdale, NY, United States) for 2 minutes at 230 RPM and incubated at 35°C for 24±2 hours. Subsequently, overnight APW and BPW enrichments were streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS) (BD Difco, Detroit, MI, United States) and Enterococcosel (BD BBL, Franklin Lakes, NJ, United States) agars, respectively, and incubated at 35ºC for 18-24 hours for identification of *Vibrio* spp. and *Enterococcus* spp., respectively. One colony with positive colony morphology (yellow or green to blue-green colonies being characteristic of *Vibrio*, and beige colonies with strong black halos being

characteristic of *Enterococcus*) was selected from each plate and streaked to purity on blood agar plates. Presumptive positives for Vibrio were confirmed to genus level via PCR using the forward primer: 5'-GGC GTA AAG CGC ATG CAG GT-3'; and the reverse primer: 5'-GAA ATT CTA CCC CCC TCT ACA G-3', as previously described in Thompson et al. (2004). *Enterococcus* were confirmed with Gram-staining for identification of Gram-positive cocci and biochemical tests (catalase negative and PYR positive) using BD BBL DrySlide™ PYR kits and following methods previously described by Aryal (2016).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was conducted on a subset of isolates comprised of 110 *Vibrio* (110/241) and 110 *Enterococcus* (110/359) isolates using the broth microdilution method with the NARMS Gram-negative (CMV3AGNF) and Gram-positive (CMV3AGPF) panels, respectively. Isolates were streaked onto selective agar plates (TCBS and Enterococcosel agar for *Vibrio* and *Enterococcus*, respectively) and incubated at 35°C for 18-24 hours. A colony with typical morphology was then restreaked onto blood agar (Thermo Fisher Scientific, Waltham, MA, United States) and incubated at 35°C for 20-24 hours.

Pure colonies on fresh overnight blood agar plates were suspended in sterile demineralized water to an optical density (OD) between 0.08 and 0.10 as measured by a spectrophotometer (BioMate 3; ThermoSpectronic, Rochester, NY) set to a wavelength of 625 nm. Aliquots of the suspension (20 uL for *Vibrio* and 10 uL for *Enterococcus*) were then transferred to 11 mL of cation-adjusted Mueller-Hinton broth (CAMHB; BD Difco, Detroit, MI, United States), and the mixture was vortexed for five to ten seconds. Subsequently, 50 uL of the CAMHB mixture was

transferred to each well of the MIC plate. Additionally, a loopful (10 uL) of the CAMHB suspension was streaked onto a blood agar plate for quality control. MIC plates and blood agar plates were then incubated at 35ºC for 18-24 hours. The minimum inhibitory concentration was recorded as the lowest concentration of each drug with fully inhibited growth in the wells and per guidelines from Clinical and Laboratory Standards Institute (CLSI) methods (Clinical and Laboratory Standards Institute, 2018).

Resulting MIC values were interpreted as susceptible, resistant, or intermediate based on CDC breakpoints for non-cholera *Vibrio* (CDC, 2019) and FDA NARMS breakpoints for *Enterococcus* (FDA, 2019), both of which are based on the CLSI clinical breakpoints for human infection treatment (CLSI, 2017). Six of the fourteen drugs in the Gram-negative panel for *Vibrio* isolates (ceftiofur, ceftriaxone, chloramphenicol, nalidixic acid, streptomycin, and sulfisoxazole) have no CLSI or NARMS breakpoints, and thus could not be included in resistance calculations. The composition of each drug panel and interpretive thresholds for each drug are listed in Supplementary Table 1. Intermediate results were counted as resistant in the analysis. Multidrug resistance (MDR) was defined as resistance to three or more classes of antimicrobial drugs (Tate et al., 2022).

Whole Genome Sequencing (WGS) and Identification of Antimicrobial Resistance Genes (ARGs)

A total of 52 *Vibrio* isolates were selected randomly from those that exhibited phenotypic resistance in AST and sent to the Food and Drug Laboratory Branch at the California Department of Public Health for sequencing. The strains were streaked onto Trypticase Soy Agar with 0.6% Yeast Extract (TSA-YE) and 3% saline for recovery as well as CHROM *Vibrio* plates for

confirmation. A single colony was restreaked on TSA-YE with 3% saline and incubated at 35°C for 18-24 hours. Genomic DNA was extracted from bacteria using the DNeasy Blood and Tissue Kit (Qiagen) and quantified using a Qubit fluorometer (ThermoFisher Scientific). DNA libraries were prepared with Illumina DNA Prep kits (Illumina Inc., San Diego, California). Whole genome sequencing was performed on the Illumina MiSeq DNA sequencing system using the MiSeq reagent kit version 2 (2 × 250-bp paired-end reads) per CDC PulseNet guidelines (PulseNet, n.d.). After the successful completion of the sequencing runs, the FASTQ files along with the corresponding metadata were submitted to the PulseNet for data analysis and upload to NCBI. Identification of antimicrobial resistance genes was done with raw reads using the ResFinder database (version 4.1, Center for Genetic Epidemiology, Kongens Lyngby, Denmark) with genes determined as present if sequences met quality control thresholds of 90% match and 60% minimum length (Bortolaia et al., 2020; Zankari et al., 2020; Clausen et al., 2018).

Statistical Analysis

Descriptive statistics for the prevalence of *Vibrio* and *Enterococcus* in shrimp samples, the distribution of resistant patterns among isolates, sample characteristics, and the prevalence of resistance genes were conducted in Microsoft Excel (version 2207, Redmond, WA, USA). Percent of isolates resistant to an antimicrobial agent was determined by dividing the number of isolates with a MIC value classified as resistant based on the appropriate CDC or FDA breakpoint criteria by the total number of isolates.

Prevalence and metadata analyses were performed using R version 4.1.2 (Vienna, Austria). Fisher's exact test with adjusted P-values was used to evaluate the associations between these

demographic factors and multidrug resistance. Figures were created in R using packages ggplot2, ggtext, and heatmap.3.

Concordance between phenotypic resistance from MIC analysis and genotypic resistance from ARGs identified via WGS were evaluated as previously described (Lee et al., 2022). Phenotype and genotype were considered concordant when an isolate with phenotypic resistance to a drug in the MIC panel also had ARGs associated with the corresponding drug (true positive, TP), or when an isolate with phenotypic susceptibility to a drug also did not contain any corresponding ARGs (true negative, TN). False negatives (FN) were defined as isolates that exhibited phenotypic resistance but did not harbor any ARGs known to confer resistance to the corresponding drug, and false positives (FP) were defined as isolates that exhibited phenotypic susceptibility to a drug but contained ARGs associated with that drug. Sensitivity was calculated as TP/(TP + FN) and specificity was calculated as TN/(TN + FP). Two *Vibrio* isolates that were speciated as *V. cholerae* were omitted from concordance analysis because phenotypic resistance was determined based on MIC breakpoints defined specifically for non-cholera *Vibrio*. Streptomycin, chloramphenicol, ceftriaxone, ceftiofur, sulfisoxazole, and nalidixic acid were omitted from the analysis due to the lack of available breakpoints to determine phenotypic resistance, and ciprofloxacin was omitted because point mutations contributing to quinolone resistance were not assessed.

Results

Prevalence of Bacteria

The overall prevalence of *Vibrio* spp. in retail shrimp samples in this study was 60.25% (241/400). Farmed samples (78.44%, 211/269) tended to have higher *Vibrio* prevalence than wild caught

samples (45.80%, 60/131). *Vibrio* prevalence also tended to be higher in imported samples (71.61%, 227/317) than domestic samples (53.01%, 44/83). *Enterococcus* spp. were present in 89.75% of all samples (359/400), including 92.94% of farmed samples (250/269), 83.21% of wild-caught samples (109/131), 91.17% of imported samples (289/317), and 84.34% of domestically sourced samples (70/83) (Table 1).

Phenotypic Resistance from Antimicrobial Susceptibility Testing

The predominant antimicrobials that the 110 *Vibrio* isolates tested for phenotypic resistance were resistant to were ampicillin (47.27%, 52/110) and cefoxitin (35.45%, 39/110). Low prevalence of resistance was observed for tetracycline (9.09%, 10/110), trimethoprim-sulfamethoxazole (8.18%, 9/110), amoxicillin/clavulanic acid 2:1 ratio (2.73%, 3/110), gentamicin (1.82%, 2/110), ciprofloxacin (0.91%, 1/110), and azithromycin (0%, 0/110) (Table 2). The number of *Vibrio* isolates with MIC values below the lowest concentration and above the highest concentration tested for those drugs in the Gram-negative panel are reported in Supplementary Table 2. Multidrug resistance was observed in 8.18% (9/110) of *Vibrio* isolates. A further 21.82% (24/110) were resistant to two antimicrobial classes, 35.45% (39/110) were resistant to one class, and the remaining 34.55% (38/110) were pansusceptible (Table 3).

Table 1. Prevalence of *Vibrio* spp. and *Enterococcus* spp. in retail shrimp samples.

Variable	*Vibrio* spp. prevalence % (n/N)	*Enterococcus* spp. prevalence % (n/N)
Production Type		
Wild caught	22.00% (53/241)[a]	30.36% (109/359)[c]
Farmed	78.00% (188/241)[a]	69.64% (250/359)[c]
Country of origin		
Argentina	2.49% (6/241)	6.41% (23/359)
Bangladesh	0.41% (1/241)	0.84% (3/359)
Canada	0.00% (0/241)	0.28% (1/359)
Ecuador	9.96% (24/241)	6.13% (22/359)
India	32.36% (78/241)	31.75% (114/359)
Indonesia	21.58% (52/241)	19.50% (70/359)
Mexico	4.56% (11/241)	5.01% (18/359)
Saudi Arabia	1.24%(3/241)	0.56% (2/359)
Thailand	7.88% (19/241)	5.57% (20/359)
USA	16.60% (40/241)	19.50% (70/359)
Vietnam	2.48% (6/241)	4.18% (15/359)
Not specified	0.41% (1/241)	0.28% (1/359)
Product source		
Domestic	16.60% (40/241)[b]	19.50% (70/359)
Imported	83.40% (201/241)[b]	47.63% (171/359)
Month of sample purchase		
Sep 2019	10.37% (25/241)	10.58% (38/359)
Oct 2019	15.76% (38/241)	15.04% (54/359)
Nov 2019	11.62% (28/241)	12.53% (45/359)
Dec 2019	14.52% (35/241)	12.53% (45/359)
Jan 2020	9.96% (24/241)	8.08% (29/359)
Feb 2020	7.47% (18/241)	5.85% (21/359)
May 2020	19.92% (48/241)	24.51% (88/359)
Jun 2020	10.37% (25/241)	10.86% (39/359)
Total	**60.25% (241/400)**	**89.75% (359/400)**

[a]*Vibrio* prevalence differed significantly between wild caught and farmed shrimp samples (P = 5.62e-11).

[b]*Vibrio* prevalence differed significantly between domestic and imported shrimp samples (P = 2.33e-5).

[c]*Enterococcus* prevalence differed significantly between wild caught and farmed shrimp samples (P = 2.6e-3).

Table 2. Distribution of *Vibrio* isolates resistant to antimicrobial agents.

Antimicrobial class	Antimicrobial agent	Number of resistant isolates	*Vibrio* resistance (%)
Aminoglycoside	Gentamicin	2	1.82
	Streptomycin	*	*
Phenicol	Chloramphenicol	*	*
Beta-lactam	Amoxicillin/clavulanic acid 2:1 ratio	3	2.73
Cephem	Cefoxitin	39	35.45
	Ceftriaxone	*	*
	Ceftiofur	*	*
Folate pathway antagonist	Sulfisoxazole	*	*
	Trimethoprim/sulfamethoxazole	9	8.18
Macrolide	Azithromycin	0	0
Penicillin	Ampicillin	52	47.27
Quinolone	Ciprofloxacin	1	0.91
	Nalidixic Acid	*	*
Tetracycline	Tetracycline	10	9.09

*Drugs in which resistance could not be determined due to lack of breakpoints

Table 3. Distribution of phenotypic resistant patterns of *Vibrio* isolates (n=110).

Resistance Pattern	**No. of isolates with pattern n/N (%)**	**Drug Classes**
AMP	21/110 (19.09%)	penicillins
AMP-FOX	20/110 (18.18%)	cephems, penicillins
FOX	11/110 (10.00%)	cephems
TET	6/110 (5.45%)	tetracyclines
FOX-SXT-AMP*	4/110 (3.64%)	cephems, penicillins, folate pathway antagonists
AMP-AUG2-FOX-SXT*	1/110 (0.91%)	beta-lactams, cephems, penicillins, folate pathway antagonists
AMP-AUG2-FOX-GEN*	1/110 (0.91%)	aminoglycosides, beta-lactams, cephems, penicillins
AMP-AUG2-FOX*	1/110 (0.91%)	beta-lactams, cephems, penicillins
AMP-SXT-GEN*	1/110 (0.91%)	aminoglycosides, penicillins, folate pathway antagonists
AMP-SXT-TET*	1/110 (0.91%)	penicillins, folate pathway antagonists, tetracyclines
AMP-SXT	1/110 (0.91%)	penicillins, folate pathway antagonists
AMP-TET	1/110 (0.91%)	penicillins
CIP-TET	1/110 (0.91%)	quinolones, tetracyclines
FOX-TET	1/110 (0.91%)	cephems, tetracyclines
SXT	1/110 (0.91%)	folate pathway antagonists
Pansusceptible	38/110 (34.55%)	-

*Patterns indicating multidrug resistance (resistance to three or more antimicrobial classes)

Abbreviations: GEN gentamicin, STR streptomycin, AUG2 amoxicillin/clavulanic acid 2:1 ratio, FOX cefoxitin, AXO ceftriaxone, XNL ceftiofur, SXT trimethoprim/sulfamethoxazole, AZI azithromycin, AMP ampicillin, CHL chloramphenicol, CIP ciprofloxacin, NAL nalidixic acid, TET tetracycline

Antimicrobial susceptibility testing of *Enterococcus* isolates revealed high prevalence of resistance to lincomycin (96.36%, 106/110), quinupristin-dalfopristin (87.27%, 96/110), ciprofloxacin (84.55%, 93/110), linezolid (78.18%, 86/110), erythromycin (52.73%, 58/110), and chloramphenicol (39.09%, 43/110) (Table 4). Only one isolate was pansusceptible to all drugs in the MIC panel and all other isolates exhibited resistance to at least one of these six drugs in addition to various combinations of the other drugs in the panel. Low levels of resistance were found for tetracycline (15.45%, 17/110), tylosin tartrate (13.64%, 15/110), nitrofurantoin (9.09%, 10/110), gentamicin (2.73%, 3/110), tigecycline (1.82%, 2/110), kanamycin (0.91%, 1/110), penicillin (0.91%, 1/110), vancomycin (0.91%, 1/110), daptomycin (0%, 0/110), and streptomycin (0%, 0/110). Of these *Enterococcus* isolates, 93.64% (103/110) were multidrug resistant, 3.64% (4/110) were resistant to two classes of antimicrobials, 1.82% (2/110) were resistant to one class, and 0.91% (1/110) were pansusceptible. The phenotypic resistance patterns of *Enterococcus* isolates were diverse, though half exhibited one of four patterns involving chloramphenicol (CHL), ciprofloxacin (CIP), erythromycin (ERY), lincomycin (LIN), linezolid (LZD), and quinupristin-dalfopristin (SYN): CIP-ERY-LIN-LZD-SYN (14.55%, 16/110), CIP-LIN-LZD-SYN (14.55%, 16/110), CHL-CIP-ERY-LIN-LZD-SYN (12.73% (14/110), and CHL-CIP-LIN-LZD-SYN (8.18%, 9/110) (Table 5).

Table 4. Distribution of *Enterococcus* isolates resistant to antimicrobial agents.

Antimicrobial class	Antimicrobial agent	Number of resistant isolates	*Enterococcus* resistance (%)
Aminoglycoside	Streptomycin	0	0
	Kanamycin	1	0.91
	Gentamicin	3	2.73
Phenicol	Chloramphenicol	43	39.09
Glycopeptide	Vancomycin	1	0.91
Lincosamide	Lincomycin	106	96.36
Lipopeptide	Daptomycin	0	0
Macrolide	Tylosin tartrate	15	13.64
	Erythromycin	58	52.73
Nitrofuran	Nitrofurantoin	10	9.09
Oxazolidinone	Linezolid	86	78.18
Penicillin	Penicillin	1	0.91
Quinolone	Ciprofloxacin	93	84.55
Streptogramin	Quinupristin / dalfopristin	96	87.27
Tetracycline	Tigecycline	2	1.82
	Tetracycline	17	15.45

Table 5. Distribution of phenotypic resistant patterns of *Enterococcus* isolates (n=110).

Resistance Pattern	No. of Isolates with Pattern n/N (%)	Drug classes
CIP-ERY-LIN-LZD-SYN*	16/110 (14.55%)	macrolides, lincosamides, oxazolidinones, quinolones, streptogramins
CIP-LIN-LZD-SYN*	16/110 (14.55%)	lincosamides, oxazolidinones, quinolones, streptogramins
CHL-CIP-ERY-LIN-LZD-SYN*	14/110 (12.73%)	lincosamides, macrolides, oxazolidinones, phenicols, quinolones, streptogramins
CHL-CIP-LIN-LZD-SYN*	9/110 (8.18%)	lincosamides, oxazolidinones, phenicols, quinolones, streptogramins
CHL-CIP-ERY-LIN-LZD-SYN-TYLT*	3/110 (2.72%)	lincosamides, macrolides, oxazolidinones, phenicols, quinolones, streptogramins
CHL-CIP-ERY-LIN-SYN*	3/110 (2.72%)	lincosamides, macrolides, phenicols, quinolones, streptogramins
CIP-ERY-LIN-SYN*	3/110 (2.72%)	lincosamides, quinolones, streptogramins
CIP-LIN-SYN*	3/110 (2.72%)	lincosamides, quinolones, streptogramins
CHL-CIP-ERY-LIN-LZD-TET*	2/110 (1.81%)	lincosamides, macrolides, oxazolidinones, phenicols, quinolones, tetracyclines
CIP-ERY-LIN-LZD-SYN-TYLT*	2/110 (1.81%)	lincosamides, macrolides, oxazolidinones, quinolones, streptogramins
CHL-ERY-LIN-LZD-SYN-TET*	2/110 (1.81%)	lincosamides, macrolides, oxazolidinones, phenicols, streptogramins, tetracyclines
CIP-LIN-LZD-SYN-TET*	2/110 (1.81%)	lincosamides, oxazolidinones, quinolones, streptogramins, tetracyclines

ERY-LIN-LZD-SYN*	2/110 (1.81%)	lincosamides, macrolides, oxazolidinones, streptogramins
CHL-CIP-LIN-NIT*	2/110 (1.81%)	lincosamides, nitrofurans, phenicols, quinolones
LIN-LZD-SYN*	2/110 (1.81%)	lincosamides, oxazolidinones, streptogramins
LIN-TET	2/110 (1.81%)	lincosamides, tetracyclines
CHL-CIP-ERY-GEN-KAN-LIN-NIT-SYN-TET-TYLT*	1/110 (0.91%)	aminoglycosides, lincosamides, macrolides, nitrofurans, phenicols, quinolones, streptogramins, tetracyclines
CHL-CIP-ERY-LIN-LZD-SYN-TET-TGC*	1/110 (0.91%)	lincosamides, macrolides, oxazolidinones, phenicols, quinolones, streptogramins, tetracyclines
CIP-ERY-LIN-LZD-NIT-SYN-TET-TYLT*	1/110 (0.91%)	lincosamides, macrolides, nitrofurans, oxazolidinones, quinolones, streptogramins, tetracyclines
CHL-CIP-ERY-LIN-LZD-SYN-TGC*	1/110 (0.91%)	lincosamides, macrolides, oxazolidinones, phenicols, quinolones, streptogramins, tetracyclines
CHL-CIP-GEN-LIN-LZD-SYN-TYLT*	1/110 (0.91%)	aminoglycosides, lincosamides, macrolides, oxazolidinones, phenicols, quinolones, streptogramins
CHL-CIP-LIN-LZD-SYN-TET*	1/110 (0.91%)	lincosamides, oxazolidinones, phenicols, quinolones, streptogramins, tetracyclines
CHL-CIP-ERY-LIN-SYN-TYLT*	1/110 (0.91%)	lincosamides, macrolides, phenicols, quinolones, streptogramins
CHL-CIP-LIN-LZD-SYN-TYLT*	1/110 (0.91%)	lincosamides, macrolides, oxazolidinones, phenicols, quinolones, streptogramins
CHL-LIN-LZD-NIT-SYN-TYLT*	1/110 (0.91%)	lincosamides, macrolides, nitrofurans, oxazolidinones, phenicols, streptogramins
CIP-ERY-LIN-LZD-NIT-SYN*	1/110 (0.91%)	lincosamides, macrolides, oxazolidinones, quinolones, streptogramins
CIP-GEN-LIN-NIT-TET*	1/110 (0.91%)	aminoglycosides, lincosamides, nitrofurans, quinolones, tetracyclines
CIP-LIN-LZD-SYN-TET*	1/110 (0.91%)	lincosamides, oxazolidinones, quinolones, streptogramins, tetracyclines
CIP-LIN-LZD-SYN-TYLT*	1/110 (0.91%)	lincosamides, macrolides, oxazolidinones, quinolones, streptogramins
CIP-LIN-NIT-TET-VAN*	1/110 (0.91%)	glycopeptides, lincosamides, nitrofurans, quinolones, tetracyclines
ERY-LIN-LZD-SYN-TYLT*	1/110 (0.91%)	lincosamides, macrolides, oxazolidinones, streptogramins
ERY-LIN-NIT-TET-TYLT*	1/110 (0.91%)	lincosamides, macrolides, nitrofurans, tetracyclines
LIN-LZD-SYN-TET-TYLT*	1/110 (0.91%)	lincosamides, macrolides, oxazolidinones, tetracyclines
CIP-ERY-LZD-SYN*	1/110 (0.91%)	macrolides, oxazolidinones, quinolones, streptogramins
ERY-LZD-PEN-SYN*	1/110 (0.91%)	macrolides, oxazolidinones, penicillins, streptogramins
LIN-LZD-NIT-SYN*	1/110 (0.91%)	lincosamides, nitrofurans, oxazolidinones, streptogramins
CIP-LIN-NIT*	1/110 (0.91%)	lincosamides, nitrofurans, quinolones
CIP-LIN-STR*	1/110 (0.91%)	aminoglycosides, lincosamides, quinolones
CIP-LIN	1/110 (0.91%)	lincosamides, quinolones
LIN-LZD	1/110 (0.91%)	lincosamides, oxazolidinones
CIP	1/110 (0.91%)	quinolones
LIN	1/110 (0.91%)	lincosamides
Pansusceptible	1/110 (0.91%)	-

*Patterns indicating multidrug resistance (resistance to three or more antimicrobial classes)

Analysis of Antimicrobial Resistance by Shrimp Sample Metadata

For analysis, origin was collapsed to domestic or imported categories due to small sample size by country. Similarly, season of collection was excluded due to small sample sizes by season. However, country of origin and time of collection are listed in Table 1. Packaging claims were excluded because few samples included claims about antimicrobial use. All claims that were found came in the form of the Global Seafood Alliance's Best Aquaculture Practices (BAP) certifications, which mandate veterinary and regulatory oversight of antimicrobial usage and prohibit the use of drugs for growth promotion (Best Aquaculture Practices, 2014). The eight farmed *Vibrio* isolates from packages with BAP certifications averaged resistance to 1.88 drugs, which was more (P = 0.015) than the 0.96 average of the 73 isolates without the certification. Thirteen *Enterococcus* isolates were sourced from farmed shrimp samples with BAP certifications, and their average resistance to 5.31 drugs did not significantly differ (P = 0.212) from the average of the 4.79 drug average for the 68 uncertified. Shrimp species was excluded as a variable for analysis because the majority of samples were whiteleg shrimp (57.0%, 228/400) or did not specify species (21.75%, 87/400), and the remainder consisted of nine different species, which limited the ability to make comparisons between samples.

No significant differences in prevalence of multidrug resistance were found in *Vibrio* or *Entercoccus* isolates (Table 6). *Vibrio* isolates from farm raised shrimp were multidrug resistant 8.64% (7/81) of the time, compared to 6.90% (2/29) for those sourced from wild caught shrimp (P = 1.0). Of *Enterococcus* isolates from farm raised shrimp samples, 95.1% (77/81) were resistant to at least one of the sixteen antimicrobial agents, compared to 89.66% (26/29) from wild caught shrimp samples (P = 0.377). No significant differences in prevalence of multidrug resistance were found between farm raised and wild caught production, either (Table 6). Domestic and imported

Vibrio isolates were multidrug resistant 5.26% (1/19) and 8.79% (8/91) of the time, respectively (P = 1.0). *Enterococcus* isolates from domestically produced shrimp were multidrug resistant 88.89% (16/18) of the time, while those from imported shrimp were multidrug resistant 94.57% (87/92) of the time (P = 0.321).

Table 6. Fisher's exact test of antimicrobial resistance in *Vibrio* and *Enterococcus* from retail shrimp.

		Vibrio		***Enterococcus***	
		No. MDR	No. not MDR	No. MDR	No. not MDR
Production Method	Farm raised	7	74	77	4
	Wild-caught	2	27	26	3
		P = 1.0		*P* = 0.3773	
Origin	Domestic	1	18	16	2
	Imported	8	83	87	5
		P = 1.0		*P* = 0.3214	

Vibrio Species Identification and Metadata Trends via Whole Genome Sequencing (WGS)

Ten of the 52 sequences submitted for WGS were excluded from subsequent analysis because one or both of species and ARG presence could not be determined by the PulseNet. For the other 42 isolates, the prevalence of species and resistance genes alongside metadata characteristics are summarized in Table 7. The most common *Vibrio* species identified by WGS was *V. metschnikovii* (24/42; 57.14%), followed by *V. parahaemolyticus* (12/42; 28.57%), *V.*

alginolyticus (3/42; 7.14%), *V. cholerae* (2/42; 4.76%), and *V. fluvialis* (1/42; 2.33%). All seven domestic isolates subjected to WGS were speciated as *V. metschnikovii*, while the majority of imported isolates were either *V. metschnikovii* (17/35; 48.57%) or *V. parahaemolyticus* (12/35; 34.29%). The domestic isolates averaged 0.29 ARGs, compared to 1.63 ARGs on average for imported isolates (Table 8). While the WGS subsample included twice as many isolates sourced from farmed shrimp than wild caught shrimp, the bacterial species compositions within the groups were similar to each other and to the full sample selected for AST.

Table 7. Distribution and characteristics of *Vibrio* isolates (n=42) by species.

Species	No. of isolates *n*/*N* (%)	Average no. ARGs	Wild caught (%)	Farmed (%)	Domestic (%)	Imported (%)
V. metschnikovii	24/42 (57.14%)	0.71	37.50	62.50	29.17	70.83
V. parahaemolyticus	12/42 (28.57%)	3.00	25.00	75.00	0.00	100.00
V. alginolyticus	3/42 (7.14%)	0.33	33.33	66.67	0.00	100.00
V. cholerae	2/42 (4.76%)	2.50	0.00	100.00	0.00	100.00
V. fluvialis	1/42 (2.38%)	0.00	100.00	0.00	0.00	100.00

Table 8. Distribution of ARG abundance and *Vibrio* species by production type and source.

Production type/source	No. of isolates *n*/*N* (%)	Avg no. ARGs	*V. metschnikovii* (%)	*V. parahaemolyticus* (%)	*V. alginolyticus* (%)	*V. cholerae* (%)	*V. fluvialis* (%)
Farmed	28/42 (66.67%)	0.93	53.57	32.14	7.14	7.14	0.00
Wild caught	14/42 (33.33%)	2.36	64.29	21.43	7.14	0.00	7.14
Domestic	7/42 (16.67%)	0.29	100.00	0.00	0.00	0.00	0.00
Imported	35/42 (83.33%)	1.63	48.57	34.29	8.57	5.71	2.86

Resistance Gene Identification via WGS

Whole genome sequencing identified 27 unique ARGs from the 42 *Vibrio* isolates. Among these resistance genes were genes corresponding to two types of aminoglycoside-modifying enzymes (AMEs), phosphotransferases (*aph(3')-Ia*, *aph(3'')-Ib*, and *aph(6)-Id*) and adenylyltransferases (*aph(2'')-Ia*). Nine unique bla_{CARB} and one bla_{VEB} ARGs were found, which are associated with resistance to beta-lactam agents including penicillins. Four ARGs associated with folate pathway antagonists were identified, three of which (dfr_{A1}, dfr_{A6}, and dfr_{A31}) are known to confer resistance to trimethoprim and one (*sul2*) known to confer resistance to sulfamethoxazole. Three ARGs encoding for tetracycline efflux pumps, two pentapeptide genes conferring resistance to quinolones, one chloramphenicol efflux pump gene, and one macrolide inactivation gene were also present in this subsample of *Vibrio* isolates. Two genes were identified that confer resistance to rifamycins, a drug class not represented on the Gram-negative panel. Cephems were the only class on the panel for which no ARGs were identified. The frequencies at which these ARGs were observed are visualized in Figure 1.

V. parahaemolyticus isolates tended to have more ARGs than other species, averaging 3.00 ARGs per isolate. They were the only isolates to contain resistance genes associated with beta-lactams including penicillins. Sixteen unique resistance genes were identified in one *V. parahaemolyticus* isolate from a wild caught shrimp originating from Vietnam. This was also the only isolate with rifamycin and macrolide resistance genes and was one of only two with aminoglycoside resistance genes. All five ARGs identified from *V. metschnikovii* isolates corresponded to either quinolones or folate pathway antagonists.

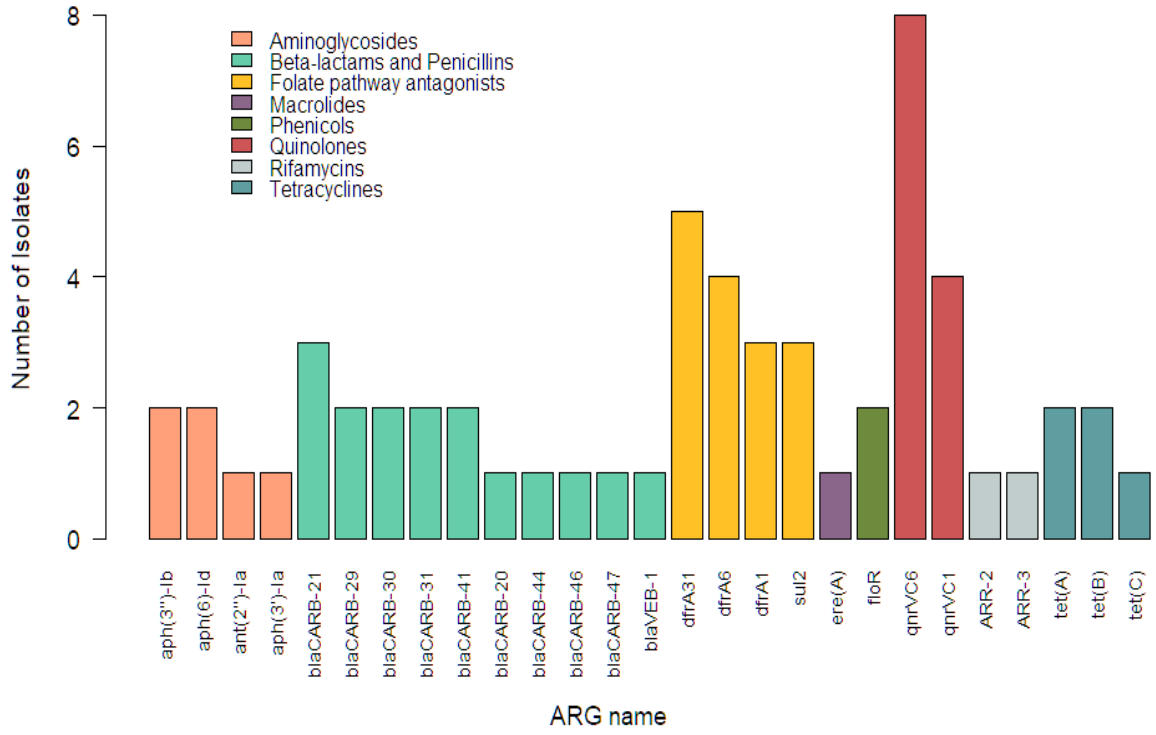

Figure 1. Distribution of antimicrobial resistance genes in *Vibrio* isolates (n=42) from retail shrimp.

Farmed isolates (28/42; 66.67%) contained between zero and five ARGs (mean = 0.93), while wild caught isolates (14/42; 33.33%) contained between zero and sixteen (mean = 2.36). The quinolone ARG *qnrVC6* was the predominant resistance gene identified in *Vibrio* isolates from farmed (4/28; 14.29%), wild caught (4/14; 28.57%), and imported shrimp. Only two ARGs were identified within the seven domestic isolates, *qnrVC6* (1/7; 14.29%) and *dfrA1* (1/7; 14.29%) (Figure 2).

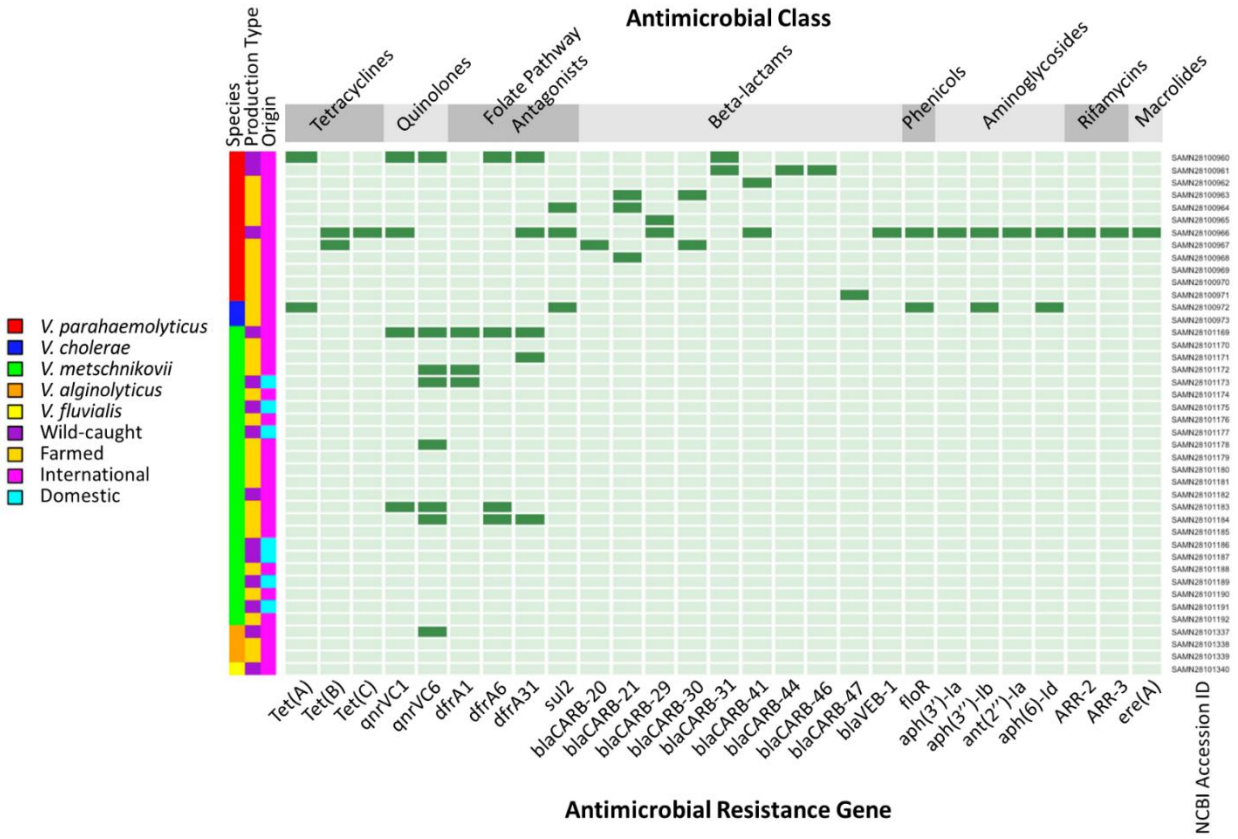

Figure 2. Heatmap of antimicrobial resistance genes (ARGs) identified through whole-genome sequencing in *Vibrio* isolates from retail shrimp. Dark green indicates the presence of an ARG and light green indicates the absence of an ARG.

Concordance of Phenotypic and Genotypic Resistance in Vibrio Isolates

Comparing phenotypic AMR and resistance genes identified from WGS in 42 non-cholera *Vibrio* isolates, the overall sensitivity and specificity were determined to be 11.54% and 96.05%, respectively. Discrepancies were observed in all drugs assessed; for each, there was at least one isolate that was categorized as phenotypically resistant but did not harbor any corresponding ARGs. None of the 17 isolates categorized as phenotypically resistant (3 resistant and 14 intermediate isolates) to cefoxitin harbored any associated ARGs. Of the four isolates categorized

as phenotypically resistant (3 resistant and 1 intermediate) to trimethoprim/sulfamethoxazole, only one had ARGs associated with both component drugs. The majority of the false positives that contributed to low specificity were for ampicillin (ampicillin associated ARGs were found in 7 of the 15 phenotypically susceptible isolates) (Table 9).

Table 9. Concordance of phenotypic and genotypic resistance of non-cholera *Vibrio* isolates from retail shrimp (n=40). Streptomycin, chloramphenicol, ceftriaxone, ceftiofur, sulfisoxazole, and nalidixic acid were omitted from analysis due to lack of available breakpoints to determine phenotypic resistance.

Antimicrobial class	Antimicrobial agent	Phenotypically susceptible (# isolates)		Phenotypically resistant (# isolates)		Sensitivity (%)[b]	Specificity (%)[c]
		Genotype: resistant (FP)[a]	Genotype: susceptible (TN)[a]	Genotype: resistant (TP)[a]	Genotype: susceptible (FN)[a]		
Aminoglycoside	GEN	1	37	0	2	0	97.37
Beta-lactam	AUG2	0	39	0	1	0	100
Cephem	FOX	0	23	0	17	0	100
	SXT	0	36	1	3	25.00	100
Macrolide	AZI	0	40	0	0	N/A[d]	0
Penicillin	AMP	7	8	3	22	12.00	53.33
Tetracycline	TET	1	36	2	1	66.67	97.30
Overall		9	219	6	46	11.54	96.05

[a]FP = false positive, TN = true negative, TP = true positive, FN = false negative
[b]Sensitivity was calculated as TP/(TP+FN)
[c]Specificity was calculated as TN/(TN+FP)
[d]Sensitivity could not be calculated for azithromycin due to lack of phenotypic resistance.
Abbreviations: GEN gentamicin, AUG2 amoxicillin/clavulanic acid 2:1 ratio, FOX cefoxitin, SXT trimethoprim/sulfamethoxazole, AZI azithromycin, AMP ampicillin, TET tetracycline

Discussion

Prevalence of Vibrio spp. and Enterococcus spp. in Retail Shrimp

This study found *Vibrio* prevalence of 60.25% (241/400) in retail shrimp meat samples of different production types and geographic origins in northern California. Other studies from around the world have reported widely varying prevalences of *Vibrio* spp. in shrimp samples, ranging from 17.1% in Iran to 88.1% in Mexico and 95.6% in Ecuador (Sperling et al., 2015; Aspargoor et al., 2018; Guardiola-Avila et al., 2020). The most directly comparable recent assessment to ours was conducted in 2022 by Tate et al. who found 40.85% (290/710) prevalence of *Vibrio* spp. in United States retail shrimp samples. In this context, the 60.25% *Vibrio* spp. prevalence we found in our 400 retail shrimp samples is not anomalous.

We observed *Enterococcus* spp. prevalence to be 89.75% (359/400) in our shrimp samples. *Enterococcus* spp. are ubiquitous bacteria common in aquatic environments and the overall prevalence in our samples, while high, was in line with expectations. Our prevalence observations were similar to those of other recent studies that measured *Enterococcus* spp. in samples of retail shrimp meat, which ranged from 58.33% prevalence in shrimp imported to grocery stores in northeastern Poland, to 66% in shrimp samples from American grocery stores, to 84.7% in shrimp imported to Denmark (Chajęcka-Wierzchowska et al., 2016; Ellis-Iverson et al., 2020; Tate et al., 2022).

Whole genome sequencing is a powerful tool in both laboratory and clinical settings and has become popular in the surveillance of foodborne pathogens for its utility in identifying the microbial species present in samples (Grundman, 2014; Köser et al., 2014). In this study we employed WGS to determine the species of a subset of 42 *Vibrio* isolates and identify the diversity

of ARGs within their genomes. While *V. cholerae*, as the causative agent of epidemic cholera, and *V. parahaemolyticus* and *V. alginolyticus*, as two of the most common causative agents of foodborne illness globally, are the species most associated with human infections that were found among our isolates, all of the species identified are among the twelve known to be associated with human infections (Morris & Acheson, 2003). The most prevalent *Vibrio* species we isolated was *V. metschnikovii* (57.14%; 24/42), followed by *V. parahaemolyticus* (28.57%; 12/42), *V. alginolyticus* (7.14%; 3/42), *V. cholerae* (4.76%; 2/42), and *V. fluvialis* (2.38%; 1/42). By comparison, in a 2011 assessment of *Vibrio* prevalences in shrimp samples in Baton Rouge, Louisiana, USA, Wang et al. observed *V. cholerae* in 17.8%, *V. mimicus* in 6.63%, *V. parahaemolyticus* in 4.57%, and other, unspecified *Vibrio* species in 21.1% of their samples.

Phenotypic Resistance of Vibrio and Enterococcus

The evaluation of *Vibrio* resistance was hindered by six drugs without defined CLSI or NARMS breakpoints on the 14-drug NARMS Gram-negative panel. One such drug was nalidixic acid, a quinolone. Quinolones are the most common class of antimicrobial agents used in aquaculture globally (Schar et al., 2020). Chloramphenicol also does not have defined breakpoints despite being highly relevant to aquaculture. It belongs to the third most commonly used class of antimicrobials in aquaculture globally, phenicols, and was the only drug in the panel approved for aquacultural use in the United States (Schar et al., 2020; FDA, 2022a).

The drugs that *Vibrio* isolates in this study were most commonly resistant to were ampicillin and cefoxitin, which is in line with previous assessments. Ampicillin resistance has been reported in *V. parahaemolyticus* and *V. vulnificus* since 1978 and 2001, respectively (Joseph et al.,

1978; Zanetti et al., 2001). The observed prevalence of *Vibrio* resistance to ampicillin in our study (40.91%; 45/110) is very similar to that observed by Akinbowale et al. (2006) in their analysis of *Vibrio* isolates collected from various aquacultural sources in Australia (40.32%, 25/62), but contrasts starkly with Raissy et al. in 2012 who found 97.2% (70/72) ampicillin resistance among *Vibrio* isolates from wild caught seafood. The prevalence of cefoxitin resistant *Vibrio* isolates (35.45%) in our study was similar to the findings of Garcia-Aljaro et al. (2014), who found 44% resistance in *Vibrio* spp. isolates from aquaculture facilities.

Antimicrobial susceptibility testing of *Enterococcus* isolates revealed resistance prevalence for multiple drugs, namely lincomycin (96.36%), quinupristin-dalfopristin (87.27%), ciprofloxacin (84.55%), linezolid (78.18%), erythromycin (52.73%), and chloramphenicol (39.09%). Interestingly, despite near-ubiquitous resistance to lincomycin among *Enterococcus* isolates in this assessment, its use in aquaculture has only been reported in China and none of our samples originated from China (Lulijwa et al., 2020). This could suggest exposure to lincomycin residues from non-aquaculture sources; lincomycin is commonly found in the waste streams of terrestrial livestock facilities and could enter surface waterways that feed directly into flow-through aquaculture systems like those that predominate shrimp farming (Boyd et al., 2022; Public Health Agency of Canada, 2022).

Co-selection of AMR, a phenomenon in which selective pressure upon exposure to one antimicrobial agent often results in the acquisition of resistance to other agents, could also have contributed to the high prevalence of lincomycin resistance (Seiler & Berendonk, 2012; Zhang et al., 2018; Imran et al., 2019). Some heavy metals, notably copper sourced from aquacultural and agricultural pollution, have been shown to cause co-selection of AMR in waterborne bacteria. Seiler and Berendonk (2012) found that exposure to high levels of copper resulted in bacterial

resistance to lincomycin, erythromycin, and vancomycin which persisted through the end of their seven day observation period. It's possible that the high rates of resistance to lincomycin observed in our assessment could have resulted from inadvertent exposure to metals such as copper.

It is also possible that the use of lincomycin in the countries of origin for these resistant isolates was unreported, or even inadvertent. Accurate tracking of antimicrobial use is a difficult endeavor that often involves non-governmental surveys which depend on the honesty and knowledge of producers, both of which can be unreliable (Shamsuzzaman & Biswas, 2012; Pham et al., 2015). Mislabeling of probiotic products in shrimp and other aquaculture has also introduced unintended and antimicrobial resistant bacteria into farms, which could be another explanation for the observed lincomycin resistance (Noor Uddin et al., 2015; Uma & Rebecca, 2018).

Ciprofloxacin is not used in aquaculture, yet many of our *Enterococcus* isolates from both farm raised and wild caught shrimp grew uninhibited in its presence (Thornber et al., 2020). Other studies have reported varied levels of ciprofloxacin resistance. Ellis-Iverson et al. (2020) found near-zero resistance to ciprofloxacin in *E. faecalis* and *E. faecium* isolates from Asian seafood imported to Denmark, while Igbinosa and Beshiru (2019) found more than 40% ciprofloxacin resistance in *Enterococcus* isolated from ready-to-eat seafood products. The prevalence observed in our study (84.55%), however, is abnormally high compared to levels of ciprofloxacin resistance in previous studies. Ciprofloxacin is classified as a critically important antimicrobial in human medicine by the World Health Organization (WHO), who cite its frequency of use and unique effectiveness against pathogenic infections as reasons that resistance could pose a significant risk to human health (WHO, 2016). While *Enterococcus* spp. are not among the pathogens of concern in this case, populations with prevalent resistance like those we observed are concerning as potential reservoirs of ARGs that could transfer to more significant pathogens.

Resistance of *Enterococcus* to linezolid has become increasingly common within the past decade, which is a growing concern in human medicine since it is used as a last resort treatment against vancomycin-resistant *Enterococcus* infections (Klare et al., 2015). Clinical studies have reported an increase in the rate of linezolid resistance among *E. faecium* samples over time, including <1% in 2008, >9% in 2014, and >20% in 2021 (Klare et al., 2015; Ma et al., 2021). Although the isolates in this assessment are not human pathogenic *E. faecalis*, the fact that 78.18% of them exhibited resistance to lincomycin and all have the capability of spreading that trait is notable. The pervasiveness of linezolid resistance in our assessment is also curious because the drug is not applied in aquaculture settings (Lulijwa et al., 2020). It is possible that this resistance could also have been acquired in a co-selection process. Pervasive, acquired resistance to a wide range of antimicrobial drugs in bacteria as adept at ARG transfer as *Enterococcus* poses a threat to human health.

Intrinsic resistance is a consideration when interpreting *Enterococcus* MIC results as well. Many *Enterococci* are known to be intrinsically resistant to aminoglycosides, although few samples were classified as resistant to streptomycin (1.82%), kanamycin (0.91%), or gentamicin (2.73%) in our susceptibility testing (Morrison et al., 1997; Harakeh et al., 2006). Some *Enterococcus* species also have unique resistances; *E. faecalis* is intrinsically resistant to streptogramins like quinupristin-dalfopristin, for example, and *E. gallinarum* and *E. casseliflavus* are intrinsically resistant to vancomycin (Morrison, 1997; Arias & Murray, 2012; Ellis-Iversen et al., 2020). These species-specific traits could not be considered in our assessment, however, since no *Enterococcus* isolates were identified beyond genus level.

Multidrug Resistance Patterns in Vibrio and Enterococcus

Prevalence of MDR in aquaculture-sourced *Vibrio* has increased in the 21st century (Han et al., 2007; Baker-Austin et al., 2009; Raissy et al., 2012; Shaw et al., 2014; Igbinosa, 2016). However, recent studies have varied substantially in their MDR observations. The prevalence of MDR for our *Vibrio* isolates (8.18%) was similar to the rates observed in aquacultured shrimp-associated bacteria by Singh et al. (2018) in their study in Punjab, India (8.4%; 10/119), and Helena Rebouças et al. (2011) conducted in northeastern Brazil (12.9%; 4/31). By contrast, Costa et al. (2015b) found that none of their 100 *Virbio* isolates from farmed shrimp in Brazil were resistant to three or more of the nine drug classes in their MIC panel, while a 2016 analysis of *Vibrio* spp. sampled from Nigerian aquaculture farms (Igbinosa, 2016) found that 57.49% of isolates (96/167) were resistant to at least three of the eight classes of antimicrobial drugs they tested. While these comparisons are valuable to contextualize the results of this assessment, it should be noted that there is variation in the composition of the drug panels between studies. The studies referenced above feature a similar number and identity of drugs and drug classes to those of our assessment, however. Moreover, the resolution of our findings was hampered by the omission of six drugs from the Gram-negative panel; many other studies assessing *Vibrio* spp. included multiple drugs within one or more classes, whereas our panel had only one representative per class among interpretable drugs.

We observed 93.64% MDR among *Enterococcus* isolates. This was driven in large part by pervasive resistance to lincomycin, quinupristin-dalfopristin, ciprofloxacin, linezolid, erythromycin, and chloramphenicol. Other recent studies have reported similar rates of MDR driven by ubiquitous or near-ubiquitous resistance to a subset of drugs. Enany et al. (2022), for instance, found that all of their 72 aquaculture-sourced *Enterococcus* isolates were resistant to

chloramphenicol, macrolides azithromycin, and erythromycin. Further, 91.6% (66/72) of their isolates were resistant to tetracycline, and all exhibited resistance or intermediate resistance to nitrofurantoin. Generally, there has been a growing trend of MDR *Enterococcus* which is a concern in clinical circles, and the results of this study reinforce that pattern (Klare et al., 2015).

Multidrug Resistance of Vibrio and Enterococcus by Shrimp Sample Metadata

The metadata analyses in the present assessment found surprising and notable results. No significant difference was found in the prevalence of multidrug resistance between isolates sourced from farm raised or wild caught shrimp for *Vibrio* ($P = 1.0$) or *Enterococcus* ($P = 0.377$). This result was unexpected because shrimp raised in a farm environment are likely to be directly exposed to antimicrobial drugs which would apply selective pressure and intuitively result in higher rates of resistance. Antimicrobial agents including many of those included on the MIC panels in this assessment have increasingly been found at detectable concentrations in coastal and estuarine ecosystems where wild shrimp are fished, and even diffuse, subinhibitory concentrations have been shown to select for AMR in environmental and shrimp-associated bacteria (Gullberg et al., 2011; Zheng et al., 2021). One other possibility is that the wild caught samples as a group were contaminated between capture and sale in a way that farmed samples were not. Still, our results imply that wild caught shrimp do not pose lower risk than farm raised shrimp of spreading AMR.

There was also no significant difference in MDR prevalence found between isolates sourced from domestically produced or imported shrimp for *Vibrio* ($P = 1.0$) or *Enterococcus* ($P = 0.321$). All domestic samples were labeled as wild caught at collection, but since no statistical difference was found between farmed and wild caught isolates, that should not affect the

interpretation of this result. The similarity between samples of different geographic origins could indicate that the United States' import monitoring has been successful in holding imported seafood to the same antimicrobial stewardship standards as domestic seafood, or that there is some overlap in the processing or distribution processes that facilitates cross-contamination of bacteria before all shrimp of any origin reach grocery store shelves (FDA, 2022b).

Results of WGS in Vibrio Isolates and Correspondence Between Genotypic and Phenotypic Resistance

Whole genome sequencing with a subset of 42 *Vibrio* isolates was performed after antimicrobial susceptibility testing to identify species and ARGs. The WGS revealed that two of the 42 isolates were *V. cholerae*, and it's possible others among the non-sequenced isolates were as well. This complicates the interpretation of the results of the MIC analysis, because, as noted in results section 3.6, the breakpoints used to classify the MIC values were specifically defined for non-cholera *Vibrio*. The confirmed *V. cholerae* isolates were still considered in the MIC analysis since the identities of those not yet sequenced are unknown. If possible, sequencing isolates before MIC testing would help ensure that this uncertainty doesn't arise in future assessments. The most common ARGs found in this assessment were *qnr* genes which encode for pentapeptide repeat proteins and confer reduced susceptibility to quinolones. Resistance to quinolones is primarily mediated by chromosomal quinolone resistance determining region (QRDR) mutations, though, and secondarily by acquired plasmid-mediated quinolone resistance (PMQR) genes like *qnrVC1* and *qnrVC6* (Zhang et al., 2018; Esmaeel et al., 2020; Lee et al., 2022). Still, acquired ARGs impart partial resistance to quinolones on their own and remain dangerous since they are highly

transmissible to other organisms and contribute to the selection of resistance-associated chromosomal mutations (Nazik et al., 2011). The raw MIC values for ciprofloxacin and nalidixic acid did not suggest that isolates with one or both of *qnrVC1* and *qnrVC6* had reduced phenotypic susceptibility to quinolones. Considering only 0.91% of *Vibrio* isolates were classified as resistant to ciprofloxacin, this likely indicates that few QRDR mutations were present in this subsample.

More unique ARGs were found related to beta-lactam and penicillin resistance than any other antimicrobial classes. Nine of the ten such genes were bla_{CARB} ARGs. The beta-lactamase protein encoded by this class of genes is a major mechanism of resistance to beta-lactam agents in *Vibrio* spp. and beyond (Potron et al., 2009; Manjusha & Bhat, 2011; Li et al, 2020). The bla_{CARB} ARGs identified in this study are almost exclusively found in *V. parahaemolyticus*, as they were herein; $bla_{VEB\text{-}1}$, the other beta-lactamase ARG found in one of our isolates, was found in *V. parahaemolyticus* in this assessment but has also been observed in *V. alginolyticus* (Alcock et al., 2020).

Genotypic resistance to the trimethoprim/sulfamethoxazole combo agent would require ARGs for both drugs to be present, though only one still confers partial resistance (Suhartono et al., 2016; Das et al., 2020; Ma et al., 2021). Among the seven isolates with one or more allelic variants of the dfr_A trimethoprim ARG without any sulfamethoxazole ARGs, there was a trend between number of unique variants and resistance to trimethoprim/sulfamethoxazole (SXT). Of the four isolates with three dfr_A variants, two expressed phenotypic resistance. In addition, of the two isolates with two dfr_A variants, one expressed intermediate resistance; the one isolate with one dfr_A variant was phenotypically susceptible to SXT. Both isolates with sulfamethoxazole-associated *sul2* gene and no trimethoprim ARGs were susceptible to the combination agent. The

other 32 isolates devoid of folate pathway antagonist ARGs were susceptible to SXT in MIC analysis.

One isolate with sixteen ARGs was the only with both a trimethoprim ARG (*drf*$_{A31}$) and a sulfamethoxazole ARG (*sul2*) and was phenotypically resistant to SXT. Among the other ARGs this isolate contained was aminoglycoside adenylyltransferase ARG *ant(2'')-Id*, which is known to confer resistance to gentamicin, though the isolate did not express phenotypic resistance to this drug in MIC testing (Ramirez & Tomalsky, 2010). Two aminoglycoside phosphotransferase ARGs, *aph(3'')-Ib* and *aph(6)-Id*, were also among the sixteen. These genes have been observed colocalized with *sul2* and other ARGs on RSF1010, an oft-transmitted plasmid (Ramirez & Tomalsky, 2010). Another isolate, a *V. cholerae* isolate from a farmed Ecuadorian shrimp, also contained *sul2*, *aph(3'')-Ib* and *aph(6)-Id*. Regardless of whether the RSF1010 plasmid is present in these isolates, the possibility highlights the high transfer potential of the ARGs identified in this study.

Tetracyclines are highly important agents for human and veterinary medicine (WHO, 2016). The tetracycline ARGs identified in this study are frequently found in bacterial genomes isolated from aquatic environments like aquaculture ponds and from crustaceans (Schmidt et al., 2001; Dang et al., 2007; Zhang et al., 2009; Kim et al., 2013). Their presence in this study means they could be spread to significant pathogens and complicate treatment in clinical settings.

Analyses of AMR should account for intrinsic resistances in the bacteria of interest (Kathleen et al., 2016). It has been shown that *V. vulnificus* and *V. parahaemolyticus* are intrinsically resistant to cefoxitin (Elmahdi et al., 2016). The twelve *V. parahaemolyticus* isolates identified by our WGS, however, did not reflect this pattern: 11/12 (91.67%) were inhibited at

cefoxitin concentrations low enough to classify them as susceptible, and the other one (8.33%) was classified as intermediate. Regardless, since not all 110 isolates subjected to antimicrobial susceptibility testing were sequenced, this would not have been accounted for in statistical analyses even if the intrinsic resistance was observed.

Chiou et al. (2015) posited that *V. parahaemolyticus* intrinsically carries the $bla_{CARB-17}$ gene which confers resistance to ampicillin, however only two of the twelve isolates identified in our study (16.67%) were phenotypically resistant to ampicillin and one (8.33%) expressed intermediate resistance. By contrast, among the other 28 non-cholera *Vibrio* isolates, 21 were phenotypically resistant (75.0%) and another was intermediate (3.57%). Further, WGS did not identify $bla_{CARB-17}$ in any of the 42 isolates, though nine other bla_{CARB} genes were found in *V. parahaemolyticus* isolates.

We observed a trend in our study that *Vibrio* from wild caught shrimp harbored a higher number ARGs on average than those from farmed samples. This is an unexpected finding given that there was no significant difference in phenotypic resistance prevalence between these two groups. Domestic isolates in this subset averaged fewer ARGs (0.29) than imported isolates (1.63), though the sample sizes and species compositions of these subgroups were distinct and limited the utility of their comparison. All seven domestic isolates were speciated as *V. metschnikovii*, whereas the 35 imported isolates had a more representative species distribution. There was also no significant difference between these two groups in phenotypic resistance prevalence.

The identification of ARGs via WGS facilitates *in-silico* predictions of phenotypic resistance (NIHR Global Health Research Unit on Genomic Surveillance of AMR, 2020; Lee et al., 2022). Phenotypic and genotypic AMR in non-cholera *Vibrio* isolates in our study correlated

with an overall sensitivity of 11.54% and specificity of 96.05%. There are a few explanations for this low sensitivity and imperfect specificity. The largest contributing factor to the low sensitivity in our study is likely the grouping of intermediate isolates with resistant isolates, particularly for cefoxitin where 14 intermediate isolates were categorized as resistant for analysis. The results from our dataset indicate that the treatment of intermediate isolates during analysis has a large impact on the assessment of phenotypic and genotypic concordance, and that the grouping of intermediate with susceptible isolates may be a better approach to optimize concordance. Other explanations include our WGS analysis of ARGs being limited to one database, so it is possible there are undetected and/or unknown AMR genetic determinants present amongst our isolates, in addition to the potential impact of cut-offs for identity and coverage used to determine the presence of ARGs. Lastly, AST and WGS in our study were conducted on separate occasions, so it is possible that plasmid loss occurred at some point, which could further contribute to incongruence of phenotypic and genotypic AMR.

Conclusion

The large-scale production and global distribution demands for shrimp results in a food production system that can be conducive to the selection and spread of antimicrobial resistance, prompting the need to better understand the occurrence of AMR in both pathogenic and commensal bacteria from these products. This present study provides food safety and public health insights on the prevalence and distribution of AMR in *Vibrio* spp. and *Enterococcus* spp. from retail shrimp in California, and highlights the importance of continued AMR monitoring of seafood

products and the value of complementing antimicrobial susceptibility testing with whole-genome sequencing for AMR assessment.

Abbreviations

AMR, antimicrobial resistance; AST, antimicrobial susceptibility testing; WGS, whole genome sequencing; FAO, Food and Agriculture Organization of the United Nations; CDC, United States Centers for Disease Control and Prevention; NARMS, National Antimicrobial Resistance Monitoring System; FDA, United States Food and Drug Administration; APW, alkaline peptone water; BPW, buffered peptone water; TCBS, thiosulfate-citrate-bile salts-sucrose; OD, optical density; CAMHB, cation-adjusted Mueller-Hinton broth; CLSI, Clinical and Laboratory Standards Institute; MDR, multidrug resistance; ARG, antimicrobial resistance gene; TSA-YE, trypticase soy agar with 0.6% yeast extract; TP, true positive; TN, true negative; FP, false positive; FN, false negative; GEN, gentamicin; STR, streptomycin; AUG2, amoxicillin/clavulanic acid 2:1 ratio; FOX, cefoxitin; AXO, ceftriaxone; XNL, ceftiofur; SXT, trimethoprim/sulfamethoxazole; AZI, azithromycin; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; KAN, kanamycin; VAN, vancomycin; LIN, lincomycin; DAP, daptomycin; TYLT, tylosin tartrate; ERY, erythromycin; NIT, nitrofurantoin; LZD, linezolid; PEN, penicillin; SYN, quinupristin/dalfopristin; TGC, tigecycline; BAP, Best Aquaculture Practices; AME, aminoglycoside-modifying enzyme; WHO, World Health Organization; QRDR, quinolone resistance determining region; PMQR, plasmid-mediated quinolone resistance; NIHR, National Institute for Health and Care Research

Authors' Contributions

BH performed AST, performed statistical analysis, and drafted the manuscript. KL performed sample collection and bacterial isolation. KYL contributed to statistical analysis and drafting of the manuscript. ERA provided oversight and laboratory resources for the study. DK performed whole genome sequencing. BB contributed to AST and bacterial confirmation. MG contributed to

AST and performed supplementary lab work. ZL and AY contributed to whole genome sequencing. XL and XY conceived of the study and participated in research coordination. The authors read and approved the final manuscript.

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Supplemental Tables

Supplementary Table 1. Summary of antimicrobial susceptibility testing (AST) drug panels and minimum inhibitory concentration (MIC) interpretive criteria.

	Abbreviation	Antimicrobial Agent	Antimicrobial Class	MIC (ug/ml) Interpretive Criteria		
				Susceptible	Intermediate	Resistant
Enterococcus	STR	Streptomycin	Aminoglycoside	1000	-	≥ 1000
	KAN	Kanamycin	Aminoglycoside	≤ 512	-	≥ 1024
	GEN	Gentamicin	Aminoglycoside	500	-	≥ 500
	VAN	Vancomycin	Glycopeptide	≤ 4	8-16	≥ 32
	LIN	Lincomycin	Lincosamide	≤ 2	4	≥ 8
	DAP	Daptomycin	Lipopeptide	≤ 4	-	-
	TYLT	Tylosin tartrate	Macrolide	≤ 8	16	≥ 32
	ERY	Erythromycin	Macrolide	≤ 0.5	1-4	≥ 8
	NIT	Nitrofurantoin	Nitrofuran	≤ 32	64	≥ 128
	LZD	Linezolid	Oxazolidinones	≤ 2	4	≥ 8
	PEN	Penicillin	Penicillin	≤ 8	-	≥ 16
	CHL	Chloramphenicol	Phenicol	≤ 8	16	≥ 32
	CIP	Ciprofloxacin	Quinolone	≤ 1	2	≥ 4
	SYN	Quinupristin / dalfopristin	Streptogramin	≤ 1	2	≥ 4
	TGC	Tigecycline	Tetracycline	≤ 4	8	≥ 16
	TET	Tetracycline	Tetracycline	≤ 4	8	≥ 16
Vibrio	GEN	Gentamicin	Aminoglycoside	≤ 4	8	≥ 16
	STR	Streptomycin	Aminoglycoside	-	-	-
	AUG2	Amoxicilin / clavulanic acid 2:1 ratio	Beta-lactam combination agent	≤ 8/4	16/8	≥ 32/16
	FOX	Cefoxitin	Cephem	≤ 8	16	≥ 32
	AXO	Ceftriaxone	Cephem	-	-	-
	XNL	Ceftiofur	Cephem	-	-	-
	FIS	Sulfisoxazole	Sulfonamide	-	-	-
	SXT	Trimethoprim / sulfamethoxazole	Sulfonamide	≤ 2/38	-	≥ 4/76
	AZI	Azithromycin	Macrolide	≤ 2	-	-
	AMP	Ampicillin	Penicillin	≤ 8	16	≥ 32
	CHL	Chloramphenicol	Phenicol	-	-	-
	CIP	Ciprofloxacin	Quinolone	≤ 1	2	≥ 4
	NAL	Nalidixic Acid	Quinolone	-	-	-
	TET	Tetracycline	Tetracycline	≤ 4	8	≥ 16

Supplementary Table 2. Distribution of *Vibrio* isolates with MICs below the lowest concentration and above the highest concentration for drugs without defined MIC breakpoints.

Class	Drug	Lowest concentration on MIC panel (ug/mL)	No. isolates with MIC at or below lowest concentration (%)	Highest concentration on MIC panel (ug/mL)	No. isolates with MIC above highest concentration (%)
Cephem	Ceftiofur	0.12	55 (50%)	8	7 (6.36%)
	Ceftriaxone	0.25	95 (86.36%)	64	0 (0%)
Phenicol	Chloramphenicol	2	105 (95.45%)	32	0 (0%)
Quinolone	Nalidixic acid	0.5	85 (77.27%)	32	5 (4.55%)
Aminoglycoside	Streptomycin	2	3 (2.73%)	64	4 (3.64%)
Folate pathway antagonist	Sulfisoxazole	16	24 (21.81%)	256	24 (21.82%)