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Probiotics and Prebiotics as Dietary Supplements for the Adjunctive Treatment of Type 2 Diabetes

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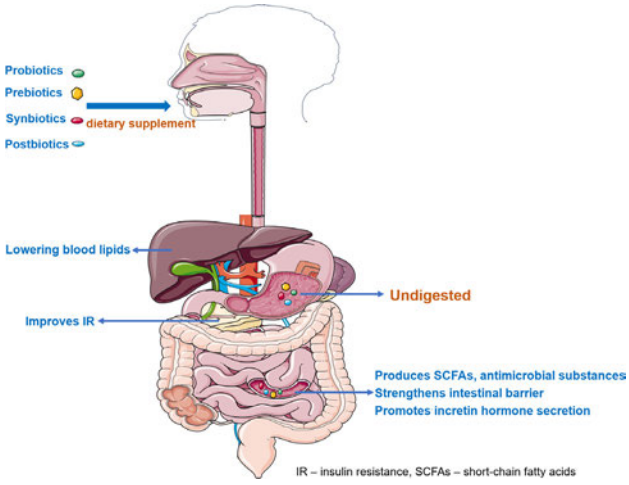
Abstract



In modern lifestyles, high-fat diets and prolonged inactivity lead to more people developing type 2 diabetes (T2D). Based on the modern pathogenesis of T2D, food, and its components have become one of the top concerns for patients. Recent studies have found that dysbiosis and gut-related inflammation are more common in T2D patients. Probiotics and prebiotics play complementary roles in the gut as dietary supplements. Together, they may help improve dysbiosis and intestinal inflammation in people with T2D, increase the production of blood glucose-lowering hormones such as incretin, and help reduce insulin resistance and lower blood glucose. Therefore, changing the dietary structure and increasing the intake of probiotics and prebiotics is expected to become a new strategy for the adjuvant treatment of T2D.

K e y w o r d s: type 2 diabetes, probiotics, prebiotics, microbiota

Introduction

Diabetes is one of the top ten causes of death globally, and 90% of patients have type 2 diabetes (T2D) (Holman et al. 2015). The International Diabetes Federation estimated that in 2015 10% of the global population had diabetes. Most patients have had hyperglycemia and/or insulin resistance (IR) for several years prior to diagnosis, and patients with undiagnosed and untreated diabetes are at higher risk of developing more severe complications than patients receiving treatment (Magliano et al. 2019). In modern society, unhealthy lifestyles such as long-term high-fat and high-sugar diets, lack of physical exercise, and excessive work pressure lead to obesity and T2D (Zheng et al. 2018; Hosomi et al. 2022). Among them, diet plays a vital role in the occurrence and development of T2D (Merino 2022). Changing unhealthy lifestyles, maintaining a healthy weight, eating healthy, maintaining physical activity, not smoking, and moderate alcohol consumption can prevent and improve T2D.

With the discovery that gut microbiota changes during the diet, exercise, traditional hypoglycemic drugs, and bariatric surgery, the relationship between gut microbiota and T2D has attracted widespread attention in the medical community. It has also been confirmed that the development of T2D is closely related to intestinal microbiota (Sonnenburg and Bäckhed 2016). The gut microbiota affects many biological functions, and its characterization has become a central area of biomedical research. Recent studies have shown that the gut microbiota plays an important role in diseases such as obesity, diabetes, and non-alcoholic fatty liver disease (Canfora et al. 2019). In many human and animal studies,

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T2D is associated with severe gut dysbiosis, and specific gut bacteria and their metabolites are significantly altered in T2D. Both the abundance of butyrate-producing microorganisms and butyrate production is reduced in diabetic patients; high-fat diet-induced T2D can activate the lipopolysaccharide (LPS) component of Gram-negative bacteria and cause endotoxemia (Sun et al. 2010). LPS plays a role in metabolic disorders by initiating innate immune mechanisms; certain antidiabetic drugs such as metformin can improve metabolic dysfunction by activating the bile acid glycoursodeoxycholic acid (GUDCA)-intestinal farnesoid X receptor (FXR) pathway through *Bacteroides fragilis* (Sun et al. 2018).

Studies of the gut microbiome have shown that the microbiota is an important regulator of host energy and substrate metabolism. Abnormal composition and/or function of gut microbes can lead to disturbances in energy metabolism, affecting the metabolism of fat, liver, muscle, and other tissues. Microbial composition and abundance depend on the integrity of the gut itself, intrinsic factors such as genetics, and extrinsic factors such as diet and drug intake (Schmidt et al. 2018). Disturbed gut microbiota is widespread in patients with T2D and contributes to the development of insulin resistance. The digestion of food is inextricably linked to the microbiome. Dietary control is selecting foods that are beneficial to the beneficial bacteria in the gut to achieve the purpose of controlling blood glucose. Altering the energy-dense diet of people with T2D to promote a diet rich in whole grains, fruits, vegetables, nuts, and legumes, and reducing intake of refined grains, red or processed meat, and sugar-sweetened beverages maybe a preventive measure and an effective method to improve T2D. A healthy diet includes a high amount of dietary fiber (in vegetables and fruits) and oligosaccharides, which belong to the category of prebiotics, and supplementation with prebiotics is beneficial in maintaining intestinal microbial balance (Kanazawa et al. 2021). In addition, clinical studies have shown that probiotics supplementation can improve the intestinal microbiota and have an adjuvant effect on T2D (Zhang et al. 2020). Specific microorganisms and their macro-, micro-, and non-nutritional components play an important role in regulating glucose homeostasis. Therefore, we review studies of dietary supplementation with probiotics and/or prebiotics in patients, which may be an adjunctive prevention and management strategy for treating T2D.

Probiotics

International Scientific Association for Probiotics and Prebiotics (ISAPP) defines probiotics as live microorganisms that, when administered in adequate numbers, confer a health benefit on the host (Hill et al. 2014). It benefits health by modulating host mucosal immune function, improving dysbiosis, or promoting nutrient absorption. The colon is where most bacteria live in the digestive tract, so the colon is the primary place for probiotics to be active. Metabolism in the colon largely depends on nutrients, the number of complex foods, the composition of the gut microbiome, and colonic transit time. The proximal colon prefers to utilize fiber, resistant starch, and prebiotics for fermentation to obtain the energy needed for survival. In contrast, the distal colon utilizes residual peptides and proteins for proteolytic fermentation, which can be further metabolized by microbial cross-feeding (Canfora et al. 2019). T2D is characterized by microbial dysbiosis, intestinal barrier damage, and a low-grade inflammatory state. A healthy microbial community generally maintains healthy barrier function, including mucosal integrity, tight junctions, and a normal mucosal immune system. Dietary changes have the most significant impact on diabetes risk and blood glucose regulation in the long run. Regarding dietary composition, probiotics, master regulators of gut microbiota, were most strongly associated with reduced risk of T2D. There are two main methods for screening probiotics with glucose-lowering potential: inhibition of α-glucosidase (Balaich et al. 2021) and inhibition of dipeptidyl peptidase IV (DPPIV). The structure of α-glucosidase inhibitors is similar to that of oligosaccharides, mainly through the competitive binding to carbohydrate-binding sites on α-glucosidase, reducing the production of monosaccharides and reducing postprandial blood glucose. Probiotics-fermented blueberry juices increased α-glucosidase inhibitory and α-amylase inhibitory activities, and enhanced antioxidant, antimicrobial, and antidiabetic activities (Zhong et al. 2021). DDPIV inhibitors induce glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) inactivation and increase their endogenous levels. The cell-free excretory supernatants and cell-free extracts of *Lactobacillus acidophilus* KLDS1.0901 has better DPP-IV inhibitory activity, antioxidative activities, and biological characteristics than *Lactobacillus rhamnosus* GG (Yan et al. 2020).

The health benefits of probiotics should also be evaluated clinically or experimentally to study their mechanisms. A total of 15 randomized controlled trials and 902 patients showed that probiotics treatment could reduce glycated hemoglobin A1c (HbA1c), fasting blood glucose (FBG), and insulin resistance levels in T2D patients quite robust and stable (Tao et al. 2020). In randomized controlled trials of probiotics intervention in 50 T2D patients, probiotics supplementation effectively reduced inflammatory factors and increased acetic acid production (Tonucci et al. 2017). Probiotics in camel milk have antidiabetic effects on T2D mice

by inducing GLP-1 secretion and increasing claudin-1 and mucin-2 proteins to repair the intestinal mucosal barrier (Wang et al. 2020). Probiotics can also regulate patients' metabolism, improve dyslipidemia and hypertension, and reduce the risk of cardiovascular disease in T2D (Kocsis et al. 2020). *Lactobacilli gasseri* and *Lactobacilli johnsonii* improve systemic glucose metabolism by restoring fatty acid β-oxidation and reducing high-fat diet-induced hepatic mitochondrial damage (Rodrigues et al. 2021). *Akkermansia muciniphila*, a bacterium present in the mucus layer, is inversely associated with T2D disease, and increasing the activity of *A. muciniphila* has a positive effect on improving metabolism and mucus layer thickness. Probiotics can lower triglyceride and total cholesterol levels in gestational diabetes mellitus (Okesene-Gafa et al. 2020). Microbial metabolites, short-chain fatty acids (SCFAs), and total bile acids can activate hypoglycemic signaling pathways, improve endotoxemia, and increase GLP-1 and peptide tyrosine (PYY) levels. Probiotics can act as an adjunct to metformin by increasing butyrate production. Delayed-release metformin relies on the dissolution of the tablet enteric coating in the distal intestine, where a high density of L cells maintains a pH of 6.5 for maximal efficacy (Buse et al. 2016). In addition, probiotics can protect β-cell function by reducing IR and oxidative stress (Kesika et al. 2019). *Ruminococcus ilealis* was significantly increased in 80% of obese patients, suggesting that this microbe may be a common pathogen in obese individuals (Wu et al. 2011). These different studies explored the relationship between gut microbes and T2D by modulating metabolism, increasing bacterial abundance, and adjusting the timing of interventions, suggesting that certain probiotics can modulate glucose metabolism and improve microbial dysbiosis.

Prebiotics

ISAPP defines a prebiotic as a selectively utilized substrate by host microorganisms conferring a health benefit (Gibson et al. 2017). Prebiotics are organic substances that are not digested and absorbed by the host but can selectively promote the metabolism and proliferation of probiotics. They can be used as probiotic nutrients or materials, regulating microbiota abundance, and improving host health. Prebiotics can enhance the effect of probiotics, and the two complement each other to maintain the homeostasis of gut microbes. In addition, probiotics themselves can regulate intestinal function by producing SCFAs through carbohydrate fermentation, improving the intestinal microenvironment and increasing the production of GLP-1 (Zhao et al. 2018). Prebiotics mainly include oligosaccharides (fructose oligosaccharides, galactooligosaccharides, xylooligosaccharides, isomaltose, soybean oligosaccharides, inulin, etc.), microalgae (such as *Spirulina*, *Arthrospira*); polysaccharides (such as *Coriolus versicolor* polysaccharide), protein hydrolyzate (such as casein hydrolyzate, α-lactalbumin, lactoferrin, etc.) and natural vegetables, fruits, herbal medicine, wild plants, and other dietary fibers (Gibson et al. 2017; Peng et al. 2020). Prebiotics are entirely (100%) translocated to the colon, which balances the microbiome and provides beneficial effects.

Prebiotics improve insulin sensitivity by altering the composition of the microbiota, reducing endotoxemia, and reducing energy harvesting. Prebiotic treatment is designed to provide a selective advantage to beneficial bacteria compared to the direct administration of probiotics. Food enters the small and large intestines through the digestion of the mouth and stomach, and the indigestible dietary fiber is fermented and decomposed by various intestinal microorganisms to generate SCFAs such as acetic acid, propionic acid, butyric acid, and reduce the metabolism of harmful compounds (such as indole and hydrogen sulfide). Fermentation of gut microbes has positive effects on reducing inflammation, controlling body weight, improving IR, and improving glucose and lipid metabolism (Zhao et al. 2018). Harmful microbiota enters the bloodstream by disrupting the intestinal mucosa, creating a low inflammatory state, and increasing IR. Supplementation of prebiotics in the diet of T2D patients, which produce SCFAs through the fermentation of intestinal microorganisms, is important for maintaining the intestinal barrier (Robertson 2020). Prebiotic supplementation normalized the abundance of *A. muciniphila* and improved abnormal glucose metabolism (Everard et al. 2013). Resistant starch from Kudzu helps restore the expression of insulin receptor substrate 1 (IRS-1), phosphorylated-phosphatidylinositol 3-kinase (p-PI3K), phospho-Akt and glucose transporter 4 (GLUT4), thereby increasing the efficiency of insulin (Song et al. 2021). *Cyclocarya paliurus* polysaccharides can reduce diabetes symptoms and increase the abundance of probiotics (Li et al. 2021). The addition of oligofructose-enriched inulin decreased the ratio of *Firmicutes* to *Bacteroidetes* while increasing the abundance of *Bifidobacterium* (de Cossío et al. 2017). Arabinoxylan promoted the growth of fiber-degrading bacteria and increased SCFAs decreased the abundance of opportunistic pathogens. Arabinoxylan also decreased 12α-hydroxylated bile acids and increased equal, indole propionic acid, and behenic acid (Nie et al. 2022).

Dietary fiber is a large group of prebiotics; it is not a single substance. Digestive enzymes do not efficiently hydrolyze them, and fibers are divided into soluble (pectin, gum, mucilage) and insoluble (cellulose, hemicellulose, lignin) according to their water solubility. The definition of dietary fiber also includes those isolated from food materials or edible carbohydrates. Fiber is

not easily digested and absorbed in the human small intestine but can be partially or fully fermented in the colon. In the gastrointestinal tract, it has been shown to improve bowel function, lower blood lipids, and lower blood glucose (Waddell and Orfila 2022). Individual differences in gut microbial composition were associated with individualized responses to dietary fiber intervention and significantly contributed to changes in probiotics abundance.

The dietary composition impacts the gut microbiota, so a specific dietary fiber diet can control the gut microbiota to a healthy gut pattern (Healey et al. 2016). While solubility is a fundamental determinant of physiological responses, viscosity, and fermentability play a more prominent role in physiological benefits, so high-viscosity dietary fiber makes chyme more cohesive and can slow glucose release (Kumar et al. 2012). In addition, soluble viscous fiber reduces gastric emptying by absorbing water to form a viscous gel and plays an important role in controlling postprandial blood glucose and insulin (Holt et al. 1979). The most substantial and most consistent effect in reducing the risk of developing T2D was observed in a diet high in insoluble and only moderately fermented grain fiber. At the same time, fruits and vegetables were important sources of soluble/fermentable fiber. The U.S. recommends 1.5–2 cup-equivalents of fruits and 2–3 cup-equivalents of vegetables daily for modern health (Lee et al. 2022). A prospective study of fruit and vegetable supplements has been shown to help reduce HbA1c in T2D patients (Gordon et al. 2022). A prospective study of 120,343 volunteers in the United Kingdom showed that increased intake of fresh fruits and vegetables reduced the incidence of T2D during an 8.4-year follow-up period (Gao et al. 2022).

Synbiotics and postbiotics

ISAPP defines synbiotics as a mixture comprising live microorganisms and substrates selectively utilized by host microorganisms that confer a health benefit on the host (Swanson et al. 2020). It not only exerts bacterial activity but also selectively and rapidly increases prebiotics, making the effect of probiotics more pronounced and long-lasting. As a new generation of micro-ecological regulators, synbiotics play the physiological functions of both probiotics and prebiotics to fight diseases and maintain micro-ecological balance. A study of 11 randomized controlled trials and 18 subjects showed that synbiotics supplementation lowered blood glucose levels and improved IR in patients with prediabetes and T2D (Naseri et al. 2022). The addition of synbiotics in T2D patients helps to improve their gut environment (Kanazawa et al. 2021) and taking synbiotics may be a potential adjunctive therapy to improve metabolism and lower HbA1c (Bock et al. 2021).

ISAPP defines a postbiotic as a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host. Effective postbiotics must contain inactivated microbial cells or cell components, with or without metabolites, that contributes to observed health benefits (Salminen et al. 2021). Postbiotics have the potential to modulate intestinal barrier stability and improve metabolic diseases. SCFAs can improve glucose homeostasis, and 24 weeks of inulin-propionate supplementation can enhance insulin release by inhibiting apoptosis while maintaining functional β-cell viability (Pingitore et al. 2017). SCFAs maintain the intestinal barrier by promoting the growth of intestinal epithelial cells, strengthening tight junctions, and modulating the activity of microbiota and immune cells (Liu et al. 2021). Butyrate alleviates dysbiosis induced by a methionine-choline-deficient diet, and the improvement of the microbiota by butyrate is closely related to intestinal barrier function (Ye et al. 2018). In addition, butyrate suppressed high-fat diet-induced obesity and IR, and the tight junction protein claudin-1 was significantly increased in the jejunum, ileum, and colon epithelium (Matheus et al. 2017).

Application of probiotics and prebiotics

In terms of probiotics supplementation, people should pay attention to the proper consumption of probiotics-containing foods, rather than relying too much on probiotics products. Fermented dairy products are the carrier of choice for probiotics. On the one hand, dairy products do not require changes in technology and manufacturing processes, and on the other hand, the dairy matrix can protect probiotics through gastrointestinal transport (Fernandez and Marette 2017). Surprisingly, minerals such as calcium and magnesium in dairy products were associated with a lower incidence of T2D (Madjd et al. 2016). Probiotic yogurt is a nutrient-dense food that may benefit patients with lactose intolerance, constipation, diarrhea, high blood pressure, cardiovascular disease, diabetes, and certain cancers. Probiotic yogurt overcomes host defense factors such as intestinal colonization resistance, reduces FBG, and improves oxidation. For lactose intolerance (Shaukat et al. 2010), *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* produce β-galactosidase, in which lactose is hydrolyzed by β-galactosidase into monosaccharides that are easily absorbed by the intestinal epithelium.

Antibiotics are one of the key therapeutic tools in the fight against bacterial infections and therefore deserve our attention in treating dysbiosis. Antibiot-

ics can sometimes be used synergically with probiotics (Al-Dulaimi et al. 2021). Antibiotics can be used to remove or inhibit harmful microorganisms, probiotics can be used to introduce missing microorganisms, and prebiotics can be applied to enhance the proliferation of beneficial microorganisms to maximize sustained changes in the microbiome's composition. Furthermore, probiotics can act as adjuvants for antibiotics, adhere to the intestinal layer to enhance barrier function, secrete antimicrobials to fight pathogen invasion, and coordinate immune function (Wan et al. 2019). Nevertheless, repeated clinical use or abuse of antibiotics can lead to the development of bacterial resistance, which will result in more significant harm to people with common bacterial infections. The use of probiotics is expected to improve this symptom, and a clinical trial evaluation showed that various probiotics (including *Bacillus* spp., *Bifidobacterium* spp., *Clostridium butyricum*, *Lactobacilli* spp.) have a protective effect against antibiotic-associated diarrhea as well as shortening the duration of diarrhea (Guo et al. 2019). For antibiotic-sensitive probiotics bacteria, altering their genetic modules may be a solution. Encoding plasmids in *Lactobacillus* to achieve independent high expression of multiple toxin-antitoxin system genes will help expand the use of *Lactobacillus* in healthcare (Dey et al. 2023).

Numerous clinical studies have shown that supplementation with probiotics, prebiotics, synbiotics, or postbiotics can reduce HbA1c and lipid levels and improve IR (Bock et al. 2021), while their use is more effective than placebo in reducing FBG, both in the short and long term (Rittiphairoj et al. 2021). While probiotics products can be taken alone, the best time to consume them is after meals (Wang et al. 2022). Food enters the stomach after meals, and the neutralization of gastric acid is more conducive to the effect of live probiotics. In addition, the intestinal microbiota should be maintained in a balanced state, not the more probiotics, the better. Excessive intake of probiotics has no positive effects, but overloading can also have serious adverse effects, wreaking havoc on the gut environment.

Conclusion

Many human and animal experiments have confirmed that probiotics can significantly regulate microbial dysbiosis, repair the intestinal barrier system, and have particular clinical significance in improving IR and reducing blood glucose. However, current research still needs in-depth characterization studies of the leading probiotic strains to treat T2D. Therefore, there is a greater need to study and explain these microorganisms' different mechanisms of action to evaluate their application in functional foods and different food matrices in the future. On the other hand, another medical and nutritional therapy challenge that should be considered is managing T2D through whole foods without any side effects. Therefore, the long-term effects of prebiotics, synbiotics, and postbiotics on glucose metabolism may be a potential hope for treating T2D.

Author contributions

Yuying Wang designed the concept and drafted the article. Yuying Wang, Lina Wen, Huazhen Tang, and Jinxiu Qu participated in the compilation of the article materials. Benqiang Rao made critical revisions to important content. All authors finally approved the submitted version.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Prevalence and Biological Characteristics of *Listeria* Species Isolated from Livestock and Poultry Meat in Gansu Province, China

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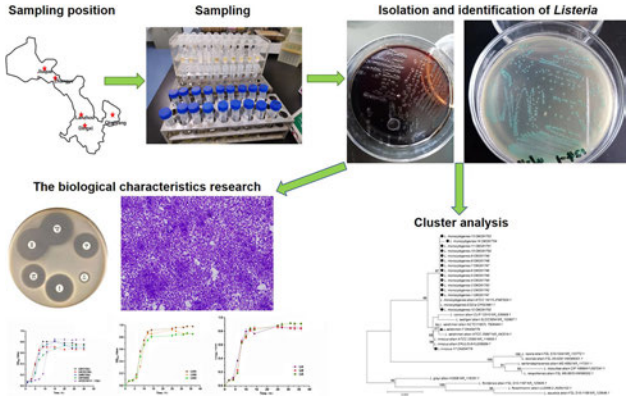
Abstract

Listeria monocytogenes is a widespread foodborne pathogen contaminating foods during their production or processing stages. Fresh meat is susceptible to such contamination if it is not properly preserved. Our study was conducted to reveal the level of contamination and prevalence of *Listeria* spp. present in livestock and poultry meat from Gansu province. A total of 1,387 samples were collected from five cities in Gansu Province according to standard sampling procedures, of which 174 samples (12.5%) were positive for *Listeria* species. Among them, 14 isolates of *L. monocytogenes* (1.0%), 150 isolates of *Listeria innocua* (10.8%), and ten isolates of *Listeria welshimeri* (0.7%) were identified by conventional bacteriological and molecular identification methods. All isolates were subjected to serological assays, antimicrobial susceptibility tests, growth curve assays, determination of biofilm-forming capacity, and cluster analysis of the 16S rRNA gene sequences. Four predominant serotypes of *L. monocytogenes* were identified, including 1/2a (35.7%, 5/14), 1/2b (14.3%, 2/14), 1/2c (42.9%, 6/14), and 4b (7.1%, 1/14). All *L. monocytogenes* isolates were resistant to tetracycline and cefoxitin. Most *L. innocua* isolates (63.6%, 14/22) and *L. welshimeri* (40%, 4/10) were resistant to tetracycline. The high biofilm-forming ability was observed among 1/2c and 1/2a serotype isolates. The cluster analysis of the 16S rRNA gene sequences revealed a close genetic relationship between the three *Listeria* species. This study fills the gap in the knowledge of livestock and poultry meat that carry *Listeria* in slaughterhouses and markets in Gansu Province.



K e y w o r d s: *Listeria monocytogenes*, *Listeria innocua*, *Listeria welshimeri*, livestock and poultry meat, slaughtering, cluster analysis

Introduction

Listeria is sporeless, facultative, and Gram-positive bacteria, widely distributed in the environment and ubiquitously found in decaying vegetation, sewage, and soil (Jadhav et al. 2012). According to the genome sequence, pathogenicity traits, and catabolism characteristics, the genus *Listeria* can be divided into thirteen species, of which *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* are most prevalent (Schmid et al. 2005). It should be highlighted that *L. monocytogenes* is a zoonotic pathogen that has been ranked among the four foodborne pathogens deemed the greatest threat to public health by the World Health Organization (WHO) in the early 1980s (Chlebicz and Slizewska 2018). Moreover, diseases caused by *L. monocytogenes* mainly occur in immunocompromised individuals and manifest as encephalitis, meningoencephalitis, and septicemia. The consequences of the pregnant woman and newborn infection are extremely severe,

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including abortion, premature birth, and pneumonia (Doumith et al. 2004; Indrawattana et al. 2011).

Recently, different studies have reported several food contamination events caused by *L. monocytogenes* worldwide. For example, the prevalence of *L. monocytogenes* in raw milk and milk products in the Samson Province of Turkey was 3.7% (Terzi Gulel et al. 2020). The prevalence of *L. monocytogenes* in sliced cheese and ham in the retail market in southern Brazil was 9.4% (Maia et al. 2019), but in the poultry production chain in Italy was higher and equal to 26.7% (Iannetti et al. 2020). The prevalence of *L. monocytogenes* in slaughterhouses and supermarkets in three neighboring provinces (Chongqing, Shanxi, and Yunnan) of China was 5.5%, and the bacterium was found in 4.0% of foods from Beijing (Fang et al. 2018). Astonishing was that *L. monocytogenes* contamination rates in food from the Sichuan province of China reached 46.9% (Fang et al. 2018; Li et al. 2018a). *L. innocua,* a non-pathogenic bacterium, is widely distributed in meat and meat products. *L. innocua* often accompanies *L. monocytogenes* and is regarded as the indicator bacteria of the latter (den Bakker et al. 2010; Yıldırım et al. 2016). *L. welshimeri* is also a non-pathogenic microorganism and does not have virulence genes, including *aad*A, *str*A, *str*B, and *pen*A (Davis and Jackson 2009). Although *L. welshimeri* lacks virulence genes for intracellular survival, similar to *L. innocua*, it is more closely related to *L. monocytogenes* than other *Listeria* species (Hain et al. 2007). Davis and Jackson (2009) found that *L. welshimeri* had the broadest resistance range compared to *L. monocytogenes* and *L. innocua*. Therefore, the potential for *L. welshimeri* exists to serve as a reservoir of transferable resistance genes (Davis and Jackson 2009).

Foodborne listeriosis outbreaks have been linked to various meat products (Matle et al. 2020). The first laboratory-confirmed invasive case of listeriosis associated with meat products was in 1988 after consuming contaminated turkey franks (Schwartz et al. 1989). In 1987–2018, worldwide listeriosis outbreaks or sporadic cases comprised of 2,087 cases related to meat products, including processed, vacuum-packaged meat products (Jensen et al. 2016; Chen et al. 2017), and polonies (Smith et al. 2019). These cases were mostly reported in the United Kingdom, Australia, France, the United States, Denmark, and South Africa. Poor hygienic practices and lack of effective monitoring during meat processing are conducive to the growth and survival of *L. monocytogenes*.

Currently, there is no report on the contamination by *Listeria* species in livestock and poultry meat in the Gansu Province, northwest China. In this study, we investigated the prevalence of *Listeria* spp. in livestock and poultry meat from the slaughterhouses and markets of the Gansu province. We conducted a preliminary study of the biological characteristics of isolated bacteria; further studies are warranted to develop effective prevention and control measures.

Experimental

Materials and Methods

Sample. From July 2020 to October 2020, 1,387 samples were randomly collected from raw pork, chicken, beef, and mutton from slaughterhouses and markets in five cities of the Gansu province (Fig. 1). Forty-one swabs were mainly derived from chopping boards, knives, and instruments from the market environment. The sampling information on 45 *Listeria* spp. isolates was shown in Table SI. The sampling followed the national criteria of meat and meat products-sampling GB/T 9695.19-2008 (2008) with moderate modifications. The meat and the environment surfaces were smeared using cotton swabs soaked in sterile saline solution for sampling, and the collected cotton swabs were put into a tube with 5 ml sterilized phosphate buffer solution (PBS). Samples were placed in the sampling box with the freezer bags and stored at 4°C after delivery to the laboratory.

Isolation and identification of *Listeria* species. *Listeria* strains were isolated according to the microbiology of food testing for *L. monocytogenes* GB 4789.30-2016 (2016) method of China with moderate modifications. The collected cotton swabs were placed into 225 ml Luria-Bertani liquid medium (LB_1) (Solarbio,

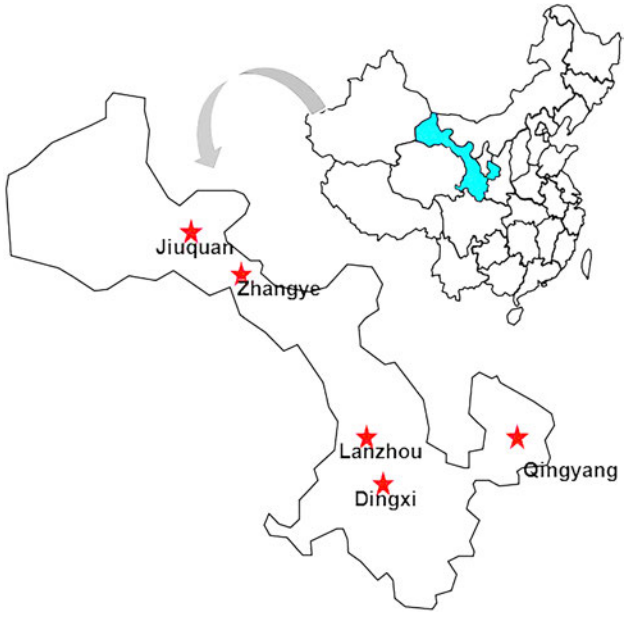

Fig. 1. Distribution diagram of sampling points in Gansu province, China. The geographic locations of samples from slaughterhouses and markets. The five-pointed stars of red represent the different sampling sites.

China) and incubated at 30°C for 36 h. Then, 0.1 ml of enrichment broth was transferred into 10 ml LB_2 for the second-step enrichment at 30°C for 48 h. The isolates were inoculated on PALCAM agar (Huankai Microbial, China) and cultured at 37°C for 36 h. Afterward, 2–3 colonies revealing the characteristic small, round, and grayish-green appearance on PALCAM agar were picked and subcultured on the selected medium for *Listeria* at 37°C for 48 h. Two or three smooth blue-green colonies were then randomly selected for purification and cultured on the solid medium of Brain Heart Infusion (BHI) (Solarbio, China) at 37°C for 8 h. Then, the bacteria were stained according to the Gram procedure. After the above operation, *Listeria* spp. were preliminarily identified. The PCR method that targets specific genes of different *Listeria* species was applied for further verification (Tao et al. 2017). Bacterial DNA was extracted from 3 ml of the above bacterial cultures using the TIANamp Bacterial DNA kit (Tiangen, China) and used as the template to identify the *Listeria* genus-specific *prs* gene by PCR. The positive isolates for the *prs* gene fragments were further identified using the primers for the *L. monocytogenes*-specific *lmo2234* gene. Then, the negative isolates were identified using the primer for the *L. innocua*-specific *lin0464* gene. After amplification, the purified PCR products were sent to the GENEWIZ Technology Co., Ltd., (China) for Sanger sequencing. The sequencing results were analyzed by BLAST in the NCBI database. The PCR primers used in this paper refer to the study of Luo et al. (2017). All the primers used in this study are listed in Table I.

***L. monocytogenes* serogroups.** The bacterial wall-breaking treatment and multiplex PCR method were used to differentiate the major serogroups of *L. monocytogenes*. The multiplex PCR primers (Doumith et al. 2004) were shown in Table I. PCR products were detected by agarose gel electrophoresis, and serogroups of the isolates were determined by analyzing the electrophoresis bands (Doumith et al. 2004). The 16S rRNA sequencing results of the different serogroup isolates were analyzed by BLAST (Basic Local Alignment Search Tool) determine the isolates' clusters further.

Antimicrobial susceptibility test. The Kirby-Bauer disk diffusion test was used to evaluate the susceptibility of *Listeria* species to ten antibiotics, including penicillin, ofloxacin, cefoxitin, sulfamethoxazole, tetracycline, gentamicin, streptomycin, erythromycin, acetylspiramycin, and fosfomycin. Finally, the diameters of the antimicrobial zone of each antimicrobial susceptibility disc were measured. Results were judged according to Reis et al. (2011).

Measurement of the growth curve. Four *L. monocytogenes* isolates of different serotypes, the reference strain ATCC® 19111™, three *L. innocua* isolates, and three *L. welshimeri* isolates were selected for the growth curve determination. *Listeria* isolates were aseptically inoculated on BHI agar plates and incubated overnight at 37°C. The single colonies were inoculated in 20 ml of BHI liquid culture at 37°C 180 × *g*. Three parallel tests were set for each sample, and one BHI medium was selected as blank control. The growth curve of the isolates was drawn by measuring the OD_{600} value of the bacterial suspension at different times. The multi-mode

Table I
Primer information.

Gene target	Primer sequence (5'-3')	Product size (bp)	Application
prs-F	GCTGAAGAGATTGCGAAAGAAG	370	*Listeria* species-specific primers
prs-R	CAAAGAAACCTTGGATTTGCGG		
Lmo2234-F	TGTCCAGTTCCATTTTTAACT	420	*Listeria monocytogenes* specific primers
Lmo2234-R	TTGTTGTTCTGCTGTACGA		
Lin0464-F	CGCATTTATCGCCAAAACTC	749	*Listeria innocua* specific primers
Lin0464-R	TCGTGACATAGACGCGATTG		
lmo0737-F	AGGGCTTCAAGGACTTACCC	691	For the identification of *Listeria monocytogenes* serogroup
lmo0737-R	ACGATTTCTGCTTGCCATTC		
lmo1118-F	AGGGGTCTTAAATCCTGGAA	909	For the identification of *Listeria monocytogenes* serogroup
lmo1118-R	CGGCTTGTTCGGCATACTTA		
ORF2819-F	AGCAAAATGCCAAAACTCGT	471	For the identification of *Listeria monocytogenes* serogroup
ORF2819-R	CATCACTAAAGCCTCCCATTG		
ORF2110-F	AGTGGACAATTGATTGGTGAA	597	For the identification of *Listeria monocytogenes* serogroup
ORF2110-R	CATCCATCCCTTACTTTGGAC		
16S rRNA-27F	AGAGTTTGATCCTGGCTCAG	1,500	Used for cluster analysis
16S rRNA-1492R	GGTTACCTTGTTACGACTT		

microplate reader with model number SpectraMax i3x (Molecular Devices Corporation, USA) was utilized for these studies.

Biofilm formation assay. The method of Hu et al. (2010) for the capacity of bacterial biofilm formation was modified. All *Listeria* isolates were aseptically inoculated on BHI agar plates and incubated overnight at 37°C. The colonies were selected and cultured in 5 ml of BHI liquid medium at 37°C 180 × *g* for 14 h. After overnight incubation, the bacterial broth was transferred to BHI at a ratio of 1:100 and incubated until the OD_{562} value reached 0.2. The bacterial solution was diluted again at a ratio of 1:100; then the bacteria diluent was transferred 200 µl of each well into a 96-well plate and incubated at 37°C for 46 h. After incubation, the suspension was discarded, and each well was washed with 200 µl sterilized saline three times to remove the planktonic bacteria. Then, the bacteria were fixed with 200 µl methanol for 30 min. After drying, 200 µl of 1% ammonium oxalate crystal violet solution was added for 30 min. Finally, 200 µl of 95% ethanol solution was added and vibrated for 20. The OD_{562} values were measured with a multi-mode microplate reader. Three replicates for each bacterial isolate were measured.

Cluster analysis. The cluster analysis of the *Listeria* genus based on 16S rRNA (Soni and Dubey 2014) was performed using MEGA 5.05 cluster analysis software (Tamura al. 2011). Thirty-three sequences were selected to analyze the *Listeria* spp. cluster relationship, including 14 sequences of *L. monocytogenes*, one sequence of *L. innocua,* one sequence of *L. welshimeri* from this study, and 17 sequences of other *Listeria* species from the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) database. The 16S rRNA sequencing data used in the experiment have been submitted to the NCBI database, and the accession numbers of all sequencing data have been collated in Table SII. Gaps were treated as missing data, with equal weightings for transitions and transversions.

Statistical analysis. SPSS 22 software (SPSS, Inc., USA) was used for statistical analyses. All experimental data were calculated by the one-way analysis of variance. Results were considered statistically significant at the p-value < 0.05 level. p-Value < 0.05 was regarded as significantly different, while p-value < 0.01 was regarded as highly significantly different. Error bars represent the standard deviation.

Results

Prevalence of *L. monocytogenes, L. innocua* and *L. welshimeri* in livestock and poultry meat. The prevalence of *Listeria* species in different samples and different regions of the province has been summarized in Table II. Specific genes of the *Listeria* species were confirmed by agarose gel electrophoresis, including *prs* (370 bp), *lmo2234* (420 bp), and *lin0464* (749 bp). In total, 12.6% (174/1,387) of samples were positive for *Listeria* spp. Among these, *L. innocua* was the most prevalent species, accounting for 86.2% (150/174) of the isolates, whereas *L. monocytogenes* accounted for 8.1% (14/174), and *L. welshimeri* for 5.7% (10/174). Among the meat samples of different categories, the

Table II
Statistic of data on the prevalence of *Listeria* spp. according to regions and sample categories.

	Number of samples	*Listeria monocytogenes* positive samples (%)	*Listeria innocua* positive samples (%)	*Listeria welshimeri* positive samples (%)	*Listeria* spp. positive samples (%)
Different regions					
Lanzhou City	298	7 (2.4^{a})	30 (10.1^{b})	2 (0.7^{b})	39 (13.1^{b})
Qingyang City	275	6 (2.2^{b})	95 (34.6^{a})	4 (1.5^{a})	105 (38.2^{a})
Jiuquan City	129	1 (0.8^{c})	2 (1.6^{d})	0 (0^{c})	3 (2.3^{d})
Dingxi City	400	0 (0^{d})	6 (1.5^{d})	0 (0^{c})	6 (1.5^{e})
Zhangye City	285	0 (0^{d})	17 (6.0^{c})	4 (1.4^{a})	21 (7.4^{c})
Total	1,387	14 (1.0)	150 (10.8)	10 (0.7)	174 (12.6)
Different categories of samples					
Pork	784	8 (1.0^{b})	81 (10.3^{d})	7 (0.9^{a})	96 (12.3^{c})
Beef and mutton	298	3 (1.0^{b})	35 (11.7^{b})	2 (0.7^{b})	40 (13.4^{a})
Chicken	264	3 (1.14^{a})	29 (11.0^{c})	1 (0.4^{c})	33 (12.5^{b})
Environment samples	41	0 (0^{b})	5 (12.2^{a})	0 (0^{d})	5 (12.2^{c})
Total	1,387	14 (1.0)	150 (10.8)	10 (0.7)	174 (12.6)

$^{a\text{-}e}$ – Different superscript lowercase letters indicate that the two sets of data in this column are highly significantly different (p-value < 0.01), and the same superscript lowercase letters indicate not significantly different values (p-value > 0.05). With the increase of a to d, the differences between groups are also gradually expanding.

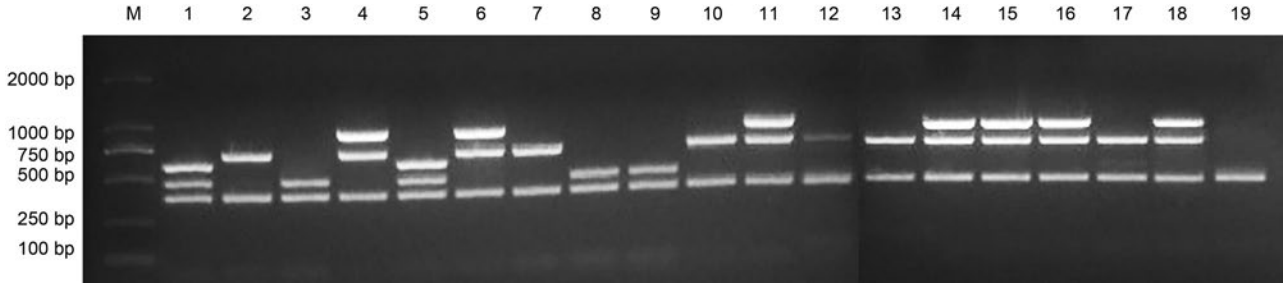

Fig. 2. Serogroups by multiplex-PCR of *L. monocytogenes* isolates.
M – DL 2000 DNA Maker, 1 – ATCC® 19115™ (serogroup 4b), 2 – ATCC® 19111™ (serogroup 1/2a), 3 – NCTC10890 (serogroup 1/2b), 4 – ATCC® 19112™ (serogroup 1/2c), 5–18 – the strains isolated in this study, 19 – *L. innocua* (negative control)

prevalence rate of *Listeria* spp. ranged from 12.2–13.4%. The prevalence rate of *Listeria* spp. in beef and mutton (13.4%) was highly significantly different than in pork (12.3%), chicken (12.5%), and environment samples (12.2%). However, the difference in the *Listeria* spp. prevalence in pork (12.3%) and environment samples (12.2%) was not statistically significant (p-value >0.05). The prevalence rate of *L. monocytogenes* in chicken (1.14%) was highly significantly different than in the other meat sample (1.0%), including pork, beef, and mutton (p-value <0.01). The prevalence rate of *L. welshimeri* in pork (0.9%) was highly significantly different when compared with that in beef (0.7%) or chicken (0.4%) (p-value <0.01). In addition, the prevalence rate of *L. innocua* in environment samples (12.2%) was highly significantly different than in pork (10.3%), beef (11.7%), and chicken (11.0%) (p-value <0.01). Furthermore, the incidence rate of *Listeria* spp. varied from 1.5% to 38.2% among different cities. The occurrence of *Listeria* spp. in Qingyang city was significantly different (p-value <0.01) compared to that in other cities. The incidence of *L. monocytogenes* was highly significantly different (p-value <0.01) in the cities of Lanzhou (2.4%), Qingyang (2.2%), and Jiuquan (1.0%).

***L. monocytogenes* serogroups.** The serogroups of *L. monocytogenes* isolates were identified by multiplex PCR (Fig. 2). *L. monocytogenes* isolates were divided into 1/2c (42.9%), 1/2a (35.7%), 1/2b (14.3%), and 4b (7.1%) serogroups. The 1/2c serogroup included two serotypes, 1/2c and 3c. The 1/2a serogroup included 1/2a and 3a serotypes. The 1/2b serogroup was composed of the 3b and 7 serotypes. The 4b serogroup primarily consisted of 4b and 4e serotypes. Combined with the results of multiple PCR and 16s rRNA sequencing analyzed by BLAST, the clusters of the isolates could be identified.

Antimicrobial susceptibility. The antimicrobial susceptibility tests were performed to examine the resistance of isolates (Table III). The results showed that all *L. monocytogenes* isolates were resistant to tetracycline and cefoxitin and sensitive to ofloxacin. Moreover, 92.9% of *L. monocytogenes* isolates indicated a high resistance to penicillin, acetylspiramycin, and erythromycin, and 78.6% were resistant to sulfamethoxazole. The resistance rate of 50.0% of *L. monocytogenes*

Table III
Result of drug susceptibility of *Listeria* spp. isolates.

Antibiotics	*Listeria monocytogenes*				*Listeria innocua*				*Listeria welshimeri*			
	Resistant isolates	Intermediate isolates	Sensitive isolates	Resistance rates (%)	Resistant isolates	Intermediate isolates	Sensitive isolates	Resistance rates (%)	Resistant isolates	Intermediate isolates	Sensitive isolates	Resistance rates (%)
Penicillin	13	0	1	92.9	2	0	20	9.1	3	0	7	30.0
Ofloxacin	1	2	11	7.1	1	13	8	4.6	2	0	8	20.0
Cefoxitin	14	0	0	100	0	0	22	0	2	0	8	20.0
Sulfamethoxazole	11	0	3	78.6	10	1	11	45.5	4	0	6	40.0
Tetracycline	14	0	0	100	14	0	8	63.6	4	0	6	40.0
Gentamicin	3	0	11	21.4	0	0	22	0	1	0	9	10.0
Streptomycin	2	1	11	14.3	5	0	17	23.7	1	0	9	10.0
Erythromycin	13	1	0	92.9	4	4	14	18.2	3	1	6	30.0
Acetylspiramycin	13	0	1	92.9	5	0	17	22.7	3	0	7	30.0
Fosfomycin	7	7	0	50.0	18	0	4	81.8	4	2	4	40.0

isolates to fosfomycin was also observed. *L. innocua* isolates were sensitive to penicillin and gentamicin, whereas they were resistant to fosfomycin (81.8%) and tetracycline (63.6%). The resistance rate for fosfomycin, tetracycline, and sulfamethoxazole in *L. welshimeri* isolates was noteworthy, with a primary resistance rate of 40.0%. In addition, only 10% of *L. welshimeri* isolates were resistant to gentamicin and streptomycin. Overall, the rate of antibiotic resistance of *L. monocytogenes* isolates was higher compared to *L. welshimeri* and *L. innocua* isolates.

Measurement of the growth curve. Four *L. monocytogenes* isolates of different serogroups entered the logarithmic growth phase after 3 h of culture. Serotypes 1/2a and 4b reached the plateau after 20 h, while serotypes 1/2b and 1/2c reached the plateau after 7 h. They apparently showed a faster growth rate compared to serotypes 1/2a and 4b. The *L. monocytogenes* reference strain ATCC® 19111™ (1/2a) entered the logarithmic phase after 8 h incubation and entered the plateau phase after 15 h. The growth rate of the reference strain was significantly slower than that of the four isolates (Fig. 3a). *L. innocua* entered the logarithmic phase between 4 and 10 h and the growth rate began to slow down after 10 to 31 h of culture, and entered the growth plateau phase after 31 h (Fig. 3b). *L. welshimeri* entered the logarithmic phase after 6 h of culture, which lasted until 12 h, entered the slow growth phase from 12 h to 14 h, and then the plateau phase after 14 h (Fig. 3c).

Biofilm formation assay. The results showed that all *Listeria* isolates of could form a biofilm (Fig. 4). Among *L. monocytogenes* isolates, LM7 formed the highest biofilm while LM14 – was the lowest. The average OD_{562} values for serotypes 1/2c, 1/2a, 4b, and 1/2b were 0.134 ± 0.016, 0.151 ± 0.037, 0.1302 ± 0.017, 0.104 ± 0.011, respectively. Thus, significant differences (p-value < 0.05) between different serotypes of *L. monocytogenes* were observed. Analysis of the biofilm formation ability of *L. innocua* isolates showed that LI22 exhibited the highest biofilm-forming ability, whereas LI19 showed the weakest biofilm formation. The average OD_{562} value for *L. innocua* was 0.09198 ± 0.01281. A highly significant difference (p-value < 0.01) was observed between LI22 and other *L. innocua* isolates. For the *L. welshimeri* isolates, LW8 was the strongest biofilm-forming isolate, and LW5 was the weakest. The average OD_{562} value of *L. welshimeri* was 0.09963 ± 0.01257. The significant differences

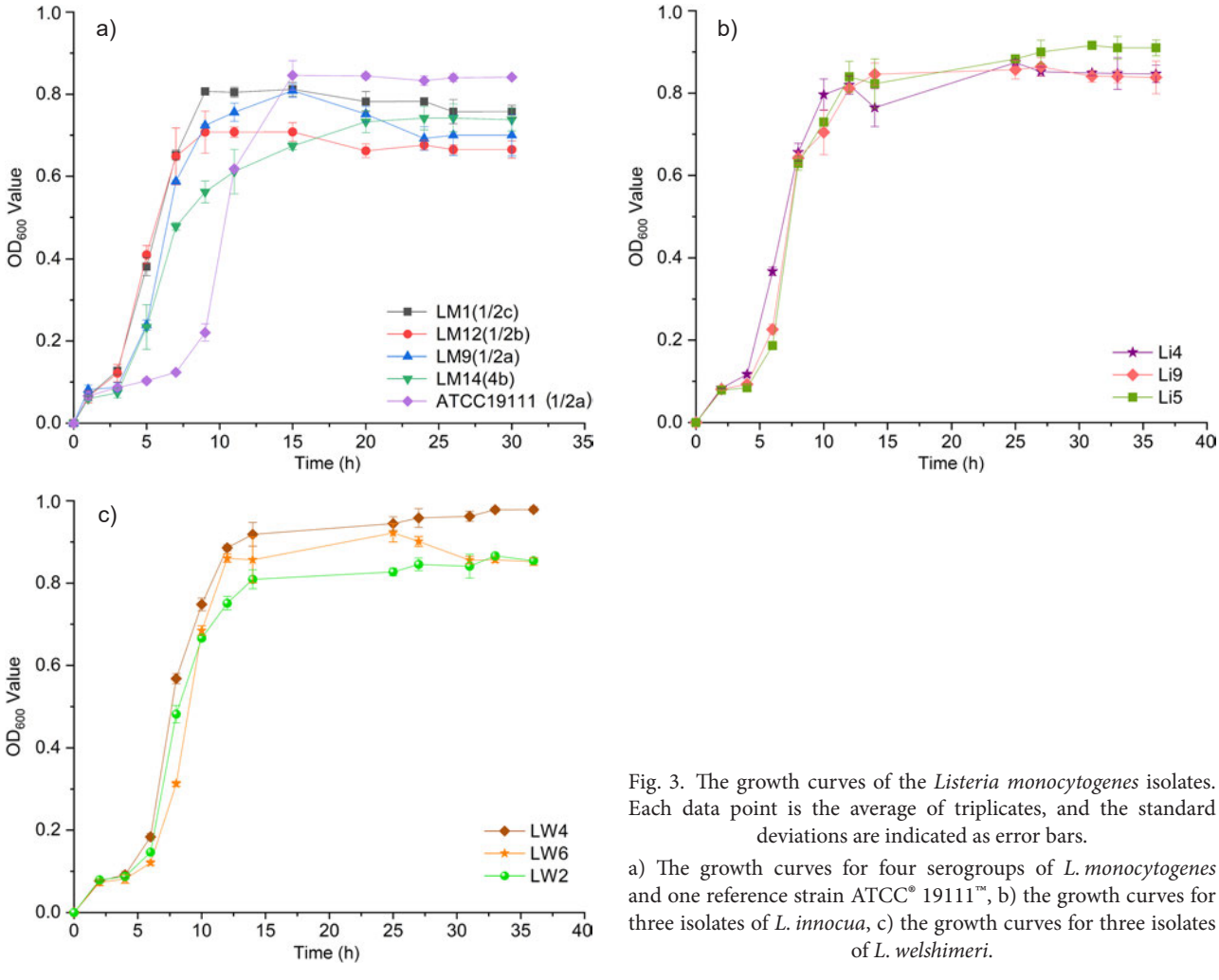

Fig. 3. The growth curves of the *Listeria monocytogenes* isolates. Each data point is the average of triplicates, and the standard deviations are indicated as error bars.

a) The growth curves for four serogroups of *L. monocytogenes* and one reference strain ATCC® 19111™, b) the growth curves for three isolates of *L. innocua*, c) the growth curves for three isolates of *L. welshimeri*.

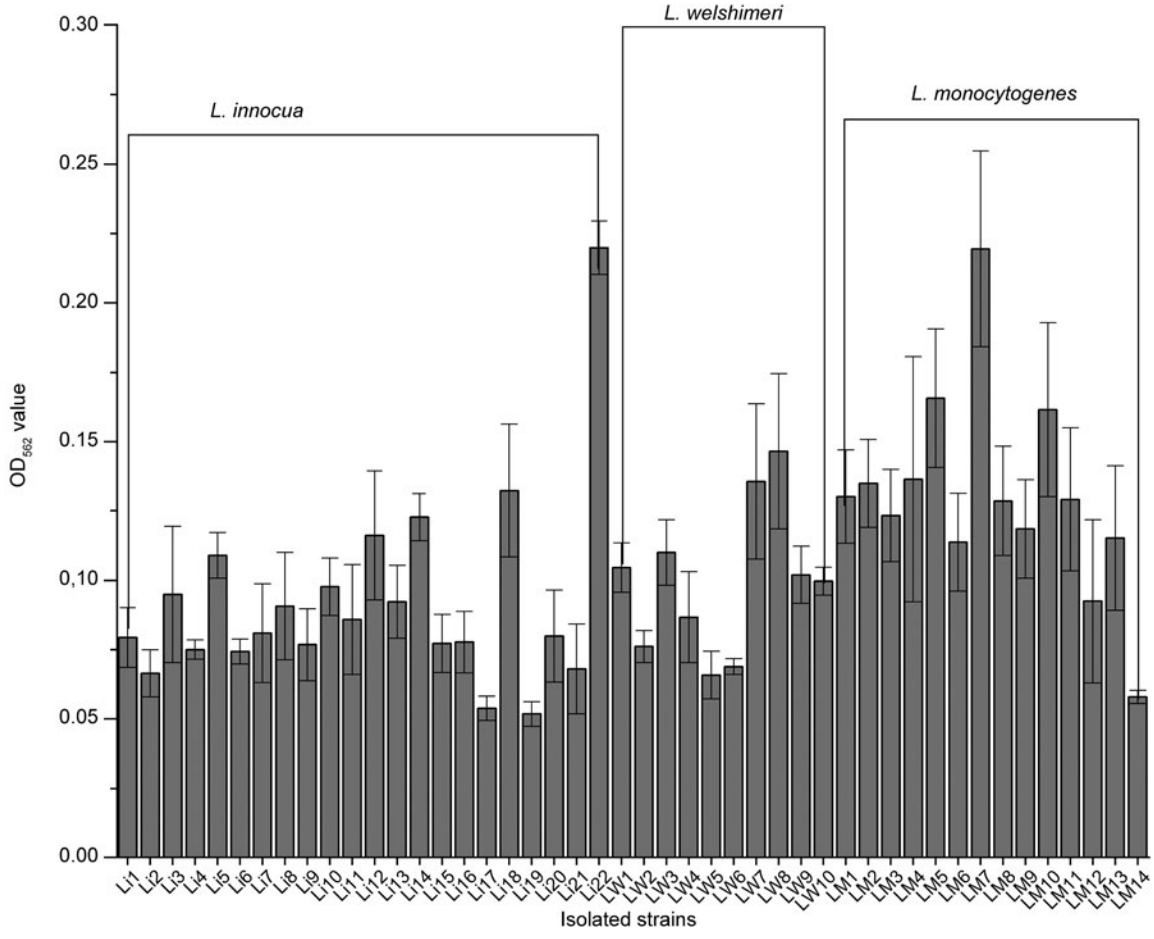

Fig. 4. Biofilm formation by *Listeria* spp. isolates. The absorbance at 562 nm was measured for 22 *L. innocua*, 10 *L. welshimeri*, and 14 *L. monocytogenes* biofilms.

(p-value < 0.05) between different *L. welshimeri* isolates were demonstrated. *L. monocytogenes* isolates formed stronger biofilm than the other two *Listeria* species.

Cluster analysis. Cluster analysis was performed on 16S rRNA sequences of 17 reference strains of *Listeria* spp. downloaded from NCBI and 16 isolates in this study (Fig. 5). The cluster analysis showed that all isolates exhibited a high level of similarity. The 16S rRNA sequence indicated that 33 strains included in genus *Listeria* were comprised of two closely related but distinct clades. The first of the clade included *L. floridensis*, *L. aquatic*, *L. grayi*, and *L. fleischmannii*, the remaining species (including *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri*) constituted the second clade.

Nevertheless, the *L. grayi* strain formed a subline distinct from the other highly interrelated species, especially from *L. innocua* and *L. welshimeri*. Fourteen species of *L. monocytogenes* isolated in this study were very similar to the *L. monocytogenes EGD-e* and *L. monocytogenes strain* ATCC® 19115™. According to the cluster analysis, all three species of *Listeria* spp. isolated in this study belonged to the same cluster as the corresponding reference strains selected from NCBI.

Discussion

Several reports have shown that *Listeria* spp. is prevalent and distributed in food processing environments in China (Du et al. 2017; Wang et al. 2017). However, the prevalence and distribution of *Listeria* spp. from livestock and poultry meat have been rarely reported in China.

In this study, we collected 1,387 samples from different areas in Gansu province. *L. monocytogenes* isolates were mainly present in pork samples in the market environment, and the prevalence of *L. monocytogenes* was 1.0% (14/1,387). This result differs from other provinces of China (Yan et al. 2016; Chen et al. 2020; Wang et al. 2021). The differences stem from the bacteria's isolation rate and the drug-resistant percentage. Differences may be caused by the distribution of microorganisms, sample sources, sampling methods, storage conditions, and hygiene conditions of retailers. We also isolated ten isolates of *L. welshimeri* (0.7%, 10/1,387) and 150 isolates of *L. innocua* (10.8%, 150/1,387) from the collected samples. The prevalence rate of *L. innocua* was much higher than that of *L. monocytogenes* and *L. welshimeri*. This finding is consistent with that of Locatelli et al.

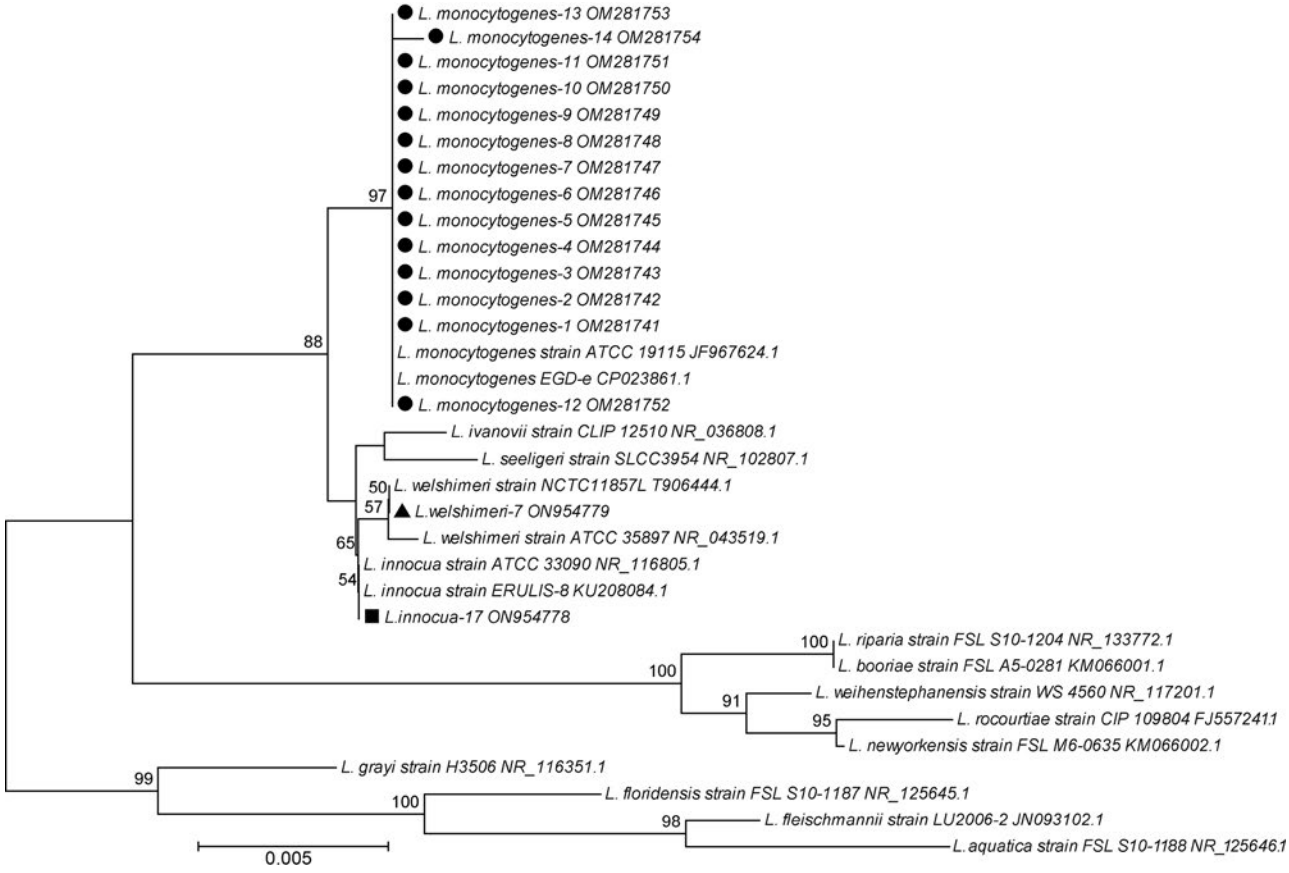

Fig. 5. Cluster analysis of *Listeria* spp. isolates based on the sequences of the 16S rRNA genes. The tree was calculated using the Neighbor-Joining method. Isolates isolated in this study are indicated with circles (*L. monocytogenes*), boxes (*L. innocua*), and triangles (*L. welshimeri*). The scale bar represents 0.005 nucleotide substitutions per character. The numbers in the tree indicate the significance (percent of outcomes) of the branches (bootstrap analysis).

(2017). Although *L. innocua* and *L. welshimeri* are non-pathogenic, the presence of these two species affects the laboratory results on the distribution of *L. monocytogenes* in the natural environment. It is related to the dominant growth of different *Listeria* species in the selective culture medium. Samples with multiple *Listeria* species coexisting during the enrichment step will inhibit *L. monocytogenes* growth (Besse et al. 2010).

Since the first strain of antibiotic-resistant *L. monocytogenes* was isolated in 1988, antibiotic resistance has become more and more serious (Heger et al. 1997). *L. monocytogenes* have special proteins on their cell membranes that can bind penicillin but cannot bind cephalosporins, so they are resistant to cephalosporins (Krawczyk-Balska et al. 2014), which was also confirmed by the results of this study. Meanwhile, all *Listeria* isolated in this study can form biofilms, which warrant bacterial persistence in the environment and antibiotic resistance development. Compared to the antibiotic resistance of *L. monocytogenes* from other places (Li et al. 2016; Fang et al. 2018; Wang et al. 2021), the isolates in this study were resistant to more than five antibiotics, indicating that they were multidrug-resistant isolates. Resistance to penicillin, tetracycline, erythromycin, and cefoxitin appears to be particularly higher in these isolates, illustrating regional variability in the use of antibiotics.

The 14 isolates of *L. monocytogenes* isolated in this study included four serotypes: 1/2a, 1/2b, 1/2c, and 4b. We combined the results of multiplex PCR and analysis of 16S rRNA sequencing on BLAST to distinguish different isolates. Among the isolated isolates, there were 11 isolates of serotypes 1/2a and 1/2c, the main serotypes of *L. monocytogenes*. Li et al. reported that strains of serotype 4b mainly exist in patients infected with listeriosis. However, this serotype was also detected in animal-derived food in this study, indicating that this serotype is widely distributed (Li et al. 2016). Among the different samples, the chicken meat was the most contaminated with *L. monocytogenes* serotype 1/2a. Most human diseases related to listeriosis, including septicemia, meningitis, and abortion were caused by only three serotypes, 1/2a, 1/2b, and 4b (Datta and Burall 2018). In China, 253 invasive listeriosis cases were reported in 19 provinces from 2011 to 2016, with a fatality rate of 25.7% (Li et al. 2018b).

The 16S rRNA gene is considered a molecular marker in bacterial molecular classification. Orsi and

Wiedmann (2016) divided *Listeria* into two groups based on their genetic relationship. One group included *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*. Another group included *L. grayi* and some other newly discovered species of *Listeria* (Orsi and Wiedmann 2016). This result is the same as that obtained from our previous study. However, some scholars believe that except for *L. grayi*, the 16S rRNA sequences of other members have a high similarity and thus are not suitable as a genetic marker gene in *Listeria* (Czajka et al. 1993). As a result, the 23S rRNA, *iap*, *ldh*, *prs* and *vclB* were also used as potential genetic markers to illustrate the phylogenetic relationship of *Listeria* (Schmid et al. 2005). With the evolution of the *Listeria* virulence gene cluster, some of the *Listeria* species have lost their pathogenic ability, such as *L. innocua* and *L. welshimeri*. The location of the *L. innocua* and *L. welshimeri* virulence gene cluster breakpoints is also consistent with this view.

Conclusions

This research investigates the prevalence of *Listeria* spp. in livestock and poultry meat in Gansu province for the first time. This study provides strong evidence for the prevalence of *Listeria* species in slaughterhouses and markets. Although the isolation rate of *L. monocytogenes* is low, four serogroups were identified with high multidrug resistance and biofilm-forming ability. The above phenomena bring great difficulty in controlling *L. monocytogenes* contamination in this area. Therefore, it is necessary to strengthen and standardize the management of processing, transportation, sales of products, rationally use antibiotics, and regular monitor the bacteria to reduce the threat of listeriosis. In addition, ecological symbiosis and phenotypic similarity existed among the three *Listeria* species. *L. innocua* and *L. welshimeri* can be used to predict the physiological behavior and contamination level of *L. monocytogenes*. Our study provides the scientific basis for preventing and controlling listeriosis in higher-risk groups and susceptible animals.

Ethical statement

All experiments were approved by the College of Veterinary Medical, Gansu Agricultural University, China.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Inactivation of *Lactobacillus* Bacteriophages by Dual Chemical Treatments

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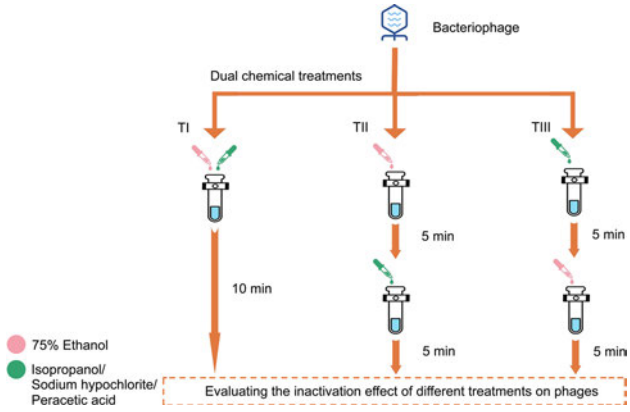
Abstract

Phage contamination is one of the significant problems in the food fermentation industry, which eventually causes economic losses to the industry. Here, we investigated the viability of *Lactobacillus plantarum* phage P1 and P2 using various biocides treatments (ethanol, isopropanol, sodium hypochlorite and peracetic acid). Results indicated that phage P1 and P2 could be completely inactivated by treatment with 75% ethanol for 5 min, followed by 400 ppm of sodium hypochlorite treatment for 5 min. Phage P2 could be completely inactivated in the reverse sequence, while 800 ppm of sodium hypochlorite was required to achieve a similar effect for phage P1. Moreover, 100% isopropanol could increase the inactivating effect of 75% ethanol. This study may provide basic information on using multiple antimicrobials for phage control in laboratories and food plants.



K e y w o r d s: *Lactobacillus plantarum* phage, combined chemical treatments, successive chemical treatments, alcohol disinfectant, peracetic acid, sodium hypochlorite

Introduction

Lactobacillus plantarum is a recognized probiotic conferring various health effects to the host, including cholesterol lowering, management of gastrointestinal disorders and diarrhea, and prevention of irritable bowel syndrome (Seddik et al. 2017). *L. plantarum* is also widely used as a starter culture in fermented food products, such as cheese, kefir, sauerkraut, grape juice, and other beverages (Wang et al. 2020; Mirmohammadi et al. 2021).

Like other lactic acid bacteria (LAB), *L. plantarum* strains are easily infected by bacteriophages, which can result in fermentation failure. Due to their ubiquitous presence and the constant maintenance in bacterial populations, bacteriophages can pose substantial financial

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losses in the food fermentation industry by altering the quality of finished products or by delaying the manufacturing processes (Połaska and Sokołowska 2019). Diverse strategies to control phages in food plants have been reported, including physical treatments (heating, filtration, high pressure, UV radiation, electro pulsing), chemical treatments (biocides, biological compounds), strain rotation or use of strains with improved phage resistance (Pujato et al. 2018). However, many reports have indicated that the inactivating effect of a single chemical biocide was limited (Guglielmotti et al. 2012b; ; Murphy et al. 2014; Briggiler Marcó et al. 2019).

Methods to improve the effect of biocides on viruses by using complex treatments, such as chemical inactivation with UV radiation, synergistic chemical inactivation, and synergistic inactivation with UV and ozone, have already been reported. Pujato et al. (2014) found that 0.15% peracetic acid and 600–800 ppm of sodium hypochlorite had a good inactivation effect on *Lactobacillus mesenterium* bacteriophage. Kim et al. (2015) reported that the single treatment of 1% trisodium phosphate or 30% ethanol for 30 min was not effective on murine norovirus 1 (MNV-1), but cotreatment showed effective inactivation of MNV-1 on stainless steel after 15 min. Therefore, the combined treatment is an alternative.

L. plantarum phage P1 and phage P2 are phages isolated from the abnormal fermentation liquid of *L. plantarum* IMAU10120. They belong to *Siphoviridae* family. Phage P1 has an isometric capsid (71.7 ± 3.0 nm) and a long noncontractile tail (272 ± 3.0 nm long, 11.3 ± 1.5 nm wide) (Chen et al. 2016). Its whole genome was composed of linear dsDNA, with 73,787 bp in length and 86 predicted CDs. The GC content was 39.17% (Guo et al. 2022). When compared to phage P1, phage P2 was smaller, with an isometric capsid about 66.7 ± 3.0 nm and a long noncontractile tail (216.7 ± 3.0 nm long, 12.3 ± 3.0 nm wide) (Chen et al. 2019). Genome analysis showed that the whole genome of phage P2 was composed of linear dsDNA, with a length of 77,937 bp, 39.28% GC content, and 96 CDs (Zhu et al. 2022). Our previous studies demonstrated that these two phages possessed relatively high resistance to temperature and a single biocide treatment. Even for the most efficient biocide (sodium hypochlorite), a high concentration (800 ppm) and a long time (30 min or 60 min) were required to inactivate the phages (Chen et al. 2017; Chen et al. 2018). In 2007, Scheffler et al. (2007) found that PAA-ethanol (PES) can efficiently inactivate pseudorabies virus (PRV) or porcine parvovirus (PPV). In 2020, Hassaballah et al. (2020) reported that the total disinfection time for the bacteriophages added to the treated wastewater could be reduced from 1 h to 12.5 min through the combined treatment of peracetic acid (PAA) and ultraviolet light. So, the objective of this study was to investigate the inactivating effects of dual chemical treatments for the control of *Lactobacillus* phages P1 and P2 in plants and laboratories.

Experimental

Materials and Methods

Bacterial strain, phages, and culture conditions. *L. plantarum* IMAU10120, phages P1 and P2 were obtained from the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, P.R. China.

L. plantarum IMAU10120 was cultured at 37°C using de Man, Rogosa, and Sharpe (MRS) broth for 24–48 h. The media were supplemented with 10 mmol/l $CaCl_2$ for phage propagation and enumeration using a double-layer plaque titration method (Capra et al. 2018).

Treatment using combined biocides (TI). Ethanol (75%, 450 µl) was added to isopropanol (450 µl) at various concentrations of 10%, 30%, 50%, and 100% in a series of microfuge tubes to obtain a final alcohol concentration as 38.3%, 47.3%, 56.3%, and 78.8%, respectively. The final concentration of the alcohol solution was calculated according to the equation described by Kalua and Boss (2008). Phage (10^8 PFU/ml, 100 µl) was mixed with each suspension (1 ml of final volume) and incubated at 25°C for 10 min. After dilution with saline buffer (0.85%), survivors were counted using a double-layer plaque titration method. Similar protocols were used with sodium hypochlorite (ppm): 100, 200, 400, and 800 or PAA (%, v/v): 0.15, 0.25, 0.45 with a ratio of 1:1.

Successive biocide treatments (TII, TIII). TII: Phages (10^8 PFU/ml, 100 µl) were mixed with ethanol (75%, 450 µl) in microfuge tubes and incubated at 25°C for 5 min. Then, 450 µl isopropanol at different concentrations (v/v, 10%, 30%, 50%, 100%) was subsequently added. After incubation at 25°C for 5 min and dilution with saline buffer (0.85%), surviving phage particles were enumerated using the abovementioned methods. Similar protocols were used with sodium hypochlorite (ppm): 100, 200, 400, 800 or PAA (%, v/v): 0.15, 0.25, 0.45.

TIII: The treatment time was the same as TII, but the inactivation sequence of 75% ethanol and isopropanol, sodium hypochlorite, or peracetic acid solutions at different concentrations, as mentioned above, was reversed, respectively.

Statistical analysis. All experiments were replicated three times. Treatments using a single biocide with the same concentration were used as control. All data were analyzed using the Originpro software (version 8.6; Originlab, USA). Means were compared using a one-

way ANOVA followed by IBM SPSS Statistics 20.0 software (IBM Corp, USA). Significance was determined at $p < 0.05$. Duncan's multiple range test was used to separate the means.

Results and Discussion

Antimicrobial effect of ethanol and isopropanol. Chemical biocides, including alcohols, sodium hypochlorite, quaternary ammonium chloride compound, and peracetic acid, sanitize and control microbial growth on the equipment and food contact surfaces.

As one of the most used biocides, ethanol has been reported to destroy or denature enzymes and microbial proteins in cell walls. The ethanol concentration, similarly to most biocides, determines whether its action is biocidal or static. Low (< 20%) concentrations will limit its effect and are usually biostatic (Kim et al. 2020). Moderate concentrations (60–85%) can quickly solidify microbial proteins resulting in a biocidal effect (Sauerbrei 2020). However, when the concentration is too high (> 90%), it can form films on the microbial surface, thereby influencing its effect (Setlow et al. 2002; Zhang et al. 2012).

Concerning phages, biocides affect the protein structure in the capsid (Sato et al. 2016). It was reported that ethanol and isopropanol could denature proteins, which is destructive to most viruses (Maillard 2002; Mahony and Sinderen 2015; Boyce 2018). However, the previous studies found that neither ethanol nor isopropanol could completely inactivate phage P1 and P2, even after a 60 min treatment. Nevertheless, the inactivation effect of isopropanol was slightly better than that of ethanol (Chen et al. 2017; 2018). To explore whether isopropanol and ethanol could mutually improve the inactivating effect on phages, the survival counts of *L. plantarum* phages P1 and P2 after the combined and successive chemical treatments with ethanol and isopropanol are shown in Fig. 1.

In dairy industries, sanitization between fermentations is a critical step in controlling the phage contamination (Hayes et al. 2017). It employs purpose-made chemical sanitizers for the physical and chemical removal of phages and other microbial contaminations (Pujato et al. 2018). For biocides to be considered eligible to use in the dairy industry, several criteria must be met. For example, in Europe, sanitizers must have a proven ability to reduce phage numbers by at least four logs under the recommended test conditions before they can be deemed suitable for phage inactivation (European Committee for Standardization (CEN), CEN/TE-216) (Bolten et al. 2022).

As illustrated in Fig. 1, combined or subsequent treatments with ethanol and isopropanol could not completely inactivate phages P1 and P2. However, isopropanol could enhance the biocidal effect of 75% ethanol to a certain extent. Ethanol (75%) or isopropanol (100%), when used alone for 10 min, reduced P1 by

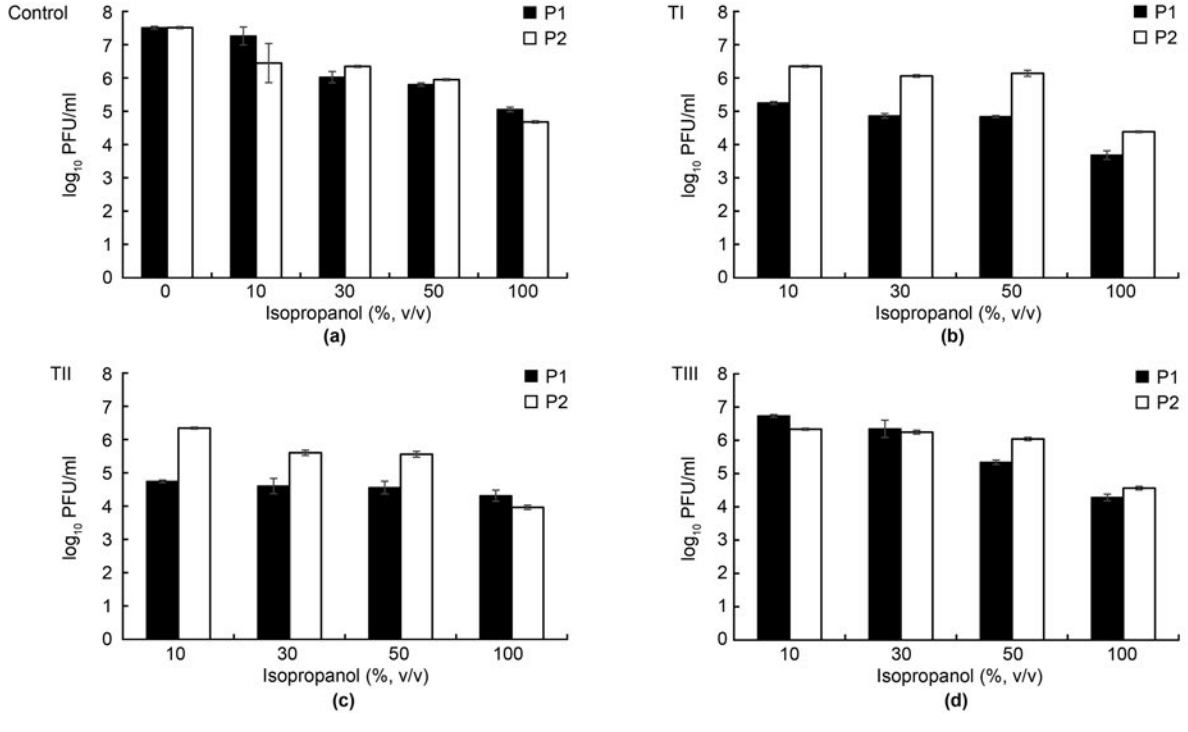

Fig. 1. Viable count of phages treated with 75% ethanol and isopropanol.
a) Treatment with isopropanol for 10 min; b) treatment with 75% ethanol and isopropanol for 10 min; c) treatment with 75% ethanol for 5 min followed by isopropanol for 5 min; d) treatment with isopropanol for 5 min followed by 75% ethanol for 5 min.

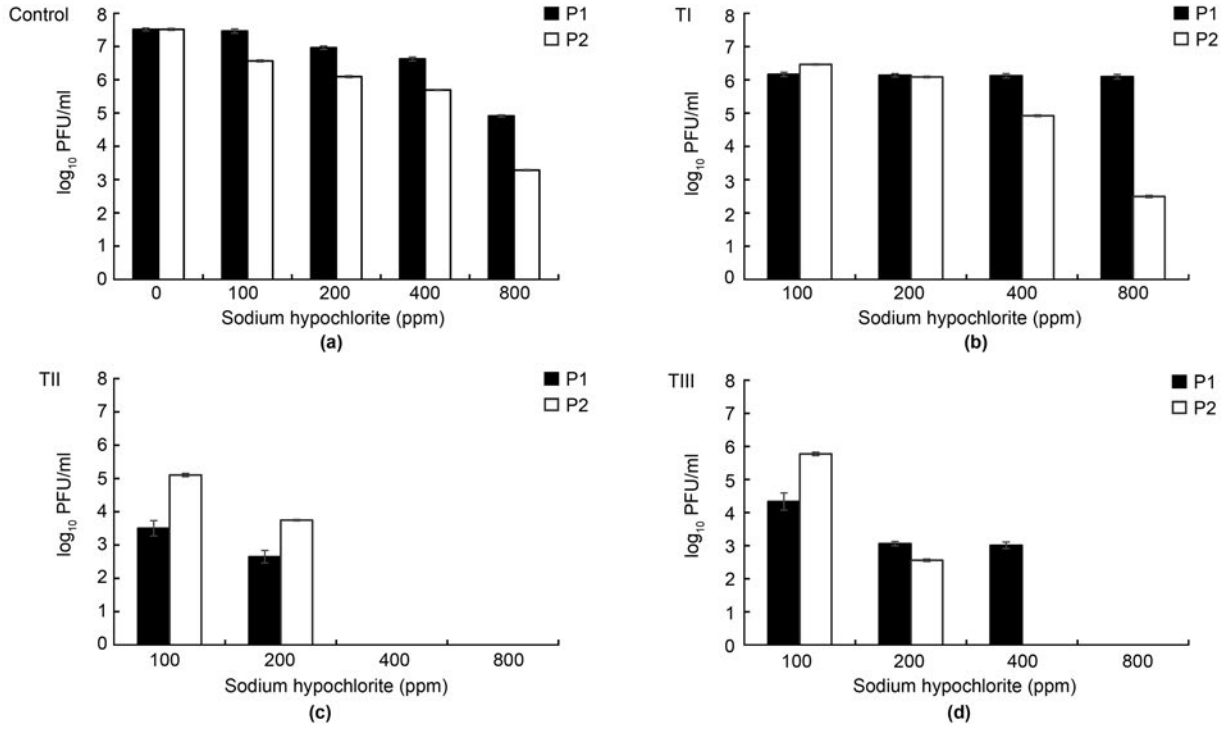

Fig. 2. Viable count of phages treated by 75% ethanol and sodium hypochlorite.
a) Treatment with sodium hypohlorite for 10 min; b) treatment with 75% ethanol and sodium hypohlorite for 10 min; c) treatment with 75% ethanol for 5 min followed by sodium hypohlorite for 5 min; d) treatment with sodium hypohlorite for 5 min followed by 75% ethanol for 5 min.

2.45 and 2.46 log (Fig. 1a), respectively. However, when phages were treated with a mixture of 100% isopropanol and 75% ethanol for 10 min, a 3.83 log reduction was obtained (Fig. 1b). Similar results were observed for phage P2, as the treatment with ethanol (75%) for 5 min, followed by the treatment with isopropanol (100%) for 5 min, was even more effective, resulting in a 3.55 log reduction (Fig. 1c).

Both ethanol and isopropanol have been reported to destroy most lipophilic viruses (Maillard 2002; Mahony and Sinderen 2015). Although isopropanol (100%) can enhance the effect of ethanol (75%) on phages P1 and P2, the augmenting effect is limited. As reported, isopropanol can interact with microorganisms in aqueous solutions to form $(CH_3)_2CHOH(H_2O)_n$, or isopropanol water clusters, where the n value represents the amount of H_2O molecules. The structures of water clusters formed by isopropanol in various electrostatic environments are different, providing differences in the structural stability of water clusters, thus having diverse effects on microorganisms (Han et al. 2017; Zhu et al. 2019). Different phages will produce different electrostatic environments in the solution. The charge density is a significant characteristic of each phage, which mainly depends on the nature of the phage capsid or tail protein and the difference in genetic material (Hernando Pérez et al. 2015; Cooper et al. 2022). In our previous research, the morphology of phages P1 and P2 were similar, but their structures were slightly different. The tail fibrin (CDs18) of phage P1 was different from that encoded in phage P2's genome (Guo et al. 2022). The two electrostatic environments caused by different tail fibrin may enable isopropanol to form isopropanol-water clusters with different stability in aqueous solution, thus affecting the biological efficacy of isopropanol on phages P1 and P2.

Antimicrobial effect of ethanol and sodium hypochlorite. The combined and successive effects of ethanol and sodium hypochlorite on phage P1 and phage P2 are shown in Fig. 2.

The phage treatment with 400 ppm of sodium hypochlorite for 10 min resulted in a 0.89 log reduction for phage P1 (Fig. 2a). As expected, the number of phage P1 survivors decreased significantly ($p < 0.05$) with increased sodium hypochlorite concentrations. Phage P1 was completely inactivated by successive treatment of 75% ethanol and 400 ppm sodium hypochlorite. The successive treatment of 800 ppm of sodium hypochlorite and 75% ethanol could completely inactivate phage P1, regardless of the chemical sequence. Similarly, for phage P2, the number of survivors significantly decreased when 75% ethanol was used with sodium hypochlorite. A 1.82 log reduction was achieved when phage P2 was treated with 400 ppm of sodium hypochlorite alone for 10 min (Fig. 2a). However, it was inactivated by a successive treatment of 75% ethanol and 400 ppm of sodium hypochlorite, regardless of the chemical sequence (Fig. 2c and 2d).

Sodium hypochlorite is a highly effective sanitizer. However, the efficacy of sodium hypochlorite is affected by several factors, such as pH, temperature, and organic matter. Lowering the pH, increasing the temperature, and reducing the organic load increase its antimicrobial effect (Cai et al. 2016). Hypochlorite acts directly on DNA. In this respect, it has been shown that *in vitro*, sodium hypochlorite can interact with DNA, forming nucleotide chloramines, leading to the destruction of the nitrogenous base ring structures (Osinnikova et al. 2019). As mentioned, our previous studies showed that sodium hypochlorite could be considered an efficient biocide that could inactivate phages P1 and P2 under high concentration and long time (phage P1: 800 ppm for 60 min; phage P2: 400 ppm for 50 min or 800 ppm for 30 min) (Chen et al. 2017; 2018). From the results showed in Fig. 2, we could observe that the successive treatments of 75% ethanol and 400 ppm or 800 ppm of sodium hypochlorite provided apparent synergistic inactivating effects on phage P1 and phage P2. It might occur due to the protein structure of the phage capsid, which was affected by ethanol, further exposing the nucleic acid within the virus head to sodium hypochlorite.

Sodium hypochlorite hydrolyzed in water form hypochlorous acid, which is lethal for most microbes. It has been demonstrated to interfere with carbohydrate metabolism, oxidize protein, and target phosphate dehydrogenase (Cho et al. 2010; Sato et al. 2016). Sodium hypochlorite also inactivates *Pseudomonas aeruginosa* phage F116 by causing structural changes in the head, tail, and overall structure. In this respect, nucleic acids were released from the damaged capsid and were detected in the surrounding medium (Maillard et al. 1998). Generally, the food industry application standard for sodium hypochlorite is 200 ppm (Briggiler Marcó et al. 2009).

As reported, 100–200 ppm sodium hypochlorite had a good inactivation effect on some phages, such as *Lactococcus lactis* phages QF12 and P001 and *Lactobacillus delbrueckii* phage Cb1/204 (Suárez and Reinheimer 2002; Ebrecht et al. 2010; Guglielmotti et al. 2012a). However, due to the specificity of phages, different phages express diverse resistance to sodium hypochlorite, and some phages possess higher tolerance to sodium hypochlorite. Quiberoni et al. (2003) reported that *L. delbrueckii* bacteriophage Ib3 isolated from yogurt could be totally inactivated when exposed to 1,200 ppm of sodium hypochlorite for 45 min. In 2010, Capra et al. (2004) showed that *Lactobacillus paracasei* bacteriophage PL-1 and *Lactobacillus casei* bacteriophage J-1 could be completely inactivated in 800 ppm of sodium hypochlorite within 5 min. Reducing the concentration to 700 ppm was insufficient to achieve a complete inactivation effect within 45 min. Murphy et al. (2014) found that nine of 11 936-type phages isolated from cheese had an average survival rate of over 96% after exposure to 800 ppm of sodium hypochlorite for 30 min. Some bacteriophages even can tolerate much higher concentrations. It has been proved that bacteriophage φpll36 isolated from Turkish dairy factory was inactivated after the exposure to 2,000–3,000 ppm of sodium hypochlorite for 20–30 min (Dilek Avsaroglu et al. 2007).

Genomic differences between phages P1 and P2 may account for the variation in resistance. As reported, sodium hypochlorite can inactivate phages by acting on their protein structures or genome. It can cause the inactivation of coliphage MS2 through genome damage. In contrast, the inactivation *Pseudomonas* Phi6 is driven by its reaction with proteins in the nucleocapsid and polymerase complex rather than with genome and lipids (Ye et al. 2018). The sensitivity of viruses to free chlorine depends on the similarity of the amino acid sequences in capsid proteins. Typical amino acid substitutions between genogroup A and genogroup B genomes of CVB4 affect the chlorine reactivity of attachment sites, resulting in different chlorine tolerance of genogroup A and genogroup B viruses of CVB4 (Torii et al. 2022).

Our previous studies have shown 11 gene differences between these two phages, including eight putative proteins, one tail fibrin, and two HNH endonucleases. Moreover, phage P2 encoded more putative proteins with unknown functional gene sequences (Guo et al. 2022; Zhu et al. 2022). The difference in protein structure and the gene sequence coding proteins may be why phages P1 and P2 have different tolerance to sodium hypochlorite. Similar to our study, Briggiler Marcó et al. (2009) reported that sodium hypochlorite (800 ppm) could completely inactivate phage B2 of *L. plantarum* in 15 min but could not inactivate phage B1, FAGK1, and FAGK2 from the same isolation source and host bacteria under the same conditions. Phages B1 and B2 shared the same host strain *L. plantarum* ATCC 8014, belonging to the *Siphoviridae* family. Genome analysis presented that the whole genome of phages B1 and B2 were composed of linear dsDNA, of a length of 38,002 bp and 80,618 bp, and GC content of 47.6% and 37.0%, containing 60 ORFs and 127 ORFs, respectively.

Interestingly, they also had tail fibrin and different HNH endonucleases. So, genomic differences may influence the tolerance of phages to chemical biocides treatments. Phages isolated from the identical source and the same host might exhibit different tolerances to sodium hypochlorite. Inactivation effects could mainly depend on the nature of the phages (Briggiler Marcó et al. 2009).

Antimicrobial effects of ethanol and peracetic acid. Compared with ethanol and chlorine, peracetic acid (PAA) is considered a relatively new sanitizer. PPA is normally used at a concentration of 0.15% (v/v) in

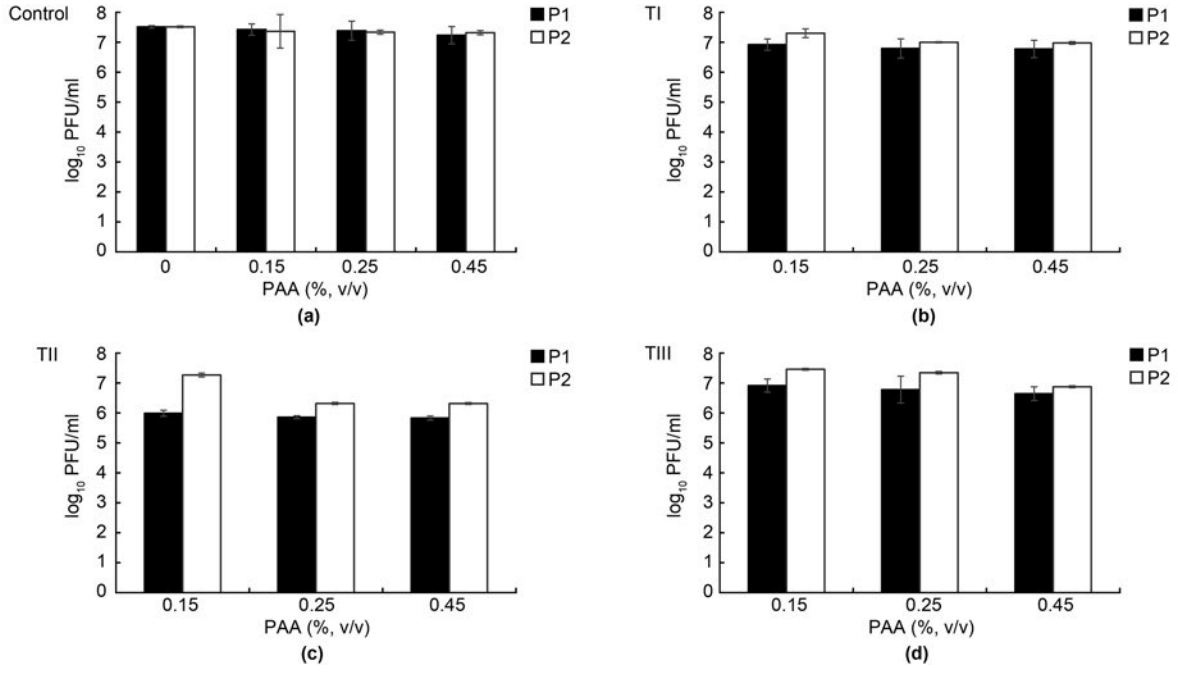

Fig. 3. Viable count of phages treated by 75% ethanol and peracetic acid (PAA).
a) Treatment with PAA for 10 min; b) treatment with 75% ethanol and PAA for 10 min; c) treatment with 75% ethanol for 5 min followed by PAA for 5 min; d) treatment with PAA for 5 min followed by 75% ethanol for 5 min.

water; the low pH of this aqueous solution (around 2) has been suggested to be responsible for phage inactivation (Mercanti et al. 2012). Nevertheless, at the same time, PAA, due to its oxidation characteristics, has a strong corrosive effect, a pungent smell, and is even dangerously explosive (Horn and Niemeyer 2022). Therefore, the use of the concentrated PAA solution has limitations.

As shown in Fig. 3, PAA was not an effective biocide against phages P1 and P2, even at a concentration of 0.45%. Phages P1 and P2 treated with the solution at a concentration of 52.6% and 63.8%, respectively, remained viable after a 10 min-treatment. Moreover, PAA did not increase the biocidal effect of ethanol (75%). As shown in Fig. 3, even the highest concentration of PAA resulted in the lowest survival rate (TII) of 2.21%. Phage P2 appeared to exhibit a higher tolerance to PAA than P1, which still exhibited a survival rate of 6.33% under the same treatment. In contrast, ethanol (75%) increased the phagocidal effect of PAA.

Peracetic acid exerts a strong oxidizing effect on proteins. It exerts a rapid bactericidal effect on various microorganisms, including spores and viruses (Yeap et al. 2015; Zonta et al. 2016). The treatment with peracetic acid (0.15%) for 60 min had little effect on the survival of P1 while increasing the concentration to 0.45% for 60 min resulted in only a 4.0 log reduction in the number of phages (Chen et al. 2017). Phage P2 demonstrated greater resistance to peracetic acid than P1. At the highest concentration evaluated (0.45%), the viability of phage P2 decreased by only 1.40 log within 60 min (Chen et al. 2018). Ethanol may accelerate the destruction of viruses by PAA, enhancing the mobility of PAA molecules in the disinfectant solutions. The combination of PAA (0.2%) and ethanol (80%) could result in a 4.0 log reduction of the poliovirus type 1 number in 1 min. A comparable virucidal effect could not be achieved with 80% ethanol, even if the exposure time was prolonged to 30 min (Wutzler and Sauerbrei 2000). However, like in the previous research results, PAA might not have a good bactericidal effect on phages P1 and P2, even when used with ethanol. In this respect, only phages with sulfur-containing amino acids (such as cysteine and methionine) in their capsid proteins show strong sensitivity to PAA (Schmitz 2021). The number and types of sulfur-containing amino acids in the capsid proteins of phages P1 and P2 must be further determined. In addition, differences in the protein configuration in the capsid brought about by secondary, tertiary, and quaternary folding may expose the different and sensitive abilities to the action of PAA (Mayer et al. 2015). The present study found that when PAA was mixed with ethanol (75%), the biocidal effect was not improved even at the highest concentration (0.45%).

In contrast, ethanol (75%) improved the biocidal effect of PAA, especially for P2. During the first 5 min of application, ethanol (75%) impacted the protein capsid allowing PAA more accessible contact with sulfur-containing groups. However, the present results also demonstrated that with the increase in PAA concentration, the biocidal effect was not increased significantly. Phages P1 and P2 may contain less PAA-sensitive target sites.

Conclusions

The dual inactivating effects of various biocides on phages P1 and P2 were evaluated. Results showed that the phages could be completely inactivated in 10 min after being treated successively with 75% ethanol and 400 ppm of sodium hypochlorite. Compared to a single biocide treatment, successive treatments could reduce the biocide's concentration and shorten the inactivation time. This study might provide some basis for controlling phage infections in laboratories and dairy plants.

Acknowledgments

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Characterization of a Plant Growth-Promoting Endohyphal *Bacillus subtilis* in *Fusarium acuminatum* from *Spiranthes sinensis*

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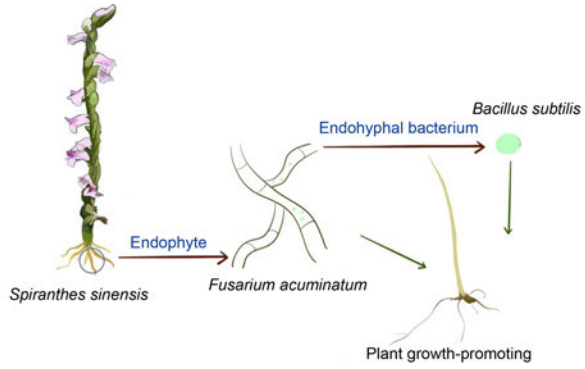
Abstract

Successful seed germination and seedling growth in orchids require an association with mycorrhizal fungi. An endophytic *Fusarium* fungal strain YZU 172038 exhibiting plant growth-promoting (PGP) ability was isolated from the roots of *Spiranthes sinensis* (Orchidaceae). The harboring endohyphal bacteria were detected in the hypha by SYTO-9 fluorescent nucleic acid staining, fluorescence *in situ* hybridization (FISH), and PCR amplification of the 16S rDNA gene's region. Consequently, one endohyphal bacterium (EHB) – a strain YZSR384 was isolated and identified as *Bacillus subtilis* based on morphology, phylogenetic analysis, and genomic information. The results indicated that the strain YZSR384 could significantly promote the growth of rice roots and shoots similar to its host fungus. Its indole acetic acid (IAA) production reached a maximum of 23.361 μg/ml on the sixth day after inoculation. The genome annotation revealed several genes involved in PGP traits, including the clusters of genes encoding the IAA (*trpABCDEFS*), the siderophores (*entABCE*), and the dissolving phosphate (*pstABCS* and *phoABDHPR*). As an EHB, *B. subtilis* was first isolated from endophytic *Fusarium acuminatum* from *S. sinensis*.



K e y w o r d s: endohyphal bacterium, *Fusarium acuminatum*, *Bacillus subtilis*, plant growth-promoting ability

Introduction

Orchid *Spiranthes sinensis* (Pers.) Ames is a Chinese herbal medicine which is rich in flavonoids, phenylpropanoids, and dihydrophenanthrene compounds (Lin et al. 2000; Peng et al. 2007; Peng et al. 2008). It can be used for inflammatory, cancer, diabetic, and other diseases (Gutiérrez 2010; Shie et al. 2015). Because the tiny seeds lack endosperm for successful germination and seedling growth, the orchids, including *S. sinensis*, require an association with endophytic fungi (Zhao et al. 2021). Endophytic fungi enhance nutrient availability to orchid roots and help them survive even upon exposure to pathogens (Sarsaiya et al. 2019). Besides, the fungi provide secondary metabolites promoting seed germination and seedling differentiation (Bhatti and Thakur 2022).

The endophytes in orchids are mostly specific fungi of Basidiomycota, such as Sebacinaceae, Ceratobasidiaceae, and Tulasnellaceae (Zettler and Corey 2018; Li et al. 2021; Wang et al. 2021). In addition, some endophytes from the root, especially *Fusarium* (Ascomycota), are thought to form mycorrhizae with orchids (Tian et al. 2022). Chen et al. (2010) found that *Fusarium* is the dominant endophytic fungi in the medicinal orchid *Dendrobium loddigesii* and could improve its growth rate and biomass. The mycorrhizal fungus *Fusarium oxysporum* of *Bletilla striata* can colonize the root of *B. striata* and *Dendrobium candidum* and promote the vegetative growth of both orchids (Jiang et al. 2019a).

Fusarium has been reported to comprise endohyphal bacteria (EHBs) (Li et al. 2010; Shaffer et al.

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2017), even in orchid root endophytic fungi (Cheng et al. 2022). EHBs inhabiting fungal hyphae were found in Mucoromycota (Desirò et al. 2018), Glomeromycota (Naumann et al. 2010), Ascomycota (Pakvaz et al. 2016), and Basidiomycota (Aslani et al. 2018). EHB can influence the ability of fungal hosts, then the interaction between fungi and their plant hosts by providing bioenergy for fungi (Ghignone et al. 2012; Salvioli et al. 2016). EHB stimulates fungi to produce more phytohormone indole-3-acetic acid (IAA), promoting plant growth (Hoffman et al. 2013; Cheng et al. 2022), regulating fungal secondary metabolism (Partida-Martinez and Hertweck 2005), and enabling fungi to fix nitrogen (Ruiz-Herrera et al. 2015).

During a screening of EHB in the root-associated endophytic fungi of *S. sinensis*, strain YZU 172038 of *Fusarium acuminatum* was found to contain EHB. The aim of this study was to verify the phenomenon of EHB harboring, the EHB isolation and identification, and to determine the PGP ability of the strain YZU 172038 and its EHB.

Experimental

Materials and Methods

EHB detection. The endophytic *F. acuminatum* YZU 172038 of *S. sinensis* was obtained from Pei et al. (2019). It was inoculated on potato dextrose agar (PDA) and cultured at 28°C to collect the mycelia. To determine the presence of bacteria in fungal hyphae, fresh mycelia were picked from two-day-old PDA colonies, stained with 100 µl of 10 µmol/l SYTO-9 green fluorescent nucleic acid solution, well mixed, and incubated for 15–30 min in the dark. Finally, the stained hyphae were observed under the Nikon ECLIPSE Ni-U fluorescence microscope (Nikon, Japan) (Obasa et al. 2017). Fluorescence *in situ* hybridization (FISH) was used to detect further the presence of bacteria in hyphae of *F. acuminatum* YZU 172038. The sample was fixed in 500 µl 4% formalin, dissolved in phosphate-buffered saline (PBS), incubated at 4°C for 5–6 h, washed twice with 500 µl PBS solution in 1.5 ml centrifuge tube, and then dehydrated in solutions of anhydrous ethanol (50%, 70%, and 95%) for 3 min. Dehydrated mycelium was mixed with 10–100 µl probe solution (400 µl formamide, 350 µl diethyl pyrocarbonate (DEPC) water, 250 µl EDTA and 1 µl 10 µM EUB338 probe) and hybridized at 46°C for 1.5 h. A universal 16S rRNA gene oligonucleotide probe of EUB338 (5'-GCTGCCTCC CGTAGGAGT-3') was labeled at the 5-end with FAM (green). Each sample was rinsed in 100 µl wash buffer (460 µl 5 M NaCl, 1 ml 1 M Tris, 50 µl 10% SDS, DEPC water, filled to 50 ml) and warmed to 46°C twice (Hoffman and Arnold 2010). Finally, the mycelium was placed on a slide with the anti-fluorescence quenching agent and observed under the fluorescence microscope.

Meanwhile, the fungal DNA was extracted by the CTAB method (Stenglein and Balatti 2006). The PCR amplification of 16S rDNA region for the genomic DNA was performed using primers of ER10 (5'-GGCGGACGGGTGAGTAA-3') and ER11 (5'-ACTGCTGCCTCCCGTAG-3') to examine its harboring endohyphal bacteria. It was carried out in a T100TM thermal cycler (BIO-RAD, USA) with the following parameters: initial denaturing for 30 s at 94°C, then 30 cycles at 94°C for 10 s, annealing at 55°C for 45 s, then at 72°C for 30 s, with the final extension for 10 min at 72°C (Cheng et al. 2022). The PCR products were visualized and displayed on 1% agarose electrophoresis gel under ultraviolet illumination.

EHB isolation and observation. For the EHB isolation, the endophytic *F. acuminatum* YZU 172038 was inoculated in potato dextrose broth (PDB) to shake at 200 rpm for 72 h at 28°C referring to Obasa et al. (2017). The mycelia were collected and washed twice with sterile distilled water. The mycelial pellets were surface-sterilized with 75% ethanol for 2 min and rinsed with sterile water five times. The fifth washing liquid was spread on Luria-Bertani (LB) agar medium cultured at 28°C for 48 h to examine the possible bacterial contamination. The resulting mycelia were re-suspended in 0.5 ml sterile water and ground well using a sterile mortar. The 100 µl abrasive solution was transferred to 5 ml LB medium containing 100 µg/ml hygromycin B, and then grown at 150 rpm at 28°C for 4 h. The obtained LB broth was diluted and spread on LB agar placed at 28°C to obtain single bacterial colonies. The experiment was repeated twice. The pure cultures were stored in glycerol solution at –80°C.

The purified EHB was inoculated on LB agar, cultured at 28°C for 24 h, and the colony morphology, color, and other traits were observed. Scanning electron microscopy (SEM) was used to observe the morphology and determine the size of EHB. The EHB was inoculated in LB medium, cultured at 28°C, 200 rpm, for 20 h, and the cultures were centrifuged at 8,000 rpm for 5 min to collect the cells. After fixing in 2.5% glutaraldehyde solution for 2–4 h, EHB's cells were washed three times with 0.1 M phosphate buffer (pH 6.8), dehydrated with 30%, 50%, 70%, 90%, and 100% ethanol in turn, for 15–20 min each, replaced twice with isoamyl acetate, and processed in the vacuum freeze dryer for 3 h. After drying, the samples were mounted on stubs, sputter-coated with gold-palladium, and finally examined with SEM (VEGA 3 SBU, Tescan, China) (Jiang et al. 2019b).

EHB identification and verification. Only one EHB strain (YZSR384) was isolated from the mycelia of endophytic *F. acuminatum* YZU 172038 and used

for further assays. It was transferred to the LB medium and incubated at 28°C for 24 h at 200 rpm. The cells were collected by centrifuging, and DNA was extracted with a bacterial genomic DNA extraction kit (OMEGA, China). Its 16S rDNA region was amplified with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3') using the condition of Galkiewicz and Kellogg (2008). The successful PCR products were sent to the TSINGKE company (Beijing, China) for purification and sequencing. The resulting sequence underwent the BLAST analysis in the NCBI database. The present EHB strain and their relevant strains derived from GenBank were aligned in by the ClustalW method implemented in MEGA software (version 7.0). The phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1,000 bootstrapped replications to estimate evolutionary distances (Kumar et al. 2016). *Pseudomonas aeruginosa* JCM 5962 was used as an outgroup.

Since the EHB appeared as *Bacillus subtilis* after the sequence analysis, a specific primer pair of EN1F (5'-CCAGTAGCCAAGAATGGCCAGC-3') and EN1R (5'-GGAATAATCGCCGCTTTGTGC-3') (Ashe et al. 2014) specific for *B. subtilis* was used to verify the existence of EHB in the endophytic fungus. Both previous fungal and EHB's genomic DNA were amplified with initial denaturing for 5 min at 94°C, then 10 cycles for 30 s at 94°C, annealing at 70°C for 20 s with the temperature decrease of 1°C every cycle, then 45 s at 74°C, followed by 25 cycles for 30 s at 94°C, annealing at 60°C for 20 s, than 45 s at 72°C, with the final extension for 10 min at 72°C. The PCR products were electrophoresed in 1% agarose gel and visualized under UV transillumination.

Plant growth-promoting (PGP) assays. To determine the PGP ability of the endophytic fungal strain YZU 172038, a mycelial plug (6 mm in diam.) was inoculated into 100 ml PDB medium incubated at 28°C for 7 d with 200 rpm rotation. After centrifugation, the supernatant was filtered with a bacterial filter of 0.22 μm pore size. Plump, healthy rice seeds were selected and sterilized with 2% sodium hypochlorite for 1 min, followed by 70% ethanol solution for 30 s, and rinsed with sterile water three times. The surface sterilized seeds were soaked in the fermentation supernatant for one day (Gholamalizadeh et al. 2017). Rice seeds (20 per plate) were transferred to sterile Petri dishes (n = 3) containing two layers of sterilized filter papers moistened with distilled water, kept in the dark incubator at 28°C for five days. PDB treatment was used as a control. The experiment was repeated two times.

To determine the PGP ability for the EHB strain YZSR384, it was inoculated into the demineralized LB medium at 28°C, 200 rpm overnight, and adjusted to an appropriate concentration ($OD_{600} = 1.0$). The previously mentioned sterile rice seeds were soaked in the bacterial culture for one day, and the PGP ability was assayed. The demineralized LB medium was used as a control.

IAA production of EHB. To determine the IAA production in EHB strain YZSR384, the Salkowski colorimetric method was used to screen (Bhutani et al. 2018). The EHB was inoculated into the LB medium containing L-tryptophan (100 mg/l) and grown at 28°C with 200 rpm rotation. 3 ml of culture broth (n = 3) was taken every day. After centrifugation, 2 ml of supernatant was treated with 2 ml of Salkowski reagent (1 ml of 0.5 mol/ml $FeCl_3$ and 50 ml 35% $HClO_4$). After incubation at room temperature in the dark for 30 min, the optical densities were measured at 530 nm with a spectrophotometer (BIO-RAD, USA). The LB medium containing 10 mg/ml L-tryptophan was used as a control. The test was repeated twice, and IAA concentration was evaluated with the standard curve resulting from different IAA concentrations.

EHB genomic analysis. The genomic DNA of EHB strain YZSR384 was extracted using the same method previously for the EHB identification. The whole-genome sequencing was completed by the BENAGEN company (Wuhan, China) using the third-generation of Nanopore sequencer and the second-generation Illumina HiSeq technology (Cock et al. 2010; Büttner et al. 2019). Freely available software Unicycler (0.4.8) (https://github.com/rrwick/Unicycler) was used to assemble the obtained data. The coding genes of the assembled genome were predicted by the Prokka software (1.1.2), and the predicted gene sequences were compared with COG, KEGG, Swiss-Prot, Refseq, and other functional databases by BLAST to obtain the gene function annotation results (Seemann 2014).

Statistical Analysis. The data were statistically analyzed by using SPSS for Windows 17.0 (SPSS Inc., USA). One-way ANOVA was applied to confirm variability and validity of the results. Duncan's multiple range test was performed to determine the significance between the treatments at $p = 0.05$.

Results

EHB existence in *F. acuminatum* YZU 172038 hyphae. Under the fluorescence microscope, many bacterial nuclei were found in the hyphae stained with the fluorescent SYTO-9 (Fig. 1A and 1B). In addition, the hybridized bacteria were also clearly observed in the hyphae of *F. acuminatum* YZU 172038 (Fig. 1C and 1D). A 255 bp PCR fragment was obtained after amplifying endophytic *F. acuminatum* YZU 172038 genomic DNA using primers ER10 and ER11 designed for a partial sequence of 16S rDNA region (Fig. 1E). These results indicated that EHB existed in the hyphae of *F. acuminatum* YZU 172038.

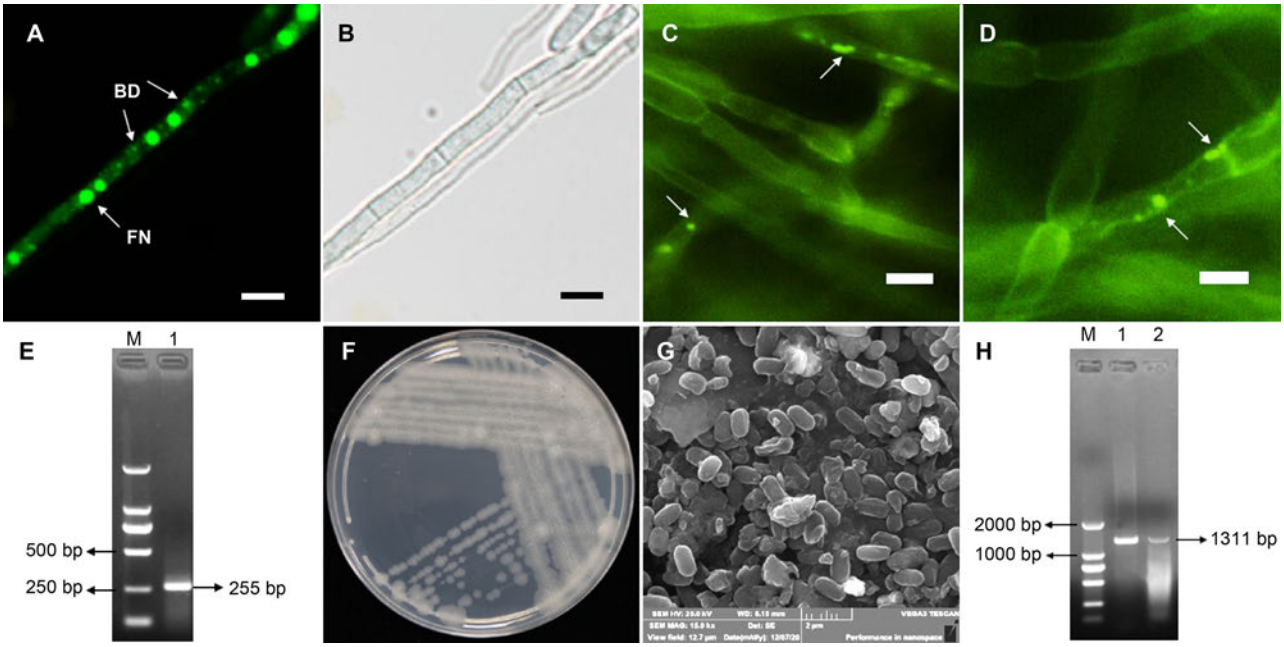

Fig. 1. The endohyphal bacteria in *Fusarium acuminatum* YZU 172038.
(A, B) Fluorescent SYTO-9 nucleic acid stained hyphae under fluorescent microscopy (FN – fungal nucleus, BD – bacterial DNA), scale bars = 5 μm; (C, D) bacteria (green signals pointed by white arrows) in hypha detected by the FISH method, scale bars = 5 μm; (E) PCR amplification of the 16S RNA gene (M – 2000 bp maker, 1 – YZU 172038); (F) colony morphology of EHB YZASR384 on LA medium; (G) scanning electron microscopy of EHB YZASR384; (H) PCR amplification of endoglucanase gene (1 – EHB YZSR384, 2 – YZU 172038).

EHB identification and verification. The colonies of EHB YZSR384 grown on the LA plate were round, with irregular edges, and milky white (Fig. 1F). Under scanning electron microscope (SEM), the EHB cells were rod-shaped, with 1–1.5 × 0.6–0.8 μm in size (Fig. 1G). The resulting 16S rDNA sequence (accession no. OP108437) had 98% identity to *B. subtilis* after BLAST searches, with a high homology to the type strain IAM 12118 of *B. subtilis* (MK267098). The phylogenetic tree (Fig. 2) showed that YZSR384 and IAM 12118 form a clade with a high bootstrap value of 98%. Therefore, based on morphological and phylogenetic analysis, the EHB YZSR384 was identified as *B. subtilis*. To further confirm the EHB existence, the genomic DNA of fungal strain YZU 172038 and its EHB YZSR384 were amplified with specific endoglucanase primer pairs of EN1R and EN1F for *B. subtilis*. The exact length of positive bands around 1,311 bp was clearly visualized under UV transillumination after electrophoresis (Fig. 1H).

Plant growth-promoting (PGP) assays and the EHB IAA production. During PGP assays upon rice seed ger-

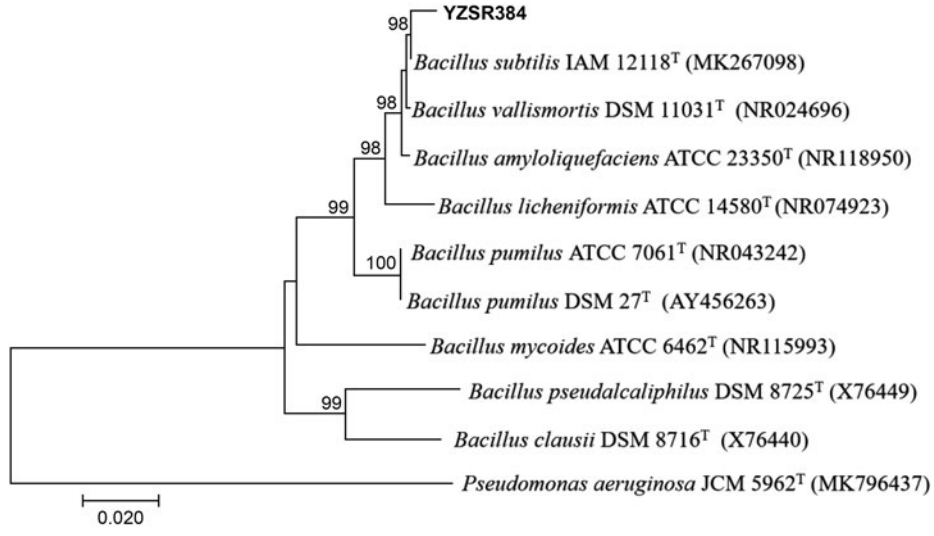

Fig. 2. Phylogenetic tree of endohyphal strain YZSR384 using the neighbor-joining method based on the 16S rRNA gene sequences. Bootstrap values (%) presented at the branches were calculated from 1,000 replications.

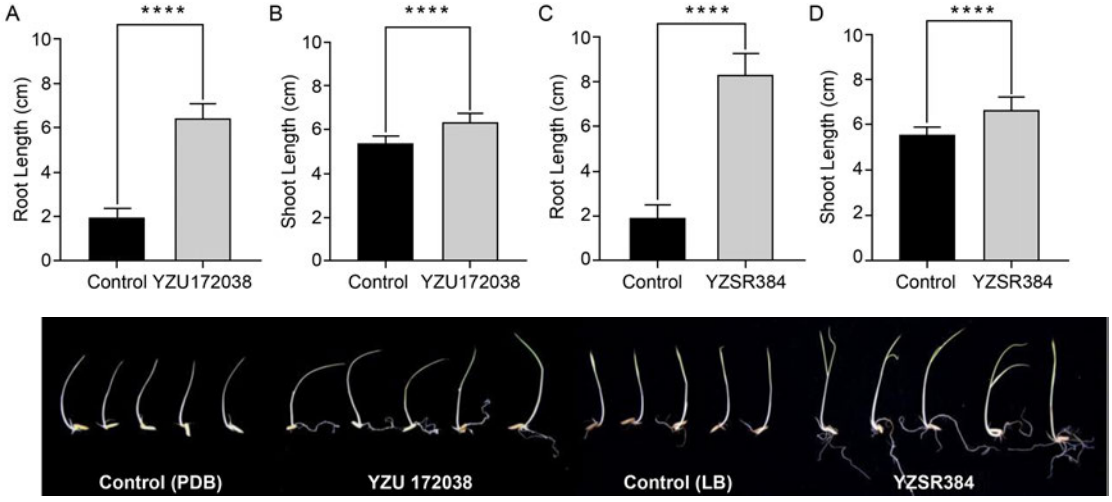

Fig. 3. Plant growth promoting (PGP) assays for rice seeds using the culture broth of endophytic *Fusarium acuminatum* YZU 172038 (A, B) and its EHB *Bacillus subtilis* YZSR384 (C, D). The controls were PDB broth (A, B) and demineralized LB liquid (C, D).

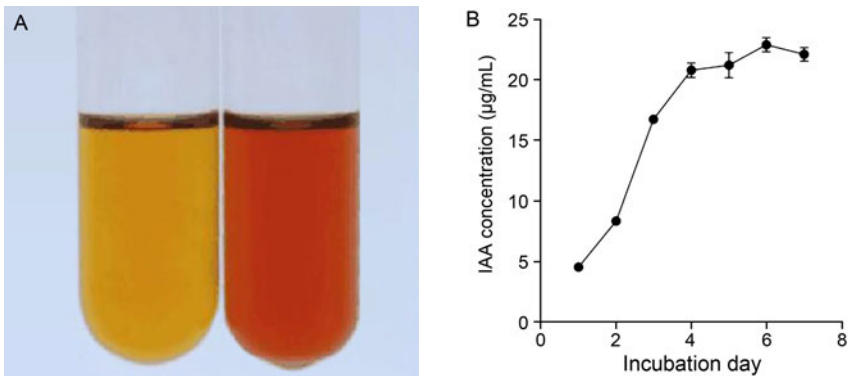

Fig. 4. Detection of the IAA production in EHB YZSR384 culture broth.
(A) Salkowski test of the negative control (left) and the EHB YZSR384 broth (right).
(B) The IAA concentration in different incubation times.

mination, the endophytic *F. acuminatum* YZU 172038 and its EHB YZSR384 cultures could promote the root and shoot growth. For the fungal strain YZU 172038, the root (6.44 ± 1.27 cm) and shoot (6.34 ± 0.82 cm) length was significantly increased compared to negative controls (1.99 ± 0.81 cm and 5.36 ± 0.57 cm, respectively, $p < 0.01$). The roots and shoots (8.32 ± 1.32 cm and 6.69 ± 0.78 cm) treated with EHB YZSR384 were significantly longer than the controls (1.93 ± 0.72 cm and 5.56 ± 0.48 cm, respectively; $p < 0.01$). The development and growth of rice roots were promoted in both strains compared to the rice shoots (Fig. 3).

The PGP-related IAA production was determined by the Salkowski method, which resulted in the color changes of the EHB broth (Fig. 4A). The results indicated that EHB YZSR384 could produce IAA. In the quantitative analysis of IAA production, it appeared that IAA was produced in the highest concentration on the sixth day, reaching 23.361 μg/ml after inoculation. Then the concentration began to decline (Fig. 4B).

Genome sequencing, assembly, and annotation. The genomic analysis of the EHB YZSR384 (accession no. CP102769) indicated that the strain's genome had a size of 4,215,636 bp with 43.51% GC content, contained 4,444 genes, 4,216 coding genes, and 118 RNA genes (including 30 rRNA genes, 87 tRNA genes, and 1 tmRNA gene). The other gene prediction is shown in Table I. These results confirmed that the EHB is *B. subtilis*.

Table I
Genome properties of EHB *Bacillus subtilis* YZSR384.

Feature	*Bacillus subtilis* YZSR384
Genome size (bp)	4,215,636
GC content (%)	43.51
Total number of genes	4444
Number of CDSs	4216
tRNA genes	87
rRNA genes	30
tmRNA genes	1
Genomic islands	4
Genes allocated to Uniprot	4,157
Genes allocated to Refseq	4,180
Genes allocated to KEGG	2,330
Genes allocated to Pfam	3,761
Genes allocated to GO	3,359
Genes allocated to COG	2,816

Table II
Genes associated with plant growth-promoting (PGP) traits for EHB *Bacillus subtilis* YZSR384.

PGP activity	Gene	Function	Chromosome location
IAA production	*trpS*	Tryptophanyl-tRNA synthetase	1218699–1217707 –
	trpA	Tryptophan synthase subunit alpha	2371911–2371108 –
	trpB	Tryptophan synthase subunit beta	2373106–2371904 –
	trpF	Phosphoribosylanthranilate isomerase	2373734–2373087 –
	trpC	Indole-3-glycerol phosphate synthase	2374491–2373739 –
	trpD	Anthranilate phosphoribosyltransferase	2375500–2374484 –
	trpE	Anthranilate synthase	2377019–2375472 –
Phosphate solubilization	*phoD*	Alkaline phosphatase	283609–285360 +
	phoB	Alkaline phosphatase	621083–619695 –
	phoA	Alkaline phosphatase	1018051–1016675 –
	phoH	Phosphate starvation-inducible protein	2615049–2614090 –
	PhoP	Alkaline phosphatase synthesis transcriptional regulatory protein	2978117–2977395 –
	phoR	Two-component sensor histidine kinase	3236753–3238354 +
	pstB	Phosphate ABC transporter ATP-binding protein	2577585–2576803 – 2578405–2577596 –
	pstA	Phosphate ABC transporter permease	2579310–2578426 –
	pstC	Phosphate ABC transporter permease	2580239–2579310 –
	pstS	Phosphate ABC transporter substrate-binding protein	2581210–2580308 –
Siderophore production	*entB*	Isochorismatase	552212–552754 + 3288208–3287270 –
	entC	Isochorismate synthase	3153313–3151898 – 3153500–3153375 – 3291891–3291106 –
	entE	2,3-dihydroxybenzoate-AMP ligase	3289855–3288236 –
	entA	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	3291080–3289884 –

Genome annotation revealed several genes that contributed directly or indirectly to PGP abilities, i.e., IAA production, phosphate solubilization, and siderophore production (Table II).

Discussion

Endohyphal bacteria (EHBs) exist in a variety of fungi which species and number are also variable within the same genus or family (Hoffman and Arnold 2010; Shaffer et al. 2016). EHBs in *Fusarium* have been studied and isolated, and the effect of EHB presence on its host fungi has already been recognized. EHB *Chitinophaga* sp. can promote the utilization of carbon sources in *Fusariun keratoplasticum* from *Fusarium solani* species complex (Shaffer et al. 2017). *Enterobacter* sp. increases *Fusarium fujikuroi* virulence (Obasa et al. 2020), and *Klebsiella aerogenes* enhances the growth-promoting ability of *F. oxysporum* (Cheng et al. 2022). In this study, EHB was isolated from an endophytic *F. acuminatum* YZU 172038 of *S. sinensis* and exhibited the plant growth-promoting (PGP) ability. This EHB was identified as *B. subtilis* based on morphological, phylogenetic, and genomic analyses. To our knowledge, it is the first time that *B. subtilis* has been found in *F. acuminatum*. The results also enrich the EHB resources of *Fusarium* fungal endophytes.

Bacillus is a worldwide bacterium that exists in various environments and can produce different compounds involved in the biological control of plant pathogens and the promotion of plant growth (Miljaković et al. 2020). Furthermore, many reports describe EHB *Bacillus* helping their host fungi in many aspects. EHB *Bacillus pumilus* can aid the maize pathogen *Ustilago maydis* to fix nitrogen (Ruiz-Herrera et al. 2015). EHBs *B. pumilus* and *B. subtilis* can improve fungal competitiveness by inhibiting other endophytes in plants (Pakvaz et al. 2016). EHBs *Bacillus* spp. (*Bacillus anthracis*, *Bacillus thuringiensis*, and *Bacillus mycoides*) in wild mushrooms exhibit biological control potential (Aslani et al. 2018). In this study, EHB *B. subtilis* YZSR384 had significant PGP effects on rice seeds to improve their development and growth of rice root and shoot. It could help improve the PGP ability of its host *F. acuminatum* YZU 172038. More work shall be conducted in the future on this topic.

Bacillus can promote plant growth in numerous ways, such as IAA production, phosphate solubilization, siderophore production, and nitrogen fixation (Kashyap et al. 2019). IAA production was found in EHB *B. subtilis* YZSR384 broth as it was determined by the Salkowski colorimetric technique. Since tryptophan is the precursor of auxin synthesis, the comprehensive genomic analysis identified the presence of the *trpABCDEFS* genes in EHB YZSR384 (Table II); tryptophan-linked genes in the genome might implicate the IAA biosynthesis (Singh et al. 2021). Furthermore, *B. subtilis* uses the phosphate-specific transport (*pst*) system for transporting free inorganic phosphate. The *pst* operon of *B. subtilis* contains the *pstS*, *pstC*, pstA, *pstB* (*pstB*1 and *pstB*2) genes (Xie et al. 2016). Besides, phosphate (Pho) regulon genes are expressed in response to phosphate starvation and regulated by the PhoP-PhoR two-component regulators (Santos-Beneit 2015). In the present study, the genome of EHB YZSR384 revealed the existence of the *pstABCS* and *phoABDHPR* genes.

The siderophores synthesized by *Bacillus* are diverse. Catecholate siderophores are synthesized in the cytoplasm when iron starvation occurs, and their biosynthesis requires seven enzymes encoding EntA-F and H. (Pakarian and Pawelek 2016). A siderophore-associated transport cluster (*entABCE*) was found in the genome of YZSR384. This genomic information underlined the PGP potential of EHB *B. subtilis* YZSR384 suggesting its role as a plant growth promoter.

EHB can assist the host fungi in surviving in unfavorable environments (Ghignone et al. 2012; Salvioli et al. 2016) and perform specific biological functions living outside the fungus (Ruiz-Herrera et al. 2015; Pakvaz et al. 2016; Aslani et al. 2018). It indicates that EHB is also a potential biological rich resource, which is worthy of further exploration and development. The present EHB could be used for the development of PGP candidates.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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A Molecular and Epidemiological Study of Cholera Outbreak in Sulaymaniyah Province, Iraq, in 2022

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Abstract

Cholera is a disease caused by a Gram-negative bacterium *Vibrio cholerae* and is among the significant threats to global public health. The disease is mainly spread in the hot months of the year; low sanitation and lack of clean water are the major causes of the disease. In this study, we conducted a molecular and epidemiological study of the recent outbreak in the city of Sulaymaniyah in Iraq. Based on the bacteriological, serological, and molecular identification of the bacterium, it was shown that *V. cholerae* O1 serotype Ogawa caused the disease. Additionally, the number of positive cholera cases were higher in June compared to July (391 positive cases in June and 23 in July). Moreover, the majority (>60%) of the cholera cases were recorded among 20–44-year-old people in both months; however, there was no significant difference in the patient genders diagnosed every month. Overall, this is the first report on the recent cholera outbreak in the city of Sulaimaniyah in Iraq.

K e y w o r d s: *Vibrio cholerae*, outbreak, bacterial infection, molecular identification, Sulaymaniyah

Introduction

Cholera is an enteric bacterial infectious disease caused by a Gram-negative, motile, flagellated bacterium *Vibrio cholerae* (Harris et al. 2012). The infection occurs from consuming vegetables and/or drinking water contaminated with the pathogen (Harris et al. 2012). The disease is particularly a problem in developing countries where poor sanitation, and lack of awareness and/or access to clean drinking water exist (Ali et al. 2015; Ramazanzadeh et al. 2015).

More than 200 serogroups of the bacteria have been identified so far, and cholera outbreaks mainly occur during the hot months of summer elicited by the O1 or O139 biotype (Ali et al. 2015; Ramazanzadeh et al. 2015). Severe watery diarrhea, known as "rice-watery" stool, odorless or of a mild fishy smell, with or without vomiting, are the major symptoms of the disease (Harris et al. 2012; Endris et al. 2022). Among different age groups, children below five years old are the most vulnerable to cholera compared to adults or the elderly (Deen et al. 2008). Additionally, people suffering from other diseases such as gastric disease and/or those individuals with type-O blood groups are at higher risk of getting cholera than other blood group types (Braunwald et al. 2001).

Cholera is considered a global health issue, and it has affected most parts of the world since the early of the nineteenth century (Harris et al. 2012). A majority (~99%) of cholera cases have been recorded in Africa and Southern Asia (Deen et al. 2008; Endris et al. 2022). In 2006, more than two million cholera cases with 6,311 deaths were recorded in 52 countries (WHO 2007a). Since 2020, 323,320 cholera cases have been reported in 27 countries, with 857 deaths (WHO 2021). In Iraq, the first recorded cholera epidemic dates back to 1820 in Basra, which resulted in many deaths (Al-Abbassi et al. 2005). Since 1966, cholera has been

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considered an endemic disease in different parts of Iraq (Hussain and Lafta 2019). In 2007, a study was conducted among 6,399 children suspected of cholera in the city of Kirkuk-Iraq and showed that 326 (5.1%) of them were infected with *V. cholerae*, of which five had died from the infection (Noaman et al. 2011). In another study, 4,667 cholera cases were reported in Iraq in 2007; 136 (2.9%) of them were recorded in the capital city of Baghdad, and about 33% of the confirmed cases were individuals between the age of (0–4) and (15–45) years old (Khwaif et al. 2010).

In 2022, a new cholera outbreak hit different cities of Iraq. According to the official data of the World Health Organization (WHO), 449 confirmed cholera cases were recorded in Iraq (WHO 2022). Here, we report the cholera outbreak data analysis and molecular identification of the pathogen in Sulaymaniyah from the beginning of June to the end of July 2022.

Experimental

Materials and Methods

The current molecular and epidemiological study was carried out on cholera cases that spread in Sulaymaniyah city, Iraq from the 1st of June 2022 to the end of July 2022. Samples and data were collected in the Shar Hospital.

Sample collections. Stool samples were collected according to the Iraqi Standard Operating Procedures for Laboratory Identification of *Vibrio cholerae* (USAID, SOP: NCL – BE 00110) (SOP 2014). In brief, stool samples were collected from suspected patients in the Cary-Blair transport medium (Sun et al. 2020). Samples were transferred to alkaline peptone water (APW, pH = 8.6) and kept for 4 to 6 hours at 37°C to identify the pathogen using bacteriological, biochemical, serological, and molecular biology methods.

Bacterial identification and biochemical tests. Colony characterization of the bacterium was carried out by sub-culturing a single colony from APW onto a selective media of thiosulfate-citrate-bile salts-sucrose (TCBS) agar and incubated at 37°C for 18–24 hours. The colonies were isolated and subjected to the following biochemical tests: oxidase test (Shields and Cathcart 2010) and Kligler's Iron Agar (KIA; Thermofisher, UK) test.

Serologic identification of *V. cholerae*. Slide agglutination test with monovalent O1 or O139 antisera (Mast group, UK) was used for serological identification of *V. cholerae* following the manufacturer's instructions. In brief, freshly grown colonies were tested against *V. cholerae* O1 and O139 serogroups monovalent antiserum. Isolates agglutinating in monovalent antiserum to the O1 serogroup were identified as *V. cholerae* O1. To identify the serotype of the serogroup, *V. cholerae* O1 was further tested for agglutination in the monovalent Ogawa and Inaba antisera.

Molecular identification of *V. cholerae*. Molecular identification of the isolates was carried out by amplifying the 16S rRNA gene following previously described protocol (Mohamedsalih et al. 2020). In brief, the bacterial DNA of three confirmed isolates randomly selected were extracted using Prlesto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan). PCR reaction mix (50 µl) contained 0.5 µM of universal 16S rRNA primers (F: 5'-AGAGTTTGATYMTGGCTCAG-3', R: 5'-ACGGYTACCTTGTTACGACTT-3') (Satokari et al. 2001), and 2 × Master mix Prime Taq Premix (GeNet Bio, South Korea). The PCR condition was as follows: initial denaturation (95°C for 2 minutes), followed by 35 cycles of denaturation (95°C for 30 seconds), annealing (58°C for 30 seconds), and extension (72°C, 30 seconds). The final extension period was for 10 min at 72°C before cooling and keeping the sample at 4°C. Aliquots (10 µl) of the amplicons were analyzed by agarose gel electrophoresis and visualized under UV light. The remaining 40 µl of PCR products were sequenced using the Sanger DNA sequencing method (Macrogen, South Korea). Analyzing and assembling the sequences were performed using Chromas software v1.0 (Technelysium software). The assembled DNA sequences were submitted to GenBank, and the accession number for each of the sequences was received using BankIt webpage (https://www.ncbi.nlm.nih.gov/WebSub).

Phylogenetic analysis. The phylogenetic tree was constructed following the method described previously by Chong et al. (2014). Assembled PCR sequences of each of the isolates were compared with closely related 16S rRNA sequences in the NCBI databank using the BLAST homology sequence searching. Multiple sequence alignment was built using ClustalW, and the tree was constructed using MEGA 7 software (Kumar et al. 2016), neighbor-joining method, the bootstrap method (1,000 replica), and the Kimura 2-parameter model.

Statistical analyses. Statistical analysis was performed using XLSTAT software (2019.2.2). The Mann-Whitney *U*-test was used to compare June and July data and analyze the differences between males and females admitted to the hospital each month. Differences were considered significant when the p-value was < 0.05.

Results

Isolation and identification of the pathogen using biochemical and serological tests. All the 414 positive cases recorded within two months study were confirmed using bacteriological and serological tests. Initially, isolation and preliminary identification of *V. cholerae* were

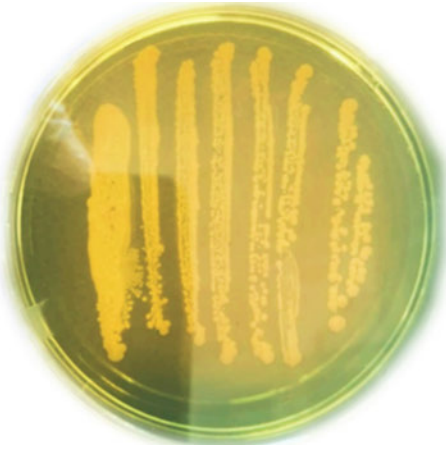

Fig. 1. An example of *Vibrio cholerae* grown on TCBS media.

carried out by culturing aliquot from APW on the TCBS media and checking for the characteristic colonies of *V. cholerae*. The preliminary indications of the *V. cholerae* were yellow, shiny, round, smooth, and slightly flattened colonies on the TCBS media (Fig. 1).

To identify the bacteria the oxidase test, KIA test, and serological tests were used. The *V. cholerae* isolates were oxidase positive (Fig. 2A); acid/alkaline, did not produce gas nor H_2S in the KIA test (Fig. 2B). All the positive tested samples were agglutination positive with monovalent O1 antiserum. The biotype O139 was not detected among the tested samples, and the majority of the samples reacted to monovalent Ogawa antiserum (Fig. 2C).

Identification of *V. cholerae* using molecular and phylogenetic analysis. The partial sequences of the 16S rRNA genes from three random bacterial isolates were used for molecular identification of the etiological agent of the outbreak. DNA sequencing and phylogenetic analysis revealed that the isolates belonged to *V. cholerae* species. The partial sequences of the three isolates were submitted to the GenBank with the following strain names: *V. cholerae* strain Suli1 (Accession number: OP470022), *V. cholerae* strain Suli2 (Accession number: OP470023), and *V. cholerae* strain Suli3 (Accession number: OP470024). Additionally, the phylogenetic tree showed that the 16S rRNA sequences of the isolates formed a clade with other *V. cholerae* strain ATCC® 14035™ (NR 119302), *V. cholerae* strain CECT 514 (NR 044853), and *V. cholerae* strain RC782 (NR 044050) (Fig. 3).

Table I
Percentage distribution of diagnosed cases according to age groups and gender for both June and July 2022.

Age group (years)	Gender	June		July	
		Number of cases	%	Number of cases	%
<1	Male	0	0.00%	0	0.00%
	Female	0	0.00%	0	0.00%
1–4	Male	0	0.00%	0	0.00%
	Female	1	0.00%	0	0.00%
5–9	Male	4	0.10%	0	0.00%
	Female	0	0.00%	0	0.00%
10–14	Male	9	0.30%	3	0.20%
	Female	25	0.70%	15	1.20%
15–19	Male	149	4.30%	66	5.20%
	Female	217	6.20%	95	7.40%
20–44	Male	1,156	33.30%	412	32.20%
	Female	980	28.20%	379	29.60%
45–64	Male	340	9.80%	90	7.00%
	Female	381	11.00%	131	10.20%
>65	Male	97	2.80%	25	2.00%
	Female	115	3.30%	64	5.00%
Total cases		3,474	100%	1,280	100%

Epidemiology of the disease. During two months of the study, 4,754 suspected cases were admitted to the Shar hospital/Sulaymaniyah city, Iraq (3,474 cases in June and 1,280 in July). The percentage of males to females in June and July were 51%:49% and 47%:53%, respectively. It was noticed that the majority of the patients were 20–44 years old (61.5% in June and 61.8% in July) (Table I). The number and the percentage of

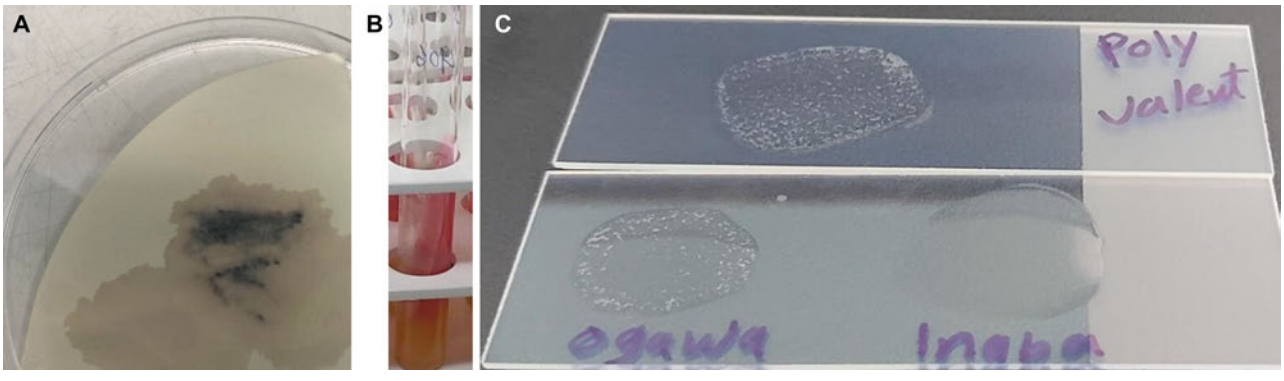

Fig. 2. The result of the bacteriological identification of *Vibrio cholerae*.
A) In the oxidase test, the purple color indicates a positive result; B) KIA test, acid/alkaline, no gas, no H_2S; C) serologic identification of *V. cholerae*, showing agglutination for polyvalent O1 antiserum and monovalent Ogawa.

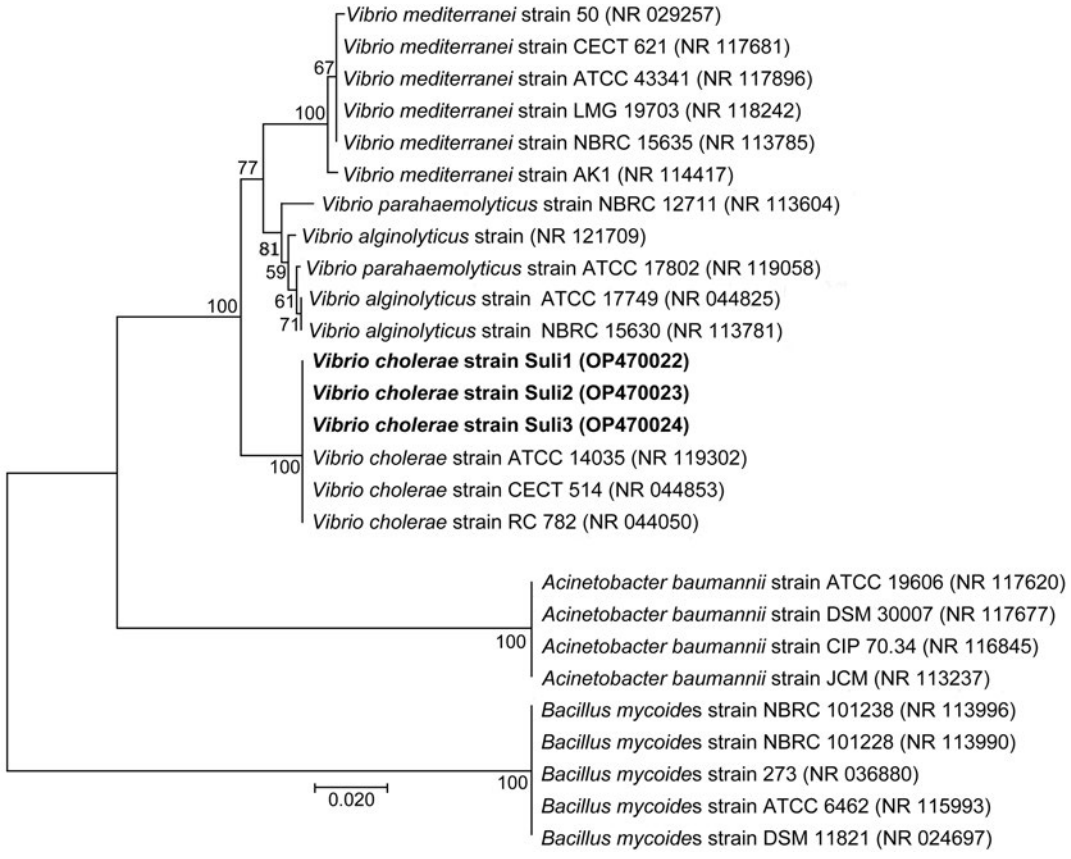

Fig. 3. The phylogenetic tree analysis is based on the similarity of the 16S rRNA sequence of the newly isolated *Vibrio cholerae* to closely related species. The tree was built using MEGA7.0 software, the neighbor-joining method, and bootstrapped 1,000 replicate runs. The newly isolated are shown in bold, and the accession numbers are shown in parentheses after the strain names. The bar indicates substitutions per nucleotide.

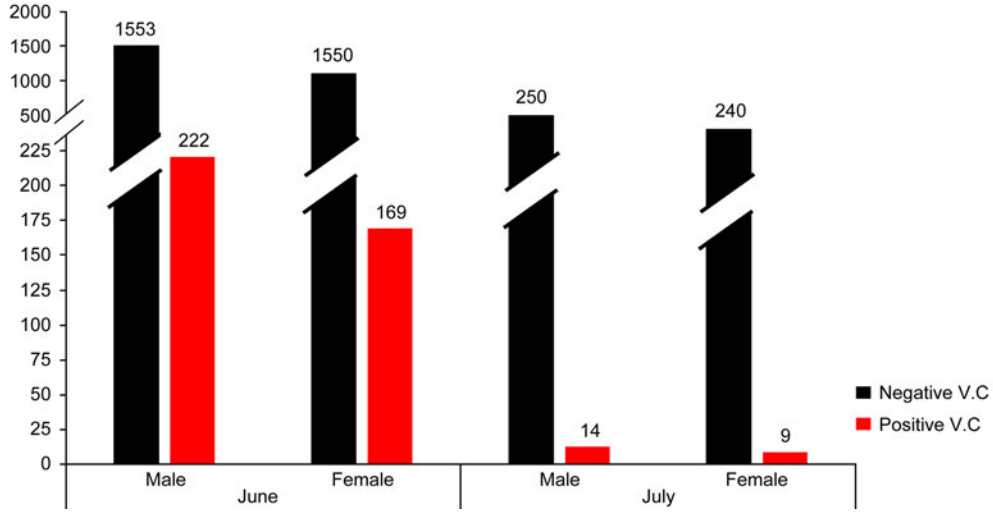

Fig. 4. The number of suspected and positive cholera cases in the Shar hospital for both genders in June and July of 2022.

the suspected age groups are shown in Table I. Out of 3,474 suspected cholera cases in June, 391 (11.3%) were diagnosed as cholera-positive, of which 222 were males, and 169 were females. Whereas the number of positive cases decreased to only 23 (1.8%) cases out of 1,280 suspected cholera cases in July of which 14 of them were male, and nine were female (Fig. 4).

The mean and the standard deviation of the male and female cholera cases were 56.61 ± 52.8 and 55.4 ± 46.37 in June, respectively. In July, the mean and the standard deviation data were 19.22 ± 7.54 and 22.06 ± 10.3 for males and females, respectively.

Statistically, the result (Mann-Whiney *U*-test) of the comparison between both genders for each

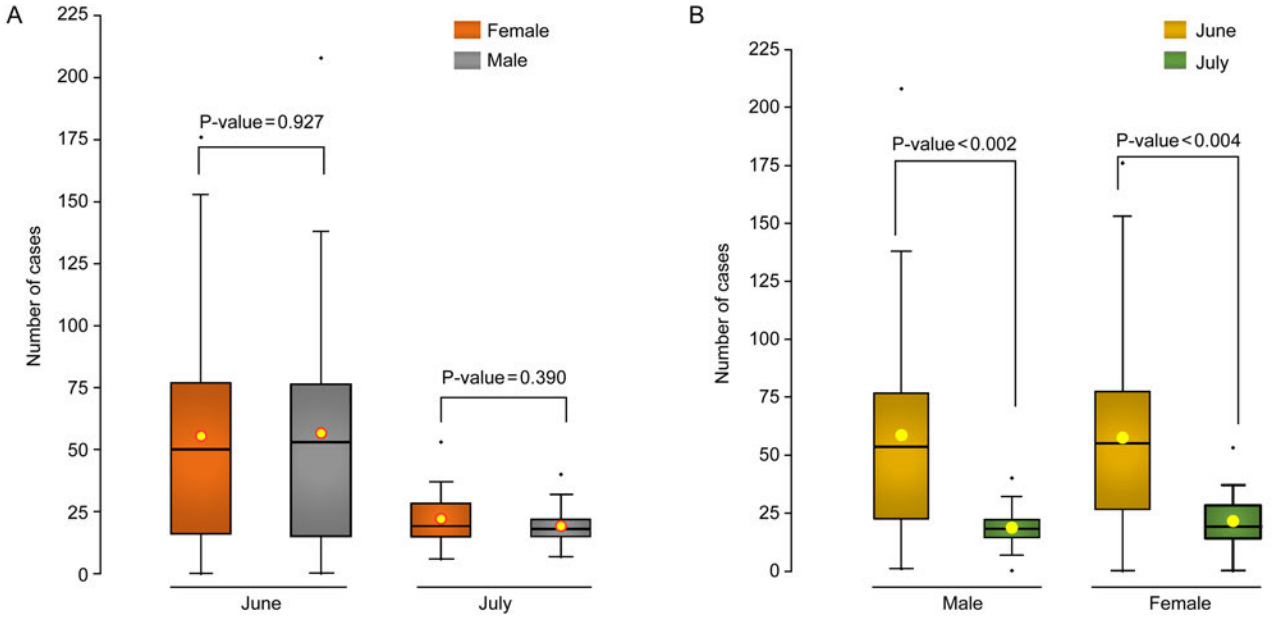

Fig. 5. Box plot showing the number of diagnosed patients admitted to the hospital based on gender; A) comparison between both genders for each month; B) differences between the number of male cases in June and July as well as female cases in June and July. The values' statistical significance (p-value) is shown above each box. The yellow dot in each box plot represents the mean value, and the horizontal line in each box represents the median value. A p-value < 0.05 is considered significant.

month showed that there is no significant difference (p-value > 0.05) among the genders (Fig. 5A).

The data of male and female genders that were admitted into the hospital in both months were further analyzed for both June and July. The results have demonstrated that there was a significant difference (p-value < 0.05) in the number of males in June and July as well as the number of females in June and July that were admitted to the hospital (Fig. 5B). Furthermore, the mean and standard deviation for the number of males in June and July were 58.50 ± 52.64 and 18.62 ± 8.16 respectively; and for females in June and July, were 57.30 ± 45.99 and 21.37 ± 10.86, respectively.

Discussion

Cholera is among the serious global health issues, particularly in Africa and southern Asia; according to WHO, it infects around 1.3 to 4 million people and causes between 21,000 to 143,000 deaths worldwide each year (Chowdhury et al. 2022). Several cholera outbreaks have been recorded in Iraq; the latest one was started at the end of May 2022, and according to a WHO report, 449 confirmed cases in Iraq by 24 July 2022 (WHO 2022). Here, we carried out a molecular and epidemiological study of the latest cholera outbreak in the Sulaymaniyah province of Iraq for the first time. The data presented here were from the public Shar hospital during the outbreak peak from the beginning of June to the end of July 2022.

Bacteriological, biochemical, and molecular identification of the isolates confirmed that all the isolates from this study belonged to the same biotype. Therefore, the etiological agent of the cholera outbreak in the Sulaymaniyah province in 2022 was *V. cholerae* biotype O1, and Ogawa was a dominant serotype. Preliminary identification showed that these bacteria were oxidase positive and changed the TCBS media's color from green to yellow; they produced acid/base, but did not produce gas nor H_2S in the KIA test (Swanson and Gillmore 1964; Lányi 1988). Moreover, serological tests revealed that isolates belonged to biotype O1, and Ogawa was a dominant serotype. Previously, it has been reported that the cholera outbreak of 2015 in Baghdad was caused by *V. cholerae* O1, biotype El Tor, and serotype Inaba (Jameel et al. 2016). Another study of 80 clinical and five environmental *V. cholerae* isolates from the outbreak in Iraq in 2007–2009 revealed that 55% of clinical isolates belonged to the Inaba serotype, 32.5% to the Ogawa serotype, and 12.5% to the Non-O1 serotype, while all the environmental isolates belonged to the Non-O1 serotype (Saleh et al. 2011). In 1995, WHO reported a cholera outbreak in Sulaymaniyah governorate that resulted in 264 confirmed cases and three deaths. The etiological agent was identified as *V. cholerae* O1 serotype Ogawa (WHO 1995). A comprehensive review of the cholera outbreaks in the Kurdistan region covering seven cholera outbreaks between 1995 and 2022 indicated that both Inaba and Ogawa serotypes of *V. cholerae*, O1, El Tor individually caused these outbreaks (Sidiq 2022). In the outbreaks in 1995, 1998, 1999, and 2012 in Iraq and the

Kurdistan Region the serotype Ogawa was dominant, while only the outbreaks of 2007 and 2015 were caused by the Inaba serotype (WHO 1995; Yassin 2002; Al-Abbassi et al. 2005; Kami 2007; WHO 2007b; Agha et al. 2008; Health Cluster 2015; Zgheir et al. 2019).

Molecular identification of the three randomly selected isolates revealed that these isolates were *V. cholerae*, and all three isolates were closely related to each other and also to *V. cholerae* strains ATCC® 14035™ (NR_119302), CECT 514 (NR_044853), and RC782 (NR_044050). The high sequence identity (> 99.9%) of the randomly selected *V. cholerae* isolates could suggest a common source origin of the pathogen before spreading in the city. Since no environmental sampling was tested in this study, the exact source of the infections remains to be determined. However, based on experimental data reported by WHO on 24 July, 11% of the water samples collected from different sources in Iraq were contaminated with *V. cholerae* (WHO 2022).

Additionally, the polluted water of the Tanjero River, which has been used to irrigate leafy vegetables in the region, can be a possible source of infection. Various waste materials and pollutants are constantly dumped into the Tanjero River in Sulaymaniyah, including sewage, industrial, and agricultural wastes (Aziz et al. 2012; Rahman et al. 2021). Previous studies have demonstrated that polluted lakes, rivers, and food were sources of cholera outbreaks in different regions of the world (Albert et al. 1997; Griffith et al. 2006; Bompangue et al. 2008; Dinede et al. 2020). It supports the hypothesis further that using polluted water of the Tanjero River for irrigation could have been the cause of the cholera outbreak in Sulaymaniyah.

The epidemiological data demonstrated that the number of people admitted to the hospital with cholera symptoms was significantly higher in June than in July: 3,474 and 1,280, respectively. The number of confirmed cholera-positive cases was also higher in June than in July: 391 and 23, respectively. Generally, acute diarrhea and vomiting conditions among people are more prevalent during summer due to contaminated food and drinking water usage. People drink more water, possibly contaminated, during summer to replace lost fluid because of the hot weather, which can also favor the growth of microorganisms on foods and cause food poisoning (Ramos-Alvarez and Sabin 1958; Anderson et al. 2020). Additionally, cholera outbreaks in summer could be due to the hot atmospheric air and low water level, which provide perfect conditions for bacterial growth (Asadgol et al. 2019). However, decreasing the number of cholera case in July could be due to the governmental action plan against the outbreak, such as prohibiting using contaminated products, treating contaminated water sources, and raising public awareness (Jensen et al. 2006; Panja et al. 2016).

Moreover, lytic bacteriophages can destroy the bacteria soon after being discharged into the water following their spread (Jensen et al. 2006; Panja et al. 2016). Some studies have found that vibrio phage can limit bacterial density and growth, and the severity of outbreaks can largely depend on the density of the phage (Jensen et al. 2006; Ujah et al. 2015; Panja et al. 2016; Asadgol et al. 2019). The transmission of cholera from person to person is more severe than those acquired from the environment (Harris et al. 2012).

In our study, only a small portion 391 (11.3%), and 23 (1.8%)) of the suspected individuals were confirmed as cholera cases in both June and July, respectively. No death was reported in the 2022 cholera outbreak in the Sulaymaniyah province; however, three deaths were recorded in the whole of Iraq; two of them were in the city of Kirkuk and one in Baghdad (WHO 2022). Besides *V. cholerae,* other pathogens such as *Entoameba histolytica* can be another causative agent of acute diarrhea and amebiasis is an important public health problem worldwide (Hameed et al. 2021). Previously, *E. histolytica* was identified among children suffering from diarrhea in the Kirkuk and Duhok provinces of Iraq (Hussein and Meerkhan 2019; Hameed et al. 2021). In addition to *E. histolytica*, *Giardia lamblia* could be a reason for acute diarrhea, and the parasite has been previously isolated from the Euphrates River in AL-Nasiriyah City in Southern Iraq (Al Jassas et al. 2022). The enteric parasites heavily contaminate the leafy vegetables in Baghdad city (Khalil 2019). Significant reasons for acute diarrhea and cholera outbreak are contaminated water sources, especially from artesian wells, house tanks, shallow wells in mosques, or water from tankers sold to people (WHO 2022). Several factors are involved in cholera's prevalence, including poor sanitation and hygiene, lack of qualified healthcare, poor infrastructure, and contamination of drinking water sources with sewage system (Ujah et al. 2015; Oguttu et al. 2017).

Moreover, our results showed that the numbers of cholera cases were slightly, but not significantly, higher among males than females; people aged 20–44 were more affected by the disease. It could be because people at this age, especially males, are more frequently eating outside, which might have increased their chances of getting exposed to different pathogens, including *V. cholerae*. A study of a cholera outbreak in Uganda in 2011 indicated that cholera cases were higher in males than females by 1.6 times (Bwire et al. 2017). A prospective study in fishing villages in Uganda between 2011–2015 covering ten cholera outbreaks showed that case-fatality ratio was significantly higher among males than females (Bwire et al. 2017).

The recurrent cholera outbreak in Iraq indicates that efforts are needed to establish safe drinking water,

proper sanitation, and treatment of sewage water, strengthen primary health care, establish disease surveillance mechanism, and improve the nation's awareness and preparedness to detect and respond to cholera outbreaks in the future rapidly.

Conclusions

A cholera outbreak is a bacterial disease caused by the Gram-negative bacterium *V. cholerae* after consuming contaminated food and water. Here for the first time, we reported molecular and epidemiological data on the 2022 cholera outbreak in the Sulaymaniyah province that revealed the incidence rate and identified the strain as *V. cholerae* Ogawa serotype. We found that the number of diagnosed people was higher in June compared to July. Also the majority (>60%) of the cholera cases were recorded among people aged 20–44 years in both months. This study provides an insight into the incidence rate of the cholera outbreak in Sulaymaniyah province during June and July 2022. It reveals the causative bacteria strain than can help authorities develop preventive strategies to stop such outbreaks in the future.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Chlorine Dioxide Reprograms Rhizosphere Microbial Communities to Enrich Interactions with Tobacco (*Nicotiana tabacum*)

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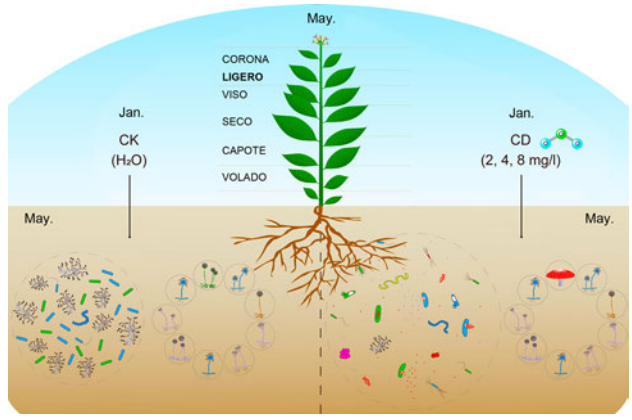
Abstract

For decades chlorine dioxide has been used in water disinfection with excellent results. As the scope of application expands, chlorine dioxide has the potential for soil disinfection. We used amplicon sequencing and gas chromatography-mass spectrometry to compare the changes of four mixed rhizosphere microbial community samples and 12 tobacco leaf volatile samples four months after the flood irrigation with chlorine dioxide in different concentrations (0, 2, 4, 8 mg/l). Phenotypic data of 60 tobacco plants were also collected. The effects of chlorine dioxide on rhizosphere microorganisms were positively correlated with dose gradients. Bacteria responded more strongly in both community structure and metabolic pathways than fungi. Five new bacterial phyla (Firmicutes, Bacteroidota, Myxococcota, Patescibacteria, Verrucomicroboata) appeared in chlorine dioxide treatment groups, while the fungal community only appeared as one new fungal phylum (Basidomycota). Alterations in 271 predicted metabolic bacterial pathways were found. However, in the fungal community were only 10 alternations. The correlations between leaf volatile compounds and rhizosphere microorganisms under the influence of chlorine dioxide treatment could be observed based on network results. However, natural connectivity had already been declining rapidly when less than 20% of the network's nodes were removed. Therefore, the microbe-metabolite network is not stable. It might be why chlorine dioxide treatments did not significantly affect tobacco quality ($p = 0.754$) and phenotype ($p = 0.867$). As a comprehensive investigation of chlorine dioxide in agriculture, this study proves the effectiveness and safety of chlorine dioxide soil disinfection and widens the application range of chlorine dioxide.



K e y w o r d s: chlorine dioxide, soil disinfection, rhizosphere microbiome, plant-microbe interactions, *Nicotiana tabacum*

Introduction

Due to its high efficiency, chemical control has been widely used to prevent rhizosphere-mediated disease (Panth et al. 2020). However, some chemicals are harmful to humans or the environment, thus limiting their application potential to the extent (Ristaino and Thomas 1997; Pesonen and Vähäkangas 2020).

Chlorine dioxide (ClO_2) is a strong oxidant with broad antimicrobial spectrum, non-toxic to human

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body, and does not pollute the environment (Gómez-López et al. 2009; Zhong et al. 2019; Jefri et al. 2022). Therefore, it plays a vital role in tap water and sewage treatment (Aieta and Berg 1986; Katz et al. 1994), medical apparatus and environment disinfection (Lowe et al. 2013; Meyers et al. 2020), and food preservation (Park and Kang 2015; Sun et al. 2017). After 12 hours of exposure to chlorine dioxide gas (4 mg/l, 0.16 mg/g), the number of yeasts, molds, *Listeria monocytogenes*, *Salmonella* spp., and *Escherichia coli* O157:H7 decreased by 3.10, 3.17, 3.94, 3.62, and 4.25 logs CFU/g, respectively (Popa et al. 2007). The antimicrobial mechanism of chlorine dioxide is the theoretical basis of application. Chlorine dioxide increases the permeability of the bacterial outer membrane and the bacterial plasma membrane, causing electrolyte leakage and eventually killing the bacteria (Ofori et al. 2018). In addition, chlorine dioxide destroys the fungal cell membrane's integrity and leads to death of fungi (Liu et al. 2020). The reaction environment in the soil is complex, which may affect the effectiveness of chemical pesticides. For example, the lower the pH, the weaker the disinfection effect of the chlorine dioxide solution. An alkaline environment is the most suitable for chlorine dioxide reactions (Ofori et al. 2018). In addition, organic matter can absorb gaseous chlorine dioxide (Ramsey and Mathiason 2020) and react with chlorine dioxide solution (Hassenberg et al. 2017). These conditions should be considered when determining the dosage of chlorine dioxide.

The application of gaseous chlorine dioxide to soil disinfection has been well studied. A field study applied to the control of *Phytophthora ramorum* showed that the maximum proportion of gaseous chlorine dioxide inactivating pathogens was 82%. Meanwhile, the disinfection efficiency increased with the soil moisture (Layman et al. 2020). Greenhouse studies indicated that the maximum *Bacillus subtilis* spores log10 reduction was 4.12 and 5.82 for the play sand and the mixed soil, respectively. In addition, as gaseous chlorine dioxide is denser than air, its concentration at the bottom was 3.8 and 3.95 times higher than at the top of the play sand and the mixed soil, respectively (Ramsey and Mathiason 2020). Since the solution is an important form of pesticide use, soil disinfection with chlorine dioxide solution is also worth studying. How does it currently perform in agricultural production? More importantly, is it safe for crops? These problems are closely related to the potential application strategy of chlorine dioxide solution in agriculture.

There are complex interactions between soil microbes and plants (Trivedi et al. 2020). Plants alter their root exudates to recruit rhizosphere microorganisms as the first barrier against soil-borne pathogens (Mendes et al. 2011; Liu et al. 2021; Song et al. 2021). Soil pathogens can also evade or suppress the plant's immune system to infect the host (De Jonge et al. 2010; Sánchez-Vallet et al. 2013; Gao et al. 2019). Therefore, rhizosphere microorganisms deserve attention. Since tobacco leaves are rich in volatile compounds (Zhang et al. 2013) and become even more abundant after flue-curing (Wahlberg et al. 1977), it is suitable for non-target metabolome analysis of the effects of chlorine dioxide on plant quality. Hence, tobacco was chosen as an indicator crop.

With these ideas in mind, we designed experiments combining amplicon sequencing and gas chromatography-mass spectrometry with studying the effects of chlorine dioxide soil disinfection on rhizosphere microbial communities and plants. We ask the following questions: 1) whether and how does chlorine dioxide solution affects rhizosphere microbial communities? 2) whether and how chlorine dioxide solution affects crops?

Experimental

Materials and Methods

Field experiments. Before tobacco seedlings were transplanted, the field experiment was conducted in Guolao Village, Baise City, Guangxi Province, China (23°02′N, 106°35′E) in January 2021. According to the Baise Climatological Survey, the average air temperature ranges from 19.0°C to 22.1°C, and the annual precipitation was 1,114.9 mm. The soil type is paddy, slightly acidic (pH 5.06), and the organic matter content is 34.35 g/kg (Table SI). Tobacco-Rice Rotation is common there; each 20-m^2 plot was planted with 40 tobaccos during the experiment. On the flat terrain, plots were distributed in three completely randomized blocks. Each block contained four plots (one control, three treatments). To ensure adjacent plots keep isolated, 0.8 m width ridges were built around them. In addition, plots in the same block were about 1 m apart, and the distance between neighboring blocks was no less than 2 m. The control plot was flood irrigated with 2,000 l of tap water (0 mg/l), equal to the chlorine dioxide solution used in other plots. The commercial powder formulation of chlorine dioxide (HS Pharma, China), consisting of passivated sodium chlorite and acid, with an active ingredient (malonic acid-iodimetry method) of 10% ± 1%, was applied in a half (powder concentration 20 mg/l, effective chlorine dioxide content 2 mg/l), in a normal (powder concentration 40 mg/l, effective chlorine dioxide content 4 mg/l) and in a twofold dose (powder concentration 80 mg/l, effective chlorine dioxide content 8 mg/l). To prevent the lack of acidic environment resulting in sodium chlorite reaction yield reduction, the mother liquor (effective chlorine dioxide content 2,000 mg/l) was first prepared in a ratio of 20 g powder to 1 l tap water and then

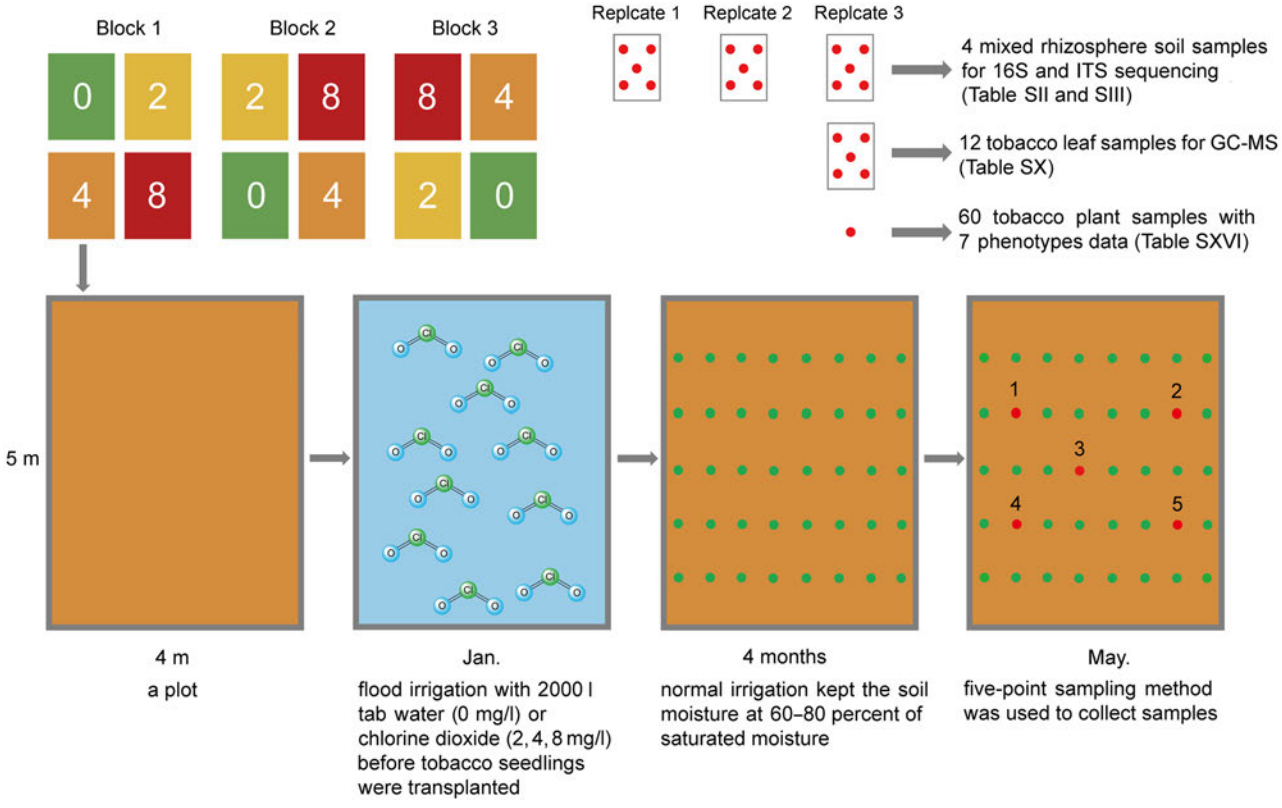


Fig. 1. Summary of experimental design.

diluted to the required concentration in a plastic tank. We used chlorine dioxide quick test paper on-site to detect the concentration and confirm that the actual content conforms to the experimental design. Regular irrigation for the next four months kept the soil moisture at 60–80 percent of saturated moisture (Fig. 1).

16S rRNA and ITS amplicon sequencing. Before tobaccos were harvested, 12 rhizosphere soil samples were collected from 12 plots with a five-point sampling strategy in May 2021. The soil auger used was 2.5 cm in diameter and 15 cm in depth. After getting enough roots with a soil auger, we shake off the loose soil from the roots and use a small brush to collect the rhizosphere soil tightly attached to the root. Three parallel soil samples of equal weight were mixed well with the sterile mortar and pestle. Three grams of the mixing sample of the group were taken. To obtain soil microbial DNA, 1 g of each mixed sample was used for extraction with the E.Z.N.A.® Soil DNA kit (D5625, Omega Bio-tek Inc., USA). All operations were performed following the manufacturer's protocols. To assess the DNA quality, OD 260/230 nm and OD 260/280 nm values were measured by the NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA). For each sample, 260/230 ratio was larger than 1.7, and 260/280 ratio was larger than 1.8.

Ten ng of DNA was used for PCR amplification (20 µl reaction, 30 cycles) in triplicate. In detail, primer sets 338F/806R, and ITS1F/ITS2R were used to amplify the 16S rRNA V3-V4 region gene and the ITS1 region gene. After that, the amplified products were sequenced with the 2 × 300 bp kit using the Illumina MiSeq platform at the Majorbio Corporation (China).

Gas Chromatography-Mass Spectrometry. Along with rhizosphere soil samples, 12 ligero leaf samples were collected with a five-point sampling strategy in May 2021. We collected the top four pieces of tobacco leaf with the stem and flue-cured them in the tobacco curing room. The third leaf from the top was chosen as the sample. These plant materials were frozen in the −80°C refrigerator before use. During pre-treatment, 50 mg of each sample was taken for immersing with liquid nitrogen and powdering using a grinding machine (LUKYM24, China). A mixture of methanol and dichloromethane (3:2) was added into the centrifuge tube containing samples to extract at 50 mg/ml concentration. After 10 min of ultrasonic treatment (KL-040ST, China) at room temperature (25°C), the supernatant was filtered through 0.22 µm membrane filtration. 200 µl of the solution was transferred to the sample bottle prepared for GC-MS analysis.

GC conditions: A Hp-5 (Agilent 19091J-413) column (30 m × 0.25 mm × 0.25 µm) (Agilent, USA) was used; the injection port temperature was 250°C. The column temperature was programmed as follows: the initial temperature at 80°C (held for 5 min), increased to 100°C at 2°C/min, increased to 180°C at 5°C/min (held for 1 min), increased to 200°C at 2°C/min, increased to 280°C at

10°C/min (held for 15 min). The injection volume of filter liquor was 0.5 µl. High-purity helium was utilized as the carrier gas. The gas flow rate was set as 1.0 ml/min.

MS conditions: An electron ionization (EI) ion source was used with 70 EV ionization energy. The mass spectra scanning range was set as 30 ~ 500 m/z. The ion source temperature was 230°C, and the GC/MS interface temperature was 280°C.

Data processing: NIST 2014 was used in the matching comparison. The screening criteria was that the matching rate should be more than 600. The relative content of each component was presented by peak area.

Bioinformatic and statistical analyses. Fastp 0.23.0 (Chen et al. 2018) was used to trim the adaptor and remove low-quality and short reads. Microbiome data were analyzed by QIIME2-2021.11 (Bolyen et al. 2019). With the q2-demux plugin, quality control and demultiplexing of FASTQ files were performed. After that, DADA2 (Callahan et al. 2016) denoising was performed using the q2-dada2 plugin. To align every ASV, mafft (Katoh and Standley 2013) was used through the q2-alignment plugin. Then, via the q2-phylogeny plugin, the phylogenetic tree was constructed with fasttree2 (Price et al. 2010). Assigning ASVs to bacteria and fungi was performed through the q2-feature-classifier (Bokulich et al. 2018) plugin, which used Scikit-learn naive Bayes classifier, against the Sliva 138 database (Quast et al. 2012) and UNITE reference sequences (Nilsson et al. 2019), respectively.

Subsequent analyses of taxon abundances were principally conducted in R software (version 4.1.3) (R Core Team 2013). Bacterial and fungal ASVs abundance tables were resampled to a median of 4,910 and 10,940 reads per sample using the phyloseq R package (McMurdie and Holmes 2013). The Newick trees were visualized using table2itol (freely available in https://github.com/mgoeker/table2itol) and Interactive Tree Of Live version 5 (Letunic and Bork 2021). In addition, Canberra dissimilarity between control and disinfectant microbial communities was measured within the R library vegan (Oksanen et al. 2013), and the construction and visualization of a hierarchical clustering tree (UPGMA) were accomplished using ggtree (Yu et al. 2017).

Due to the ability of PICRUSt2 software (Douglas et al. 2020) to predict metabolic pathways according to taxonomy annotation of ASVs, the calculation of metabolic pathways and orthologs abundances was executed in default settings. We wrote a script of Python 3 to analyze predicted MetaCyc pathways (Caspi et al. 2020), and its visualization was accomplished using the R package networkD3 (freely available at https://christophergandrud.github.io/networkD3). We developed an R script to analyze predicted KEGG ortholog (KO) and significantly differentially abundant KOs ($|\log2FC| > 1$, $p < 0.01$) visualization was accomplished using the R package ggplot2 (Kanehisa and Goto 2000). KEGG pathway enrichment analysis was performed using the R package Clusterprofile (Wu et al. 2021).

To unveil microbe-plant links possibly caused by chlorine dioxide, we developed a co-occurrence network analysis R script. All networks were constructed based on Spearman correlations between normalized family abundances of rhizosphere soil microorganisms, and metabolite abundances of ligero leaves measured by GC-MS (absolute correlation ≥ 0.8, p-value ≤ 0.1). Network visualization was accomplished by Gephi version 0.9.2 (Bastian et al. 2009). Using the R package igraph version 1.2.11 (Csardi and Nepusz 2006), various network topological indices were calculated to characterize the topological structure of co-occurrence networks: modularity, degree, weight degree, closeness centrality, betweenness centrality, eigenvector centrality. In addition, within-module connectivity, among-module connectivity, and network stability comparison were calculated by another R script. Principal coordinates analyses (PCoA) on basis of Bray-Curtis distance of the node topological properties matrix was performed within the R library vegan (Oksanen et al. 2013). A natural connectivity analysis was carried out using an R script to analyze network robustness. According to the importance of nodes in the network, nodes were removed in order. At the same time, the natural connectivity of the incomplete network was computed. The trend of value variation could reflect the robustness of the network.

During the flood irrigation, plastic tanks used as water sources were placed on the side of the road. To analyze the relationship between the inlet distance of flood irrigation and tobacco phenotype, we selected 16 tobacco plants from four plots for the t-test (Fig. S1).

Results

Bacterial and fungal communities. We analyzed the soil microbiome by 16S rRNA and ITS amplicon sequencing to investigate whether the rhizosphere microorganisms changed with chlorine dioxide dose. All bacterial and fungal communities were influenced by chlorine dioxide treatment (Fig. 2). Diversities of each treatment group were higher than the control group. At the same time, the change degree was lower in fungal communities than in bacterial communities. In addition, changed microorganisms also remained stable. Thus, the most abundant several taxa kept the same order along with increasing chlorine dioxide. Hierarchical clustering results revealed that the difference between the blank group and the treatment group was more significant in bacterial microbiome (Fig. 3).

Bacterial and fungal functional potential. To evaluate the potential function of rhizosphere microbial

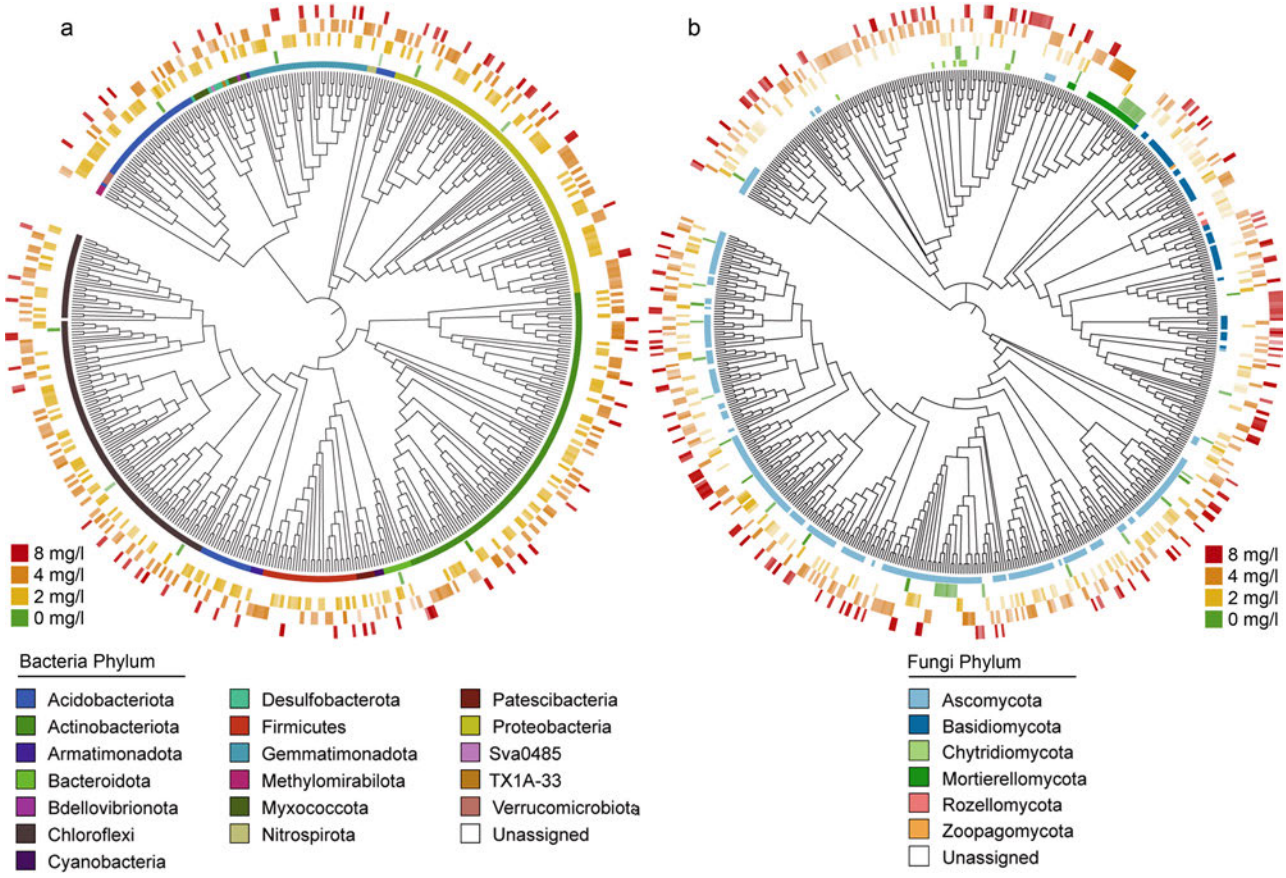


Fig. 2. ASVs found in a) bacterial and b) fungal microbiomes of different groups.
From inside to outside: taxonomic dendrogram showing each group's bacterial and fungal microbiome. The first color ring identifies microbial phyla within the rhizosphere soil. Other color rings represent the relative abundance of ASVs in different groups.

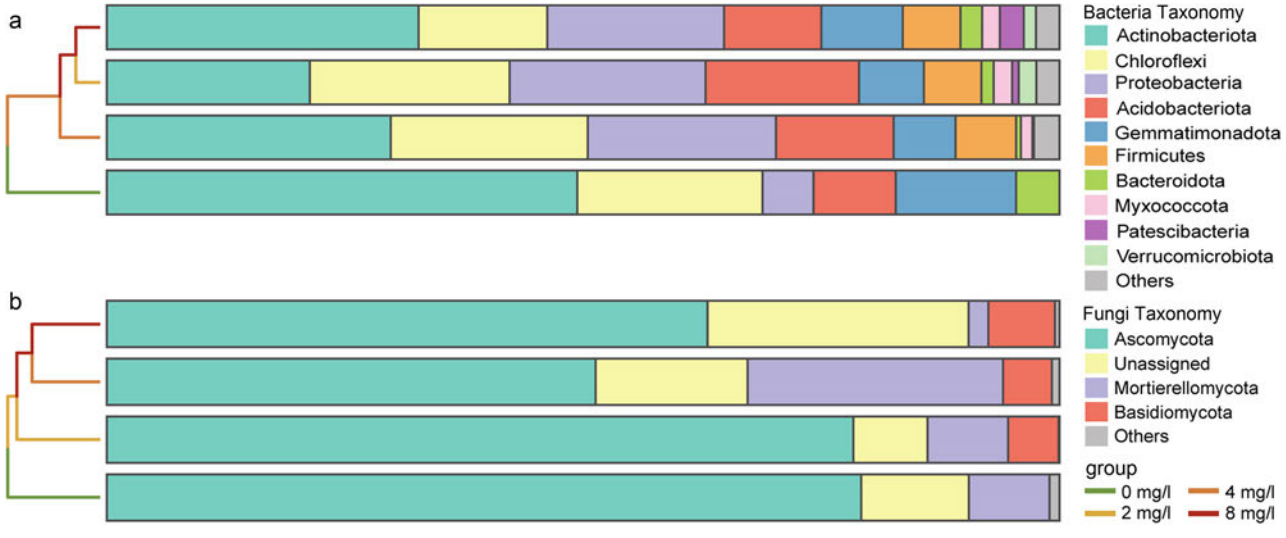


Fig. 3. Taxonomy of a) bacterial and b) fungal microbiome in different groups.
Phyla that accounted for less than 0.6% of total abundance within the four groups were classified as "Others".

communities, we used PICRUSt2 to calculate the abundances of metabolic pathways and orthologs. Corresponding to the changes in the diversity of the microbial community, bacterial metabolic pathways changed significantly, and fungal pathways remained relatively stable (Fig. 4). Alterations in 271 predicted metabolic bacterial pathways were found. These pathways belonged to the bacterial MetaCyc biosynthesis (n = 137), degradation/utilization/assimilation (n = 32), generation of precursor metabolites and energy (n = 33), superclasses (n = 67), and others (n = 2). Almost half (44.57%) of bacterial metabolic pathways were changed,

Fig. 4. Alteration of predicted metagenomic pathways in a) bacterial and b) fungal microbiome.

UP – $p < 0.05$, log2FC > 1; DOWN – $p < 0.05$, log2FC < –1; STABLE – p > 0.05 or –1 < log2FC < 1; D/U/A – Degradation/Utilization/Assimilation; GoPMaE – Generation of Precursor Metabolites and Energy. One-sample *t*-test was used to evaluate the significance of differences between the control and treatment groups.

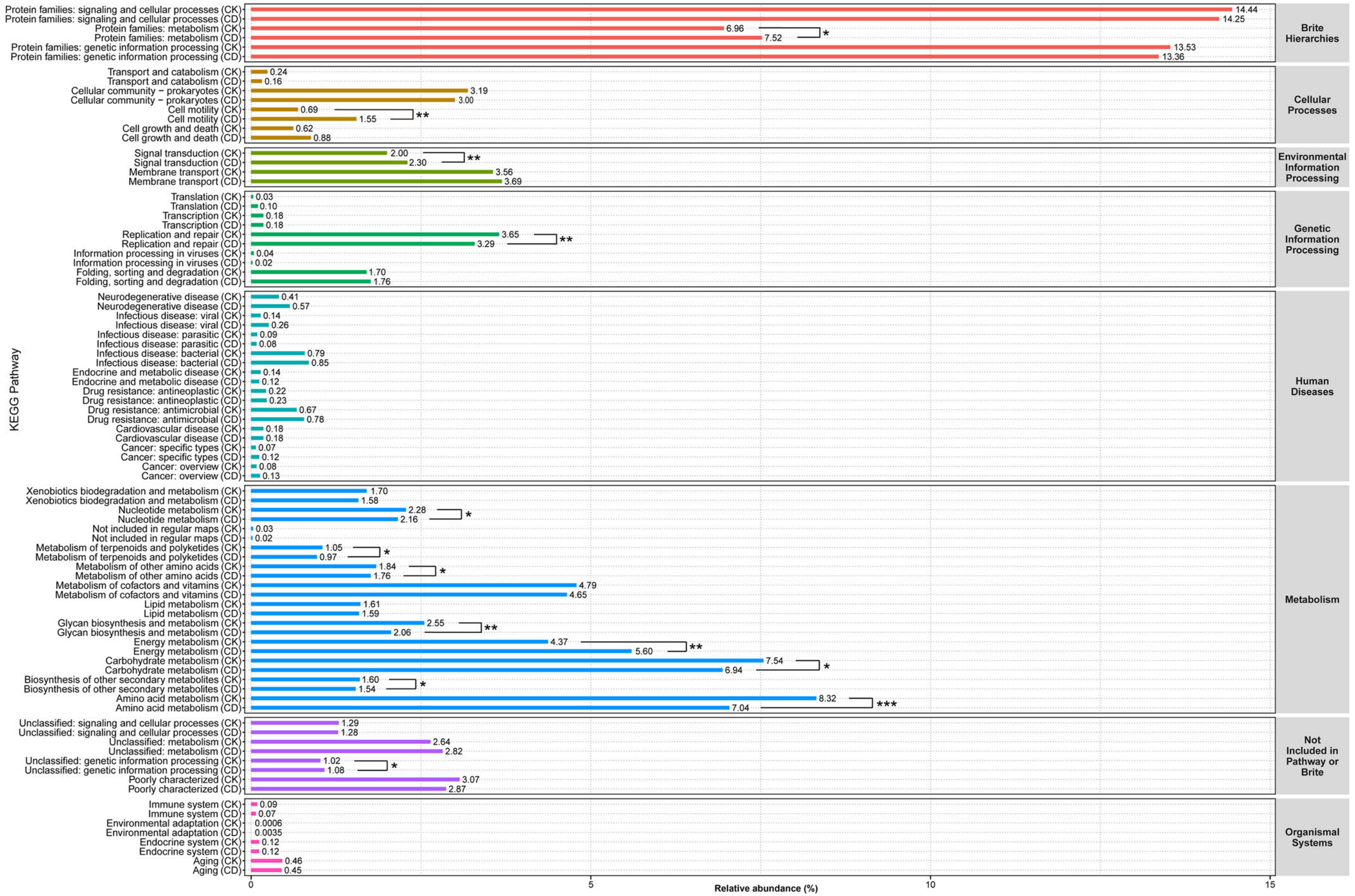


Fig. 5. Relative abundance of bacterial KEGG pathways in levels 1 and 2.

CK – H_2O treatment; CD – chlorine dioxide treatment.

* $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

One-sample *t*-test was used to evaluate the significance of differences between the control and treatment groups.

and nearly all (98.89%) of them increased in number (Table SIV and SV). Alterations in 10 predicted metabolic fungal pathways were found. These pathways belonged to the fungal MetaCyc biosynthesis (n = 2), degradation/utilization/assimilation (n = 4), generation of precursor metabolites and energy (n = 1), and superclasses (n = 3). Only a small number (8.13%) of fungal metabolic pathways were changed, and all increased in number (Table SVI and SVII).

Further analysis of bacteria was conducted with the KEGG Pathway database. 29.54% of level 2 pathways were significantly changed (Fig. 5). A comparison of KEGG ortholog (KO) abundance led to the identification of 1612 KOs with different abundance. Of these, 1,377 genes increased, and 235 genes decreased in abundance (Table SVIII). Many KOs were missing as few in the control group, which resulted many asymmetric points with high fold-change values (Fig. 6a).

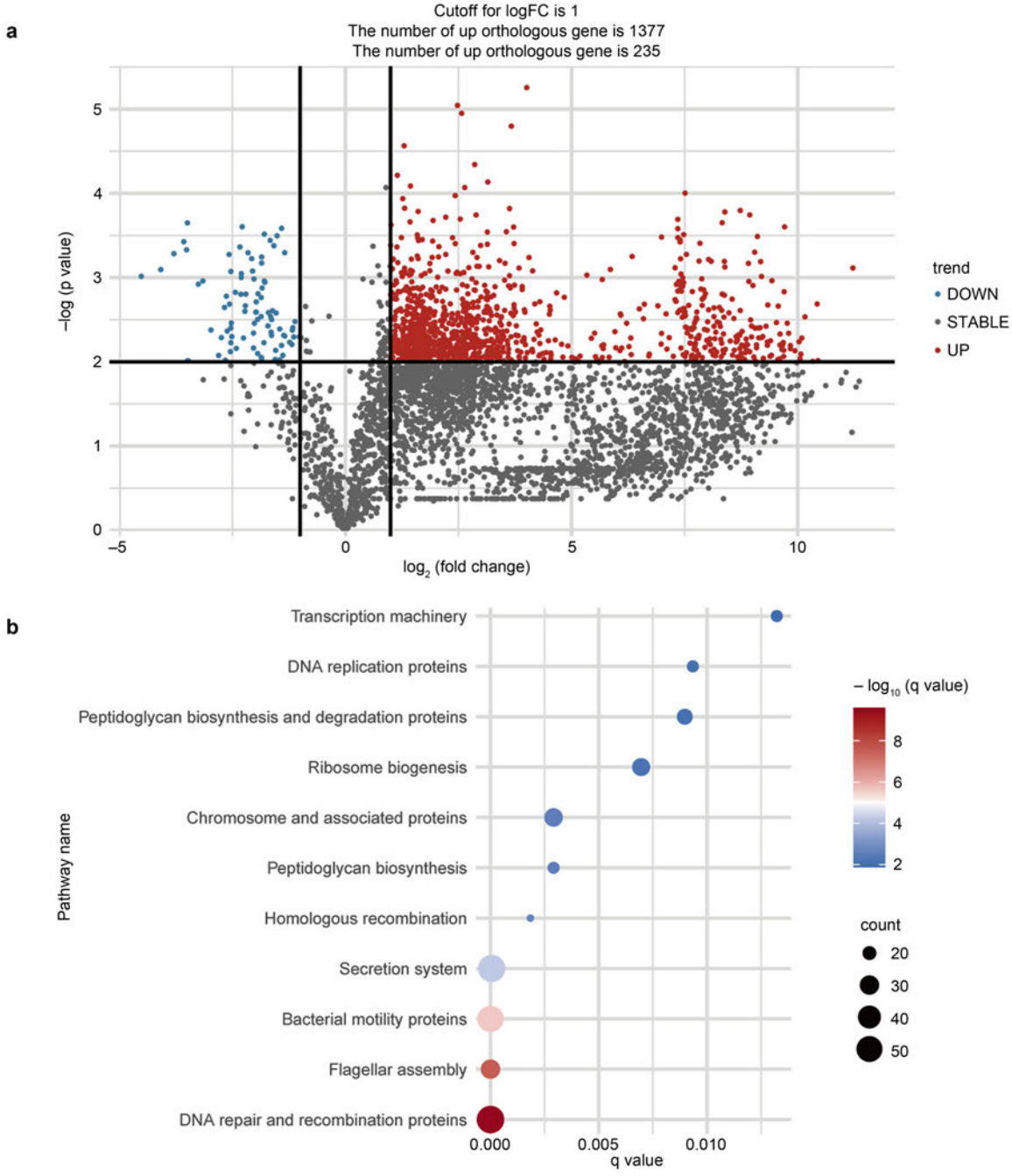


Fig. 6. KEGG enrichment analysis of bacterial communities.

a) Volcano plots of DEGs between the control and the treatment group. The horizontal and vertical lines indicate a significance threshold ($p < 0.01$, $|\log2FC| > 1$). Red dots represent upregulated DEGs, and blue dots represent downregulated DEGs.

b) Bubble diagram of the upregulated and downregulated DEGs in the KEGG database. The size of a bubble represents the number of DEGs. The color of a bubble represents the enrichment value of DEGs. One-sample *t*-test was used to evaluate the significance of differences between control and treatment groups.

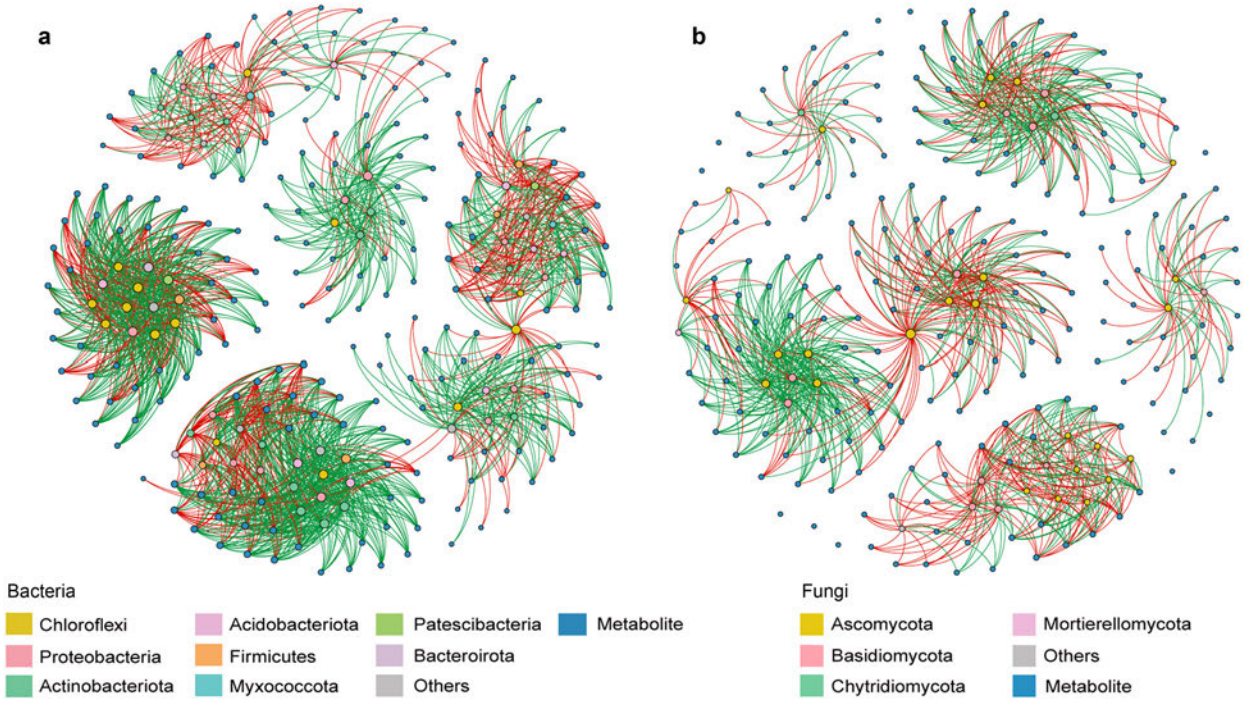


Fig. 7. Visualization of the a) bacteria-metabolite and the b) fungi-metabolite network.
Network construction is based on Spearman correlation calculation results. Blue dots represent volatile compounds. The other dots represent different family-level microbial taxonomy, respectively. Bacterial dots with different colors represent different bacterial phyla. Fungal dots with different colors represent different fungal phyla. Red lines represent positive correlations, and green lines represent negative correlations (absolute correlation ≥ 0.8, p-value ≤ 0.1).

The KEGG analysis showed that many movement-related, reproduction-related, and metabolism-related pathways were significantly enriched, which was similar to the results of bacterial MetaCyc metabolic pathways (Fig. 6b and Table SIX).

Microbe-metabolite networks. We employed a co-occurrence network analysis to assess the effects of the rhizosphere microbial community on the volatile component of tobacco leaves under the influence of chlorine dioxide. Bacteria-metabolite network was denser than the fungi-metabolite network. There were more negative correlations in the bacteria-metabolite network. Meanwhile, metabolite and microbial nodes appeared with different distribution regularities in the network. Within the same module, metabolite nodes were distributed along the edge all the time, and microbe nodes always were located in the center (Fig. 7). All 207 metabolites were correlated with bacteria. In contrast, 11 of these were not correlated with fungi. In addition, 66 bacterial families and 40 fungal families exist in the network, which accounted for 56.4% of all 117 bacterial families and 59.7% of all 67 fungal families (Table SX, SXI, and SXII).

In addition to distribution regularity, metabolite and microbial nodes they also had different topological properties. The degree of metabolite nodes was usually lower than microbe nodes. Among-module connectivity of metabolite nodes was often greater, while within-module connectivity showed an opposite trend. Thus, metabolite nodes always were connectors, and module hubs always were microbe nodes (Fig. 8). PCoA results of node properties showed distribution difference between microbe nodes and metabolite nodes (Table SXIII and SXIV, Fig. 9a and 9b). Natural connectivity analysis suggested that the robustness of microbe-metabolite networks was fragile, although their trends slightly differed (Fig. 10a). PCoA results of volatile metabolite showed no significant difference between the groups (Fig. 10b). PCoA results of tobacco phenotype demonstrated no significant differences among the groups (Fig. 10c). According to the t-test results, the distance from the entrance of flood irrigation had no significant effect on the phenotype of tobacco plants ($p > 0.05$) (Fig. S4).

If we define phyla that appeared in the taxa bar after chlorine dioxide treatment as a "new-phylum", bacterial new-phyla (Firmicutes, Bacteroidota, Myxococcota, Patescibacteria, and Verrucomicroboata) accounted for 11.3% of the bacterial community. Fungal new-phylum (Basidomycota) accounted for 5.8% of the fungal community (Table SII and SIII). However, according to the importance of nodes in the network, four of ten nodes (40%) removed from the bacteria-metabolite network belonged to bacterial new-phylum, and three of ten nodes (30%) removed from the fungi-metabolite network belonged to fungal new-phylum (Table SXV).

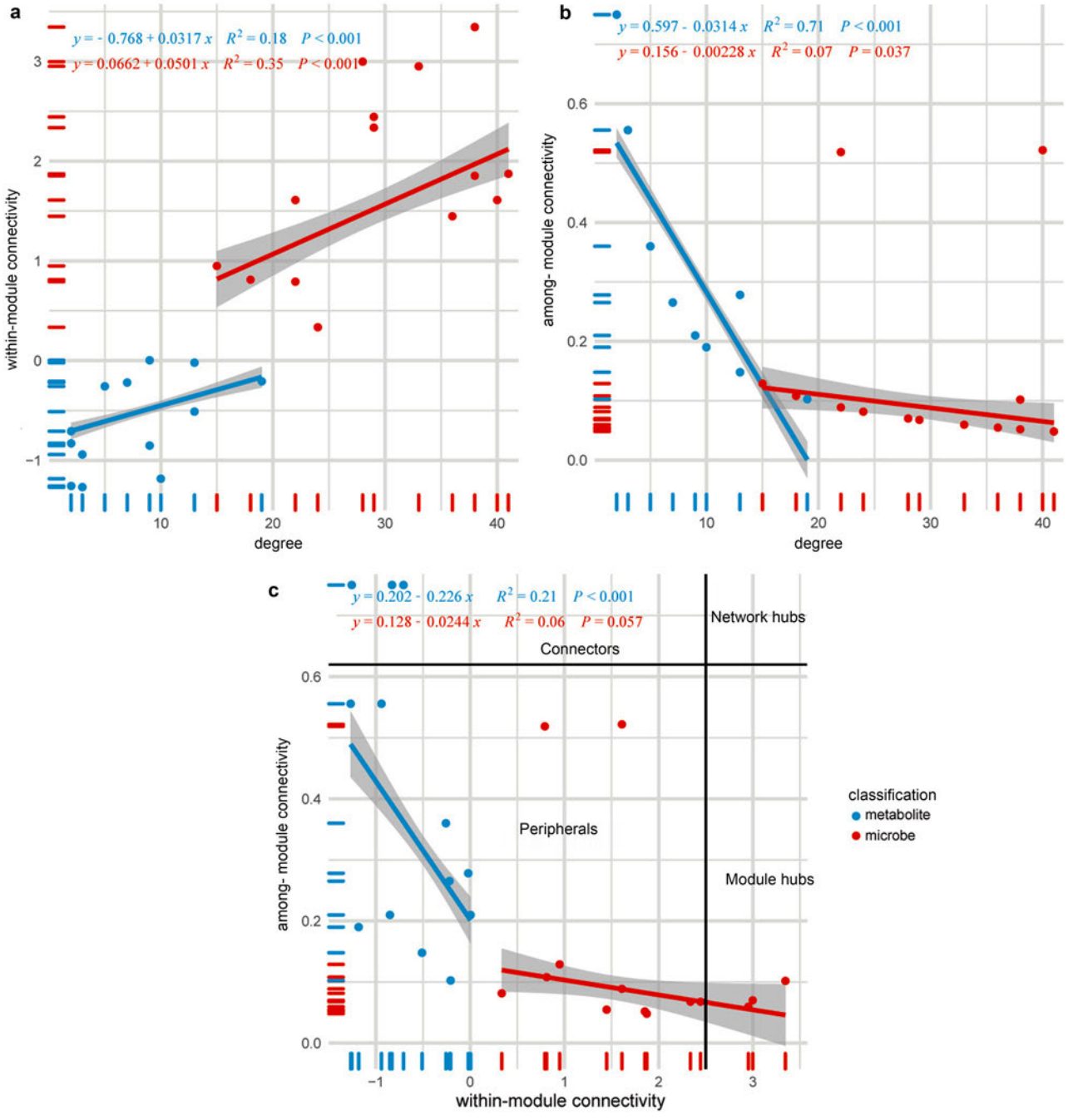
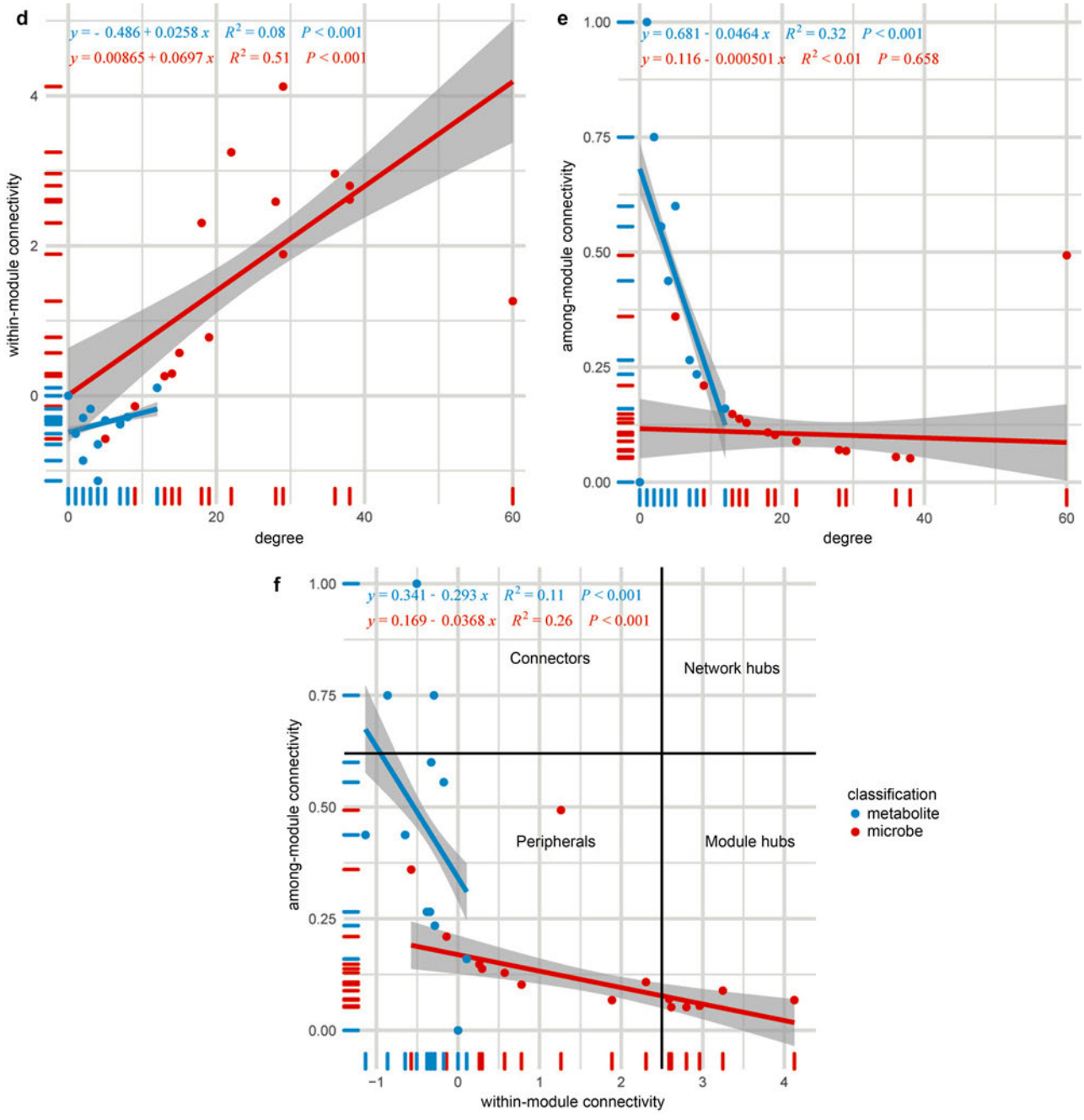


Fig. 8. Relationships among partial network node attributes.

a), d), Relationships between degree and within-module connectivity (Z_i) in a) bacteria-metabolite and d) fungi-metabolite network; b), e), relationships between degree and among module-connectivity (P_i) in b) bacteria-metabolite and e) fungi-metabolite network; c), f), keystone taxa were speculated based on their topological node features in c) bacteria-metabolite and f) fungi-metabolite network. Blue dots represent metabolite nodes, and red dots represent microbe nodes. A node was identified as a module hub if its $Z_i \geq 2.5$, as a connector if its $P_i \geq 0.62$, and as a network hub if it had $Z_i \geq 2.5$ and $P_i \geq 0.62$. *F*-tests were performed to evaluate whether models could adequately describe the data.

Discussion

According to microbial community structure and function analysis results, chlorine dioxide had an effect on rhizosphere microorganisms, and the effect on microbial community structure was enhanced with the increase of the dose (Fig. 2, 3, and 4). The disinfection treatment was only carried out once before transplanting, while the effects were not eliminated when the tobacco was harvested. KEGG enrichment analysis of rhizosphere bacterial communities revealed more details of the stress response. Chlorine dioxide might induce chemotaxis in soil bacterial community. Flood irrigation brings excess water, and soil pores are filled with water, while soil particles are less affected (Haghnazari et al. 2015). Because of chemotaxis, bacteria



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tend to escape chlorine dioxide by entering the interior of soil particles. Bacteria with well-developed flagella and strong motility are more likely to survive. Therefore, pathways of flagellar assembly and bacterial motility proteins are enriched in the bacteria kingdom suffering from chlorine dioxide disinfection (Fig. 6b and S2). The peptidoglycan synthesis pathway was also enriched since the P ring of flagellar is embedded in the peptidoglycan layer (Gupta and Gupta 2021) (Fig. 6b and S3). Simultaneous enrichment of flagellar assembly and peptidoglycan synthesis pathways was reported in the transcriptome analysis of the biocide stress effect on *L. monocytogenes* as well (Casey et al. 2014).

Chlorine dioxide might have a better inhibitory effect on bacterial soil-borne diseases. We observed that bacteria respond more strongly than fungi in terms of community structure (Fig. 3) and metabolic pathways (Fig. 4). In addition, pathways related to reproduction

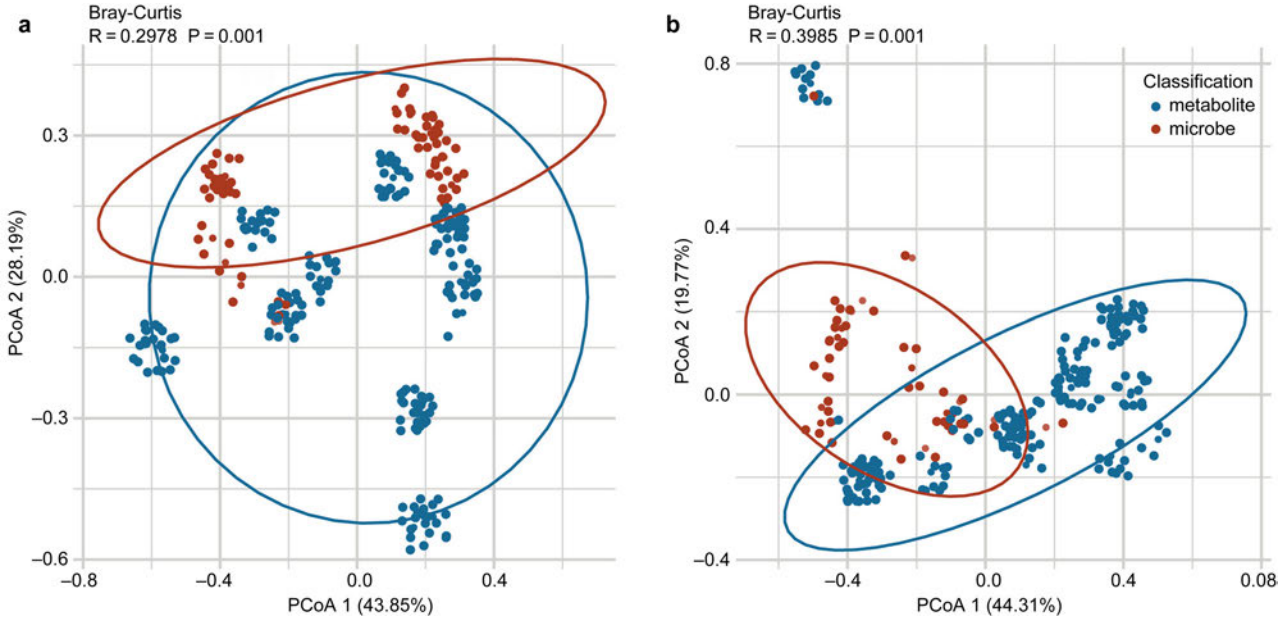

Fig. 9. Node attributes analysis.

Seven topological node parameters in a) bacteria-metabolite and b) fungi-metabolite network (listed in Table SXII and SXIII) were used for a pharmacy curriculum outcomes assessment (PCoA) analysis. Blue dots represent metabolite nodes, and red dots represent microbe nodes. In order to remove the influence of point overlap on observation, a random offset of 0.05 in the horizontal and vertical directions was added to each point. Analyses of similarity (ANOSIM) were performed on the Bray-Curtis distance matrix to evaluate whether there are differences between the metabolite node group and the microbe node group.

were also enriched in the bacterial community after disinfection treatment (Fig. 6). These phenomena suggest that bacteria were sensitive to chlorine dioxide and inactivated massively. As a result, the internal competitive pressure decreased. This difference was consistent with the previous observations that gaseous chlorine dioxide disinfection reduced bacterial concentration levels more than fungi (Popa et al. 2007; Hsu and Huang 2013). We hypothesize that a combination of the antioxidant enzyme and endomembrane systems could explain the different responses of bacteria and fungi to chlorine dioxide. Contemporary microbes inherited iron-dependent enzymes from their anoxic ancestors (Khademian and Imlay 2021). Therefore, oxidative stress induced by reactive nitrogen and oxygen species can also be an immune defense strategy against pathogenic microorganisms (Staerck et al. 2017). Microbes evolved the antioxidant enzyme system to fend off the destructive actions of oxidizing reactions (Khademian and Imlay 2021). Since eukaryotic cells have numerous endomembrane structures which improve the efficiency of cellular physiological and biochemical reactions significantly, tolerance of the antioxidant enzyme system could be better than bacterial one in terms of chlorine dioxide disinfection.

There were correlations between the soil microbial community and volatile tobacco metabolites under the influence of chlorine dioxide. We observed that several enriched pathways (flagella assembly, bacterial motility, bacterial secretion) were also associated with endophytic colonization. Once soil microorganisms perceive plant-derived signals, they move to the root through flagella and attach to the surface. To colonize the plant, they need to form biofilms and produce substances such as microbial detoxification enzymes and lytic enzymes subsequently (Trivedi et al. 2020). Microbe-metabolite co-occurrence network analysis showed that microbe nodes and metabolite nodes are not randomly combined (Fig. 8). In terms of bacteria and fungi, the different modes of topology attributes between two nodes types were also diverse (Fig. 9a and 9b), suggesting potential connections between rhizosphere microorganisms and plant metabolites. In addition, robustness results showed that the association is relatively fragile (Fig. 10a). It may be a reason for the lack of significant difference in tobacco quality and phenotype (Fig. 10b and 10c). Since plants interact with functional traits rather than a taxonomy (Trivedi et al. 2020), functional changes of rhizosphere microbial communities caused by chlorine dioxide might not be enough to affect plants significantly (Fig. 5). Therefore, chlorine dioxide soil disinfection is safe for agricultural application.

According to the ecological community theory, the emergence of "new-phylum" in treatment groups might be the result of "disturbance" and "dispersal" (Vellend 2010). Chlorine dioxide disturbs the soil microbial community, reducing the species' density and making the resources relatively adequate. For the experiment's convenience, the blocks' location was selected near the road, river, and tobacco curing room. Therefore,

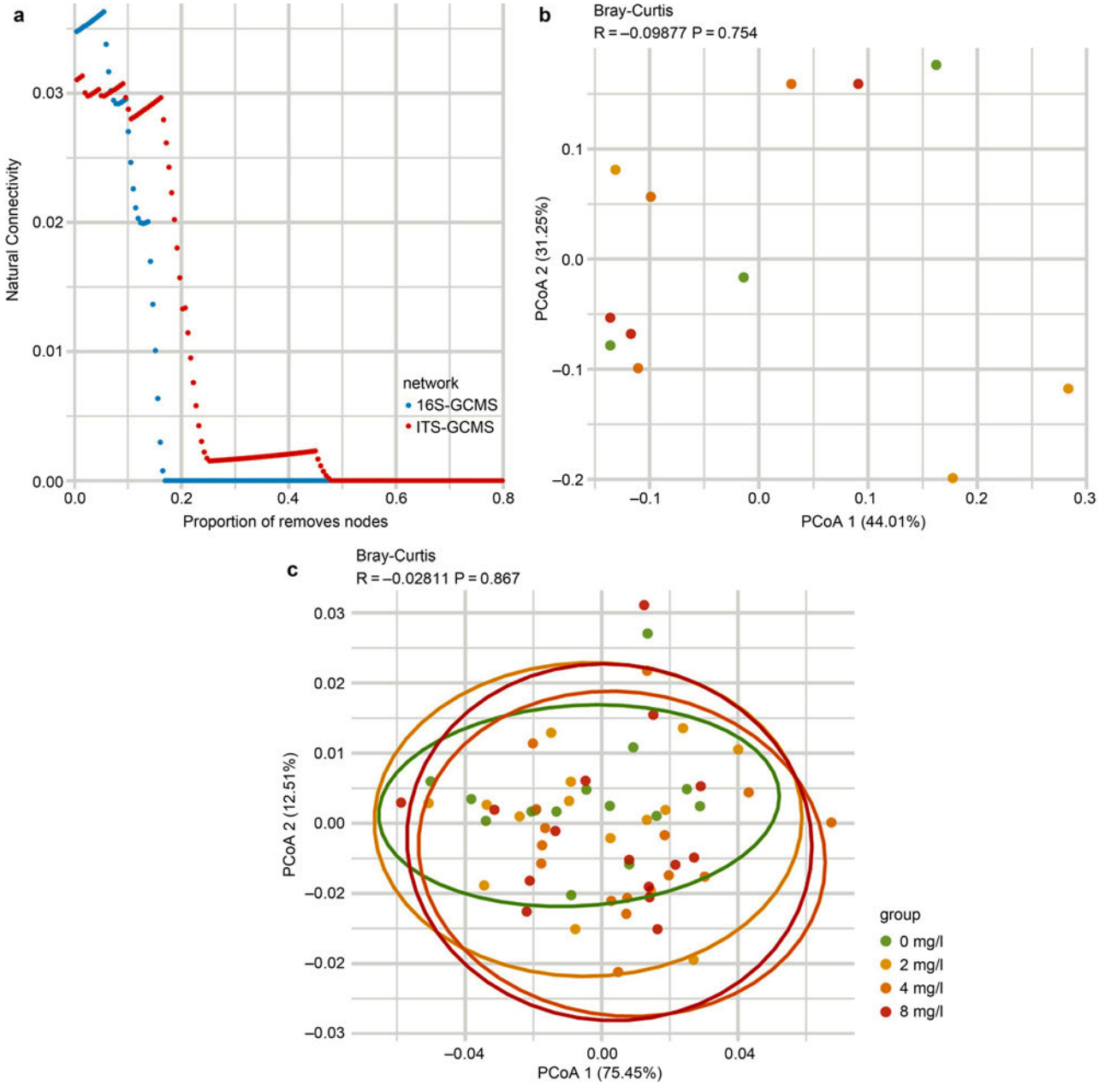

Fig. 10. Network robustness, volatile metabolite, and tobacco phenotype analysis.

a) The natural connectivity of microbe-metabolite networks. Blue dots represent the bacteria-metabolite network, and red dots represent the fungi-metabolite network. b) Abundance of 207 volatile components from 12 plots was used for a pharmacy curriculum outcomes assessment (PCoA) analysis. c) Data on seven phenotypes of 60 tobacco plants were used for a pharmacy curriculum outcomes assessment (PCoA) analysis. The analysis of similarity (ANOSIM) was performed on the Bray-Curtis distance matrix to evaluate whether there are differences between groups.

there was a regional species pool around blocks, and alien species could enter plots through dispersal. In the control group, all the ecological niches were occupied, and colonization by foreign microorganisms was challenging. Treatment groups are the opposite due to the diminished intraspecific and interspecific competition. Plant roots recruited rhizosphere microorganisms from the soil; thus, "new-phylum" appeared in treatment groups. Some beneficial microbes also emerged in treatment groups, such as *Sphingomonas*, which can enhance plant disease resistance (Matsumoto et al. 2021) and nitrogen-fixing *Bradyrhizobium* (Zimmer et al. 2016) (from 0.00% to 0.92%, 1.16%, 1.61%) (Table SII). Newly emerging microbes played a more important role in the network than the resident microorganisms (Table SXV), meaning that they had been screened and filtered through the new environment. Interactions between rhizosphere microorganisms and tobacco were enriched.

There have been some critical studies of chlorine dioxide disinfection in agriculture applications (Layman et al. 2020; Ramsey and Mathiason 2020). We aim to extend their achievements by assessing the effects on the rhizosphere microbial community and plants after

flood irrigation with chlorine dioxide. The advantage of our field experiments is that effects can be evaluated through rhizosphere microbes and crops at different dose gradients. However, a more persuasive mechanism of the presented generalization requires further research with more samples from various sites, using various crops and elucidating temporal variation in soil microorganisms and crop quality.

Availability of data and material

The raw sequences have been deposited with NCBI under the BioProject accession number PRJNA877362.

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Author contributions

Conceptualization: Z.H. and S.Q.; methodology: J.Y., P.L. and S.Q.; software: S.Q.; validation: J.Y. and J.W.; formal analysis: S.Q.; investigation: Z.H., X.L. and Q.S.; resources: J.Y.; data curation: S.Q.; writing – original draft preparation: S.Q.; writing – review and editing: J.Y., M.X. and J.L.; visualization: S.Q.; supervision: J.Y., J.W., Z.C., J.Z. and J.L; project administration: J.Y.; funding acquisition: J.Y. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Insight into the Antibiotic Resistance of Bacteria Isolated from Popular Aquatic Products Collected in Zhejiang, China

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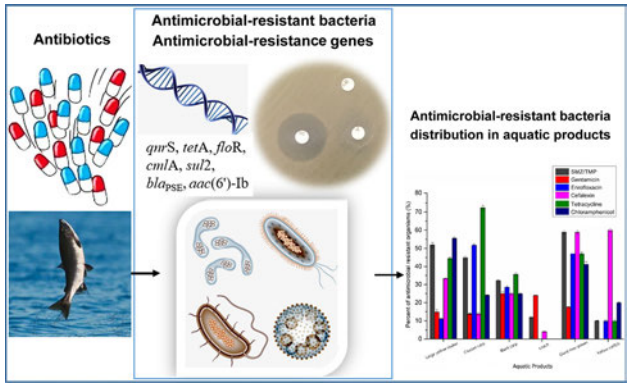
Abstract

The present study was aimed to obtain a close insight into the distribution and diversity of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) among the aquatic products collected in Zhejiang, China. A total of 136 presumptive ARB picked up from six aquatic samples were classified into 22 genera and 49 species based on the 16S rDNA sequencing. *Aeromonas* spp., *Shewanella* spp., *Acinetobacter* spp., *Myroides* spp., *Pseudomonas* spp., and *Citrobacter* spp. accounted for 80% of the ARB. Among them, 109 isolates (80.15%) exhibited resistance to at least one antibiotic. Most isolates showed resistance to not only the originally selected drug but also to one to three other tested drugs. The diversity of ARB distributed in different aquatic products was significant. Furthermore, the resistance data obtained from genotypic tests were not entirely consistent with the results of the phenotypic evaluation. The genes *qnr*S, *tet*A, *flo*R, and *cml*A were frequently detected in their corresponding phenotypic resistant isolates. In contrast, the genes *sul*2, *aac*(6')-Ib, and bla_{PSE} were less frequently found in the corresponding phenotypically resistant strains. The high diversity and detection rate of ARB and ARGs in aquaculture might be a significant threat to the food chains closely related to human health.



K e y w o r d s: antibiotic resistance bacteria, antibiotic resistance gene, aquaculture, food safety

Introduction

According to the data from the Food and Agriculture Organization, aquaculture has provided nearly half of the fishery products for human consumption, which has fulfilled the huge requirement for edible proteins and nutrients around the world (Liu et al. 2019). China is the largest aquaculture products provider in the world and yielded more than 52 million tons of farmed food aquatic products till 2020 (Bureau of Fisheries 2021). However, accompanied by the growing intensive production mode, the aquaculture system also suffers from frequent outbreaks of infectious diseases and high mortality (Reverter et al. 2020). To deal with bacterial infection, enormous amounts of antibiotics were applied in the aquaculture industry for the purpose of combating or preventing bacterial infection. In 2017, China took the largest share of global antibiotic consumption in aquaculture at 58% and was projected to remain the largest antibiotic consumer up to 2030 (Schar et al. 2020).

There is no doubt that the overuse and misuse of antibiotics in aquaculture could not only stimulate a rapid emergence of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) but also lead to a widespread of ARGs in the aquatic environment and reduce the antibiotics' therapeutic potential against human and animal pathogens (Jia et al. 2020; Preena et al. 2020). The antibiotics and their corresponding ARGs were prevalent in major mariculture sites in China, and the number and proportion of

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antibiotic-resistant bacteria were significant, which may pose a grave threat to the marine environment and human health. (Gao et al. 2018). In Dongshan Bay marine aquaculture, 175 multi-drug-resistant isolates possessed at least three ARG subtypes, and ten percent of multiple-drug-resistant microorganisms belonged to potentially pathogenic genera or species (Cui et al. 2022). Freshwater aquaculture is an important source of ARGs too. Polymerase chain reaction results revealed that the *sul*1 and *sul*2 genes were the most reported ARGs, and tetracycline resistance genes had the most often reported subtype in Chinese freshwater aquaculture ponds (Wang et al. 2021). The high prevalence of ARB and ARGs in the aquaculture environment highlights the roles of aquatic products as potential sources of ARB and ARGs. It raises the need for controlling their transmission to humans through the food chain.

Liu et al. (2017), summarizing the antibiotics applied in the Chinese aquaculture industry from 2003 to 2016, found that quinolones were the most frequently detected class in aquatic products, followed by sulfonamides and macrolides. Sulfonamides, macrolides, fluoroquinolones, and chloramphenicol also were detected in the mariculture ponds (Zhang et al. 2021). Furthermore, fluoroquinolones and tetracyclines have gradually become the dominant antibiotics in aquaculture since 2006 (Dang et al. 2007; He et al. 2016; Wang et al. 2021). Based on the previous reports and our previous research works concerning the aquaculture environment in Zhejiang (Yuan et al. 2017; Ye et al. 2019), six frequently detected antibiotic compounds, including sulfonamides, tetracyclines, enrofoxin, cephalexin, gentamincin, and florfenicol, were selected for their related ARB and ARGs investigated in this study. The objective of this study was to assess the status of distribution and diversity of the ARBs and ARGs among the popular aquatic products in Zhejiang, China. Furthermore, the phenotypic and genotypic antibiotic resistance properties were investigated among the ARB isolates. A deep insight into the ARB and ARGs associated with aquatic products is beneficial in understanding their risks to aquatic organisms and humans.

Experimental

Materials and Methods

Samples. Aquaculture products, including crucian carp (*Carassius auratus*, 400–500 g), black carp (*Mylopharyngodon piceus*, 3,000–4,000 g), yellow catfish (*Pelteobagrus fulvidraco*, 150–200 g), loach (*Misgurnus anguillicaudatus*, 50–60 g), and giant river prawn (*Macrobrachium rosenbergii*, 35–40 g) were collected from different aquaculture farms in the Linghu, Huzhou (120.03°–120.14°E, 30.39°–30.47°N) in the north of the Zhejiang province, China. Large yellow croaker (*Larimichthys crocea*, 300–350 g) was collected from Dachen Dao, Wenzhou (121.90°E, 28.49°N), southeast of the Zhejiang province. Five samples were collected at random for each species. All the samples were transported to the lab in an icebox within 6 h after collection.

Isolation of bacteria. Initially, epidermal mucus, gills, and gut contents were collected. All the tissue samples were pooled and homogenized together by a sterile homogenizer (Yetuo, YT-PJ-400, Shanghai) immediately. Then, 100 µl of 10-fold serial dilutions up to 10^{-7} were plated onto nutrient agar (2216E Agar for large yellow croaker; Qingdao Hope Bio-Technology Co., Ltd., China) containing sulfadiazine (30 µg/ml), gentamicin (15 µg/ml), enrofloxacin (5 µg/ml), cephalexin (30 µg/ml), tetracycline (30 µg/ml), and chloramphenicol (30 µg/ml). The concentration of antibiotics added to the media was selected based on the CLSI (2018) breakpoints and modified slightly according to our previous studies (Yuan et al. 2017). After the cultures were incubated at 30°C for 48 h, at least five colonies from suitable dilution plates of each sample were randomly picked up and purified by streak plating twice. The purified culture was preserved in 30% glycerol at –80°C.

Taxonomy of the isolates based on the 16S rDNA sequencing. The isolates were identified by sequencing their the 16S rRNA gene. The genomic DNA of the isolates was extracted using a Bacterial DNA Extraction Kit (Tiangen Biotech Co. Ltd., China). The 16S rDNA sequences were amplified using primers 27F and 1492R with the protocol described by Yuan et al. (2017). All the amplicons were sent directly to the Shanghai Sangon Biotechnology Co. Ltd. for sequencing. The DNA sequences were analyzed using the sequence alignment tool BLAST (Bacteria 16S ribosomal RNA sequences database of GenBank), available on the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). The BLAST results with E-values of 0.0, good query coverage (98–100%), and 98–100% identity could be confirmed to a species level.

Testing of antibiotic susceptibility. Tests for susceptibility to six antibiotics were carried out using the disk diffusion method (CLSI 2018) at the following concentrations: 25 µg sulfamethoxazole (1.25/23.75, TMP/SMZ), 10 µg gentamicin, 4 µg enrofloxacin, 30 µg cephalexin, 30 µg tetracycline, 30 µg chloramphenicol (Hangzhou Microbial Reagent Co., Ltd., China). After incubation at 35°C for 24 h, the zones of inhibition surrounding the disks were measured, and the strains were recognized as susceptible or resistant based on interpretative criteria according to the CLSI guidelines (CLSI 2018). *Escherichia coli* ATCC® 25922™ was used as the quality control strain.

Analysis of antibiotic-resistance genes. The antibiotic-resistance genes from all the isolates were amplified.

Templates for PCR were prepared by boiling as follows: about 1–2 colonies from a fresh culture were picked up and suspended in 50 µl deionized sterile water. After boiling for 5 min, the suspension was immediately incubated on ice for 2 min. Then, the suspension was centrifuged at 5,000 × *g* for 1 min. About 2 µl of the supernatant was extracted and used as a PCR template. PCR assays were performed in 50 µl reaction mixture containing 1.5 µl of 10 × LA Buffer (Mg^{2+}), 2.0 µl of 2.5 mmol/l dNTP Mix, 1 µl of each primer (10 µmol/l), 0.1 µl of 5 U/µl TaKaRa LA Taq™ DNA Polymerase (TaKaRa, Japan), and 2 µl of DNA template. The primers for the target genes *sul*2, *aac*(6')-Ib, *qnr*S, *bla*PSE, *tet*A, *cml*A, and *flo*R are described in Table SI. The target genes were amplified using the following protocol: 94°C for 5 min, followed by 35 cycles of 94°C for 60 s, appropriate annealing temperatures for each primer pair for 40 s, and 72°C for 60 s, followed by a final extension step of 72°C for 10 min. All the amplicons were resolved using electrophoresis in a 1.5% agarose gel and checked by illumination under UV light after staining with ethidium bromide. The PCR products were purified using the Gel Mini Purification Kit (Zomanbio, China) and submitted for sequencing to the Shanghai Personalbio Biotechnology Co. Ltd. The sequencing data were analyzed using the BLAST tool in the NCBI database.

Statistical analysis. All the analyses were performed in triplicate, and statistical analysis was performed using the programs Origin 2019 (Origin Lab Corporation, USA) and AutoscriptProlog (IBM® SPSS® Statistics 21.0). The data were expressed as mean values ± standard deviation.

Results and Discussion

Identification of antibiotic-resistant bacteria. Colonies were obtained in the nutrient agar plates without antibiotics for each sample, and the distribution of antibiotic-resistant bacteria varied among different samples according to the number of colonies exhibited in Table SII. In total, 136 cultivable bacteria resistant to the tested drugs were picked up and purified. All the isolates could be classified into 22 genera and 49 species (Table SIII) according to 16S rDNA sequencing data. The corresponding GenBank accession numbers were as follows, MN216250-MN216297, MN220510-MN220522, MN220538-MN220542, and MN220557-MN220626. The predominant microbiota was represented by 106 isolates within six genera, including 41 *Aeromonas* spp., 19 *Shewanella* spp., 14 *Acinetobacter* spp., 13 *Myroides* spp., 10 *Pseudomonas* spp., and 9 *Citrobacter* spp., accounting for 80% of the total isolates.

Aeromonas spp., the major pathogens causing zoonotic diseases and was distributed naturally in diverse aquatic ecosystems and aquaculture products, was the predominantly isolated genera (31%). The antibiotic-resistant *Aeromonas* spp. could be frequently isolated from both healthy and disease-out broken aquaculture environments or farmed products (Zdanowicz et al. 2020; Dien et al. 2023). In this study, ten antibiotic-resistant *Aeromonas* species were isolated. Among them, *Aeromonas veronii* took the highest ratio (28 strains, 21%), and most isolates were recovered from crucian carp (17 strains). *A. veronii* is a widely distributed novel pathogen that can cause sepsis in fish, leading to high mortality and severe economic losses to aquaculture (Sun et al. 2017). Generally, *Aeromonas* spp. is regarded as an indicator of antibiotic resistance in the aquatic ecosystem since considerable antibiotic resistance was frequently found in *Aeromonas* infections (Patil et al. 2016). In this work, presumptive drug-resistant *Aeromonas* spp. were detected in all samples, which suggested that an excess of the tested antibiotics might be applied or exist in the aquaculture environment.

Shewanella spp. was the dominant ARB genus in large yellow croaker (14 strains, 52%), and the most frequently recovered species was *Shewanella baltica* (Table SIII). *S. baltica* and *Shewanella putrefaciens* are specific spoilage organisms of several chilled marine fish (Gu et al. 2013). Antibiotic-resistant *Shewanellaceae*, mainly the species *Shewanella algae*, were often recovered together with *Vibrio* spp. from the aquatic environments (Zago et al. 2020), and sometimes those species were closely related to specific diseases (Cao et al. 2018). The antibiotic-resistant *Shewanella* spp. in the farmed large yellow croaker added more risk to food safety.

Apart from the two genera mentioned above, other commensal and pathogen bacterial including *Acinetobacter* spp., *Myroides* spp., *Pseudomonas* spp., and *Citrobacter* spp. also accounted for a large proportion of all the isolates, while the remaining ARB recovered in this study belonged to 16 other genera. The diversity of the ARB also revealed that the ARGs might horizontally transfer between different genera and species (Fu et al. 2022).

Phenotypic characterization of antibiotic resistance. Phenotypic antibiotic resistance was checked accordingly to the CLSI (2018) (Fig. S1). A high percentage of resistance to tetracycline and sulfadiazine was shown for 38% and 37% of the total isolates (Table SIV). It was consistent with the results obtained in previous research works (Gao et al. 2012; Huang et al. 2017). Similar resistance rates to chloramphenicol (38 isolates, 28%), cephalexin (37 isolates, 27%), and enrofloxacin (35 isolates, 26%) were observed in this study. Furthermore, 24 (18%) isolates exhibited resistance to gentamicin.

Multiple drug-resistant bacteria were widely distributed in the samples of this study, which confirmed the widespread multi-drug resistance (Deng et al. 2020;

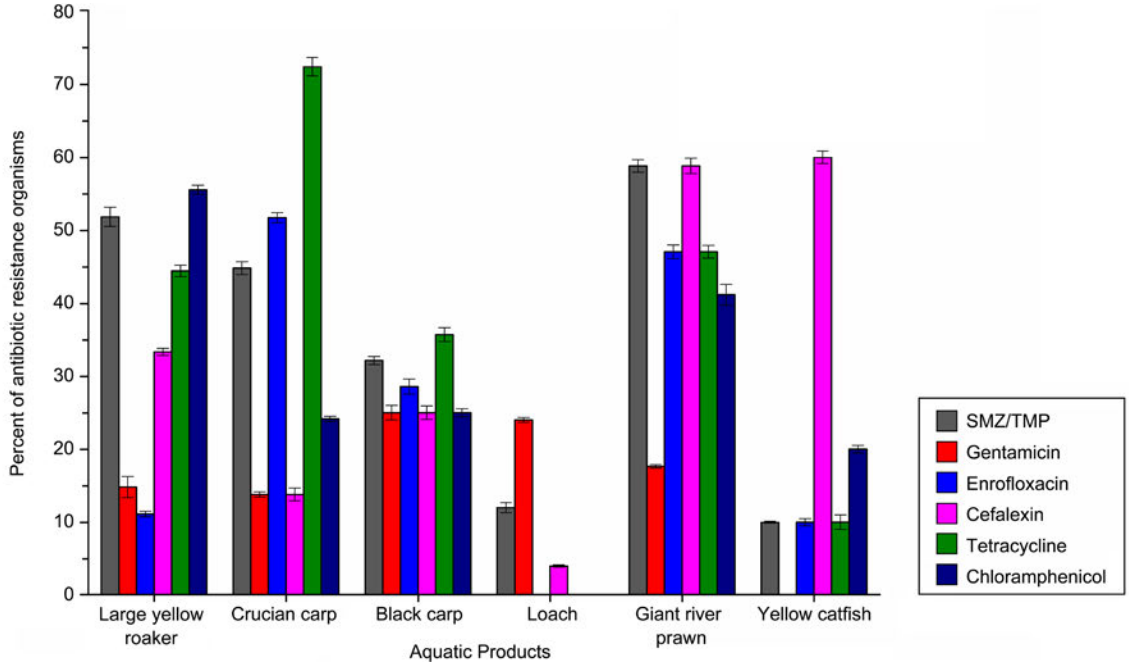

Fig. 1. The resistance rates of the isolates originated from different aquatic products to the six tested antimicrobials.

Wang et al. 2021). In total, 109 isolates (80%) exhibited resistance to at least one antibiotic, whereas 27 isolates were sensitive to all six tested drugs (Table SIV). Twenty-seven isolates exhibited resistant to only one tested drug, but the numbers of double resistant isolates and triple resistant isolates had reached to 46 and 25 respectively, which in total account for 52% of the isolates. Furthermore, eight isolates were resistant to four different drugs, while two isolates were even resistant to five. These results were consistent with previous findings of Sun et al. (2017), who reported that the multi-drug resistance rate of digestive tract bacteria in cultivated abalone was as high as 66%. The heavy or alternate use of multiple antibiotic complexes during aquaculture production might confer high multi-drug resistance to bacteria (Gao et al. 2018).

The distribution of antibiotic resistance among different samples showed great diversities (Fig. 1), which might be related to the disease outbreak frequency and administration of the antibiotic drugs. Even in the same pond, the number of ARB varied largely (Wu et al. 2019). Here in this study, cultivable bacteria from the giant river prawn displayed high resistance to sulfadiazine (59%), enrofloxacin (47%), cephalexin (59%), tetracycline (47%), and chloramphenicol (41%). The isolates from the large yellow croaker displayed high tolerance to sulfadiazine (52%), chloramphenicol (56%), and tetracycline (44%). Bacteria isolated from crucian carp also showed high resistance to sulfadiazine (45%), enrofloxacin (52%), and tetracycline (72%). Isolates from yellow catfish only had high resistance to cephalexin (60%). The resistance rates of the bacteria from black carp to the tested antibiotics were relatively low but ubiquitous, ranging between 25% and 36%. Bacteria from loach were sensitive to the tested antibiotics and showed no resistance to enrofloxacin, tetracycline, and chloramphenicol.

Genotypic characterization of antibiotic resistance. The existence of specific ARGs distributed on the conjugative plasmids, including *qnr*S, *tet*A, *flo*R, *sul*2, *aac*(6')-Ib, bla_{PSE}, and *cml*A in the 109 isolates, was checked by PCR amplification. The percentages of the amplicons were presented in Fig. 2. The prevalence

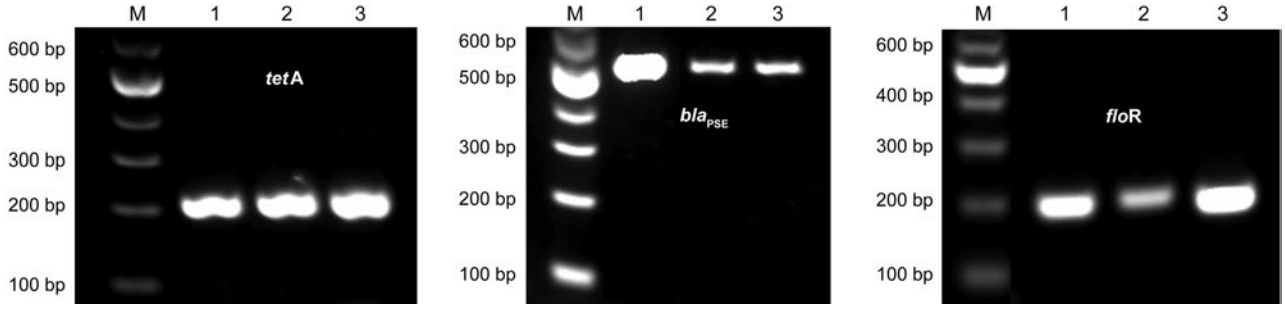

Fig. 2. Amplicons of partial antimicrobial resistance genes.

Table I
The coincidence of the resistant phenotype and genotype among the isolates.

Antimicrobial	No. of ARB isolates	ARGs	No. of the ARGs carrier	Coincidence rate (%)
Sulfadiazine	50	*sul*2	10	20%
Gentamicin	24	*aac*(6')-Ib	10	42%
Enrofloxacin	35	*qnr*S	26	74%
Cephalexin	37	bla_{PSE}	12	32%
Tetracycline	52	*tet*A	35	67%
Chloramphenicol	38	*flo*R	30	79%
		*cml*A	30	79%

of ARGs of the corresponding ARBs was presented in Table I. The genes *qnr*S, *tet*A, *flo*R, and *cml*A were principally found in isolates of phenotypically resistant characteristics, whereas *sul*2, *aac*(6')-Ib, and bla_{PSE} were less frequently detected, even in the isolates of the resistant phenotype.

The chloramphenicol resistance-related genes *flo*R and *cml*A had the highest detection rate in ARBs (79%). Although chloramphenicol has been prohibited in aquaculture since 1999 in China, chloramphenicol-resistant pathogens or environmental bacteria remain prevalent. The application of the chloramphenicol derivative florfenicol in aquaculture productions might be a direct cause. Moreover, the chloramphenicol-resistant genes left over from history also could transfer between different microorganisms in aquatic environments without a high selective pressure (Yoo et al. 2003; Wang et al. 2011). The presence of the *qnr*S gene among the ARB was over 74%, which to some degree corroborated with the fact that the quinolone-related ARGs such as *qnr*A, *qnr*B, *qnr*S were frequently discovered in the farming water, sediments, aquatic products, and pathogens causing disease in fish (Wang et al. 2021), as a result of the widespread and frequent application of quinolone and fluoroquinolone drugs (Chenia et al. 2016).

In this work, *tet*A was distributed among 67% of tetracycline-resistant ARBs. Many studies revealed that among different tetracycline-resistance *tet* genes, *tet*A was the most frequently detected in samples, including water and sediment in fish farms, fresh vegetables, and fish (Xiong et al. 2019). The *tet*A gene was also suggested to be a potential indicator of the abundance of tetracycline resistance genes in specific aquaculture modes in the Pearl River Delta (Huang et al. 2017). The gentamicin-related gene *aac*(6')-Ib, β-lactam-resistance gene bla_{PSE}, and the sulfonamide-resistance gene *sul*2 showed a lower detection rates of 42%, 32%, and 20%, respectively.

The inconsistency between the phenotypic and genotypic resistant data could owe to the following reasons. Firstly, the mechanism of drug resistance of bacteria was complicated, and diverse antibiotic resistance mechanisms against the particular antibiotic exist. For example, the plasmid-mediated quinolone resistance (PMQR) genes included *qnr*, *qep*A, and *aac*(6')-Ib genes (Suzuki, 2012). However, in this study, only the *qnr*S was selected to reflect fluoroquinolone-related gene pollution, which might be the main reason for the inconsistency between the phenotypic and genotypic resistant ratio. Secondly, the expression of some ARGs could be impressed by other factors during the dissemination (Wardenburg et al. 2019). Last but not least, limited samples in this study may not fully reveal the objective rules.

Overall, the ARB from different aquatic products showed varying resistance to the tested antibiotics, which might be related to the differential application of antibiotics during the farming process. The results also confirm that high rates of multi-drug resistance were prevalent within the isolates from all the tested samples.

The prevalence of drug-resistant opportunistic pathogens such as *A. veronii* and *Aeromonas hydrophila* indicates a potential safety hazard. The previous studies indicated that most ARGs come from plasmids that originate from *Vibrio* and *Aeromonas* (Liu et al. 2019). The abuse of antibiotics and the horizontal transfer of ARGs have increased the prevalence of multi-drug-resistant bacteria in the aquatic environment. All the tested ARGs responding to the six tested drugs could be detected among the ARBs. The widespread ARGs may increase the antibiotic resistance of pathogenic bacteria and reduce antibiotic efficacy.

Conclusions

This work showed a great diversity of ARB and ARGs in popular farmed aquatic products in Zhejiang, China. Since the potential pathogen *Aeromonas* spp. and spoilage bacteria *Shewanella* spp. were the predominant ARB in the collected samples, more concerns need to be put on the antibiotic administration. Multi-drugs resistant isolates consisted of more than half of the isolates and

were distributed widely among all the collected samples. The ARGs corresponding to the common antibiotics applied in aquaculture were detected easily, though the inconsistency between the phenotypic and genotypic antibiotic resistance was detected. The data obtained in this work could improve understanding of the risks to aquatic organisms and human beings.

Author Contributions

Jiajia Wu and Zhiyuan Dai designed the experiment; Fan Ye finished the experimental work; Jiong Qu and Jiajia Wu analyzed the data and Jiajia Wu wrote and revised the manuscript.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Prevalence of Closely Related *Candida albicans* Species among Patients with Vulvovaginal Candidiasis in Southern Poland Based on the *hwp*1 Gene Amplification

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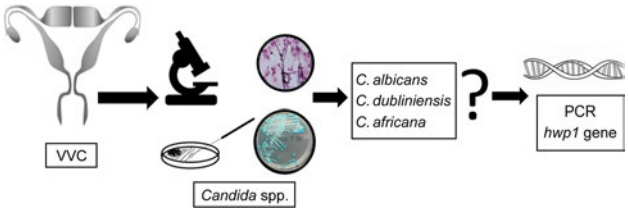
Abstract

Candida albicans remains the most common species isolated from women with vulvovaginal candidiasis. However, closely related species such as *Candida africana* and *Candida dubliniensis* may also occur, although they are often misidentified. The aim of the study was to confirm the phenotypic identification of *C. albicans* and its closely related species isolated from women with genital tract infections by amplification of the *hwp*1 (hyphal wall protein 1) gene in a PCR assay. We report a detailed molecular identification of *C. albicans* and its closely related species among 326 patients in the Małopolska region, Poland. Initial phenotypic identifications were confirmed by amplification of the *hwp*1 gene. Based on molecular analysis, we revealed 307 strains (94.17%) as *C. albicans* and 17 as *C. dubliniensis* (5.22%). No strain of *C. africana* was detected. Two patients had co-infection with *C. albicans* and *C. dubliniensis* (0.61%). A PCR assay targeting the *hwp*1 gene was reliable for correctly identifying species among the *C. albicans* complex.



Keywords: *Candida albicans*, *Candida africana*, *Candida dubliniensis*, vaginitis, vulvovaginal candidiasis

Introduction

Despite advances in the treatment of fungal infections, vulvovaginal candidiasis (VVC) remains a common problem among women of childbearing age worldwide (Sustr et al. 2020). This disease affects women to varying degrees, regardless of ethnic origin, and social or living conditions. This ailment can be induced by various factors ranging from host factors such as age, estrogen levels, sexual activity, exposure to stress, allergic diseases, or medications (Farr et al. 2021). However, most cases do not have an identifiable trigger. It is estimated that 70–75% of women will be diagnosed with vulvovaginal candidiasis at least once in their lifetime (Hedayati et al. 2015), and 5–8% of adult women have recurrent vulvovaginal candidiasis (RVVC), which is defined as four or more episodes per year (Sobel 1992). VVC affects approximately 138 million women annually (in the range of 103 to 172 million), with a global annual prevalence of 3,871 per 100,000 women; 372 million women are affected by RVVC during their lifetime (Denning et al. 2018). More than 40 species of the *Candida* genus are capable of causing infection (Pfaller et al. 2014), but *Candida albicans* remain a significant cause of VVC (Sobel and Sobel 2018). The incidence of *C. albicans* vulvovaginitis is 70% to 90%.

The taxonomy of some *Candida* species has undergone significant changes due to the development of

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identification techniques, primarily molecular biology. Due to difficulties in identification, the newly described related species were grouped into complexes and named cryptic yeasts. The *C. albicans* complex includes three closely related species *C. albicans* sensu stricto, *Candida dubliniensis*, and *Candida africana* (Sullivan et al. 1995; Tietz et al. 2001). *C. dubliniensis* and *C. albicans* have many phenotypic resemblances, such as the capacity to form pseudohyphae, chlamydospores on cornmeal agar, and germ tubes in serum (Moran et al. 2012). Phylogenetic studies indicate that *C. dubliniensis* is most closely related to *C. albicans* (McManus et al. 2008), and it is often difficult to discriminate between these two species in clinical samples. The second species from the *C. albicans* complex, *C. africana*, had previously been considered an atypical *C. albicans* strain. However, in 2001 it was proposed to isolate a separate species of *C. africana* that produces pseudohyphae but not chlamydospores (Tietz et al. 2001). Several other features, such as the inability to grow in 42°C, the incapacity to assimilate the aminosugars N-acetyl glucosamine and glucosamine, as well as the disaccharide trehalose, and the organic acid DL-lactate, could be helpful in proper identification, but during routine diagnostics misidentification often occurs in mycological laboratories (Sullivan et al. 2004). To avoid these drawbacks of classical techniques, molecular analyses have been applied in multiple studies to identify and distinguish between *Candida* species (Byadarahally Raju and Rajappa 2011; Neppelenbroek et al. 2014). A singleplex PCR assay using a single primer pair targeting the hyphal wall protein 1 (*hwp*1) gene encoding a mannoprotein specific for the filamentous form of *C. albicans*, has been proposed to distinguish *C. albicans* sensu stricto, *C. dubliniensis*, and *C. africana* according to the distinct size of the amplicon (Romeo and Criseo 2008; 2009).

The available clinical and epidemiological data show that *C. africana* and *C. dubliniensis* are isolated from patients all over the world; however, there are scant data on the frequency of occurrence of these species in Poland, identified as the cause of VVC. Due to limited knowledge concerning its prevalence, this study aims to confirm the phenotypic identification of *C. albicans* and its closely related species isolated from the genital tract by amplifying the *hwp*1 (hyphal wall protein 1) gene in the PCR assay.

Experimental

Materials and Methods

Materials. A present study covered 326 vaginal yeast isolates, presumptively identified as *C. albicans*, by phenotypic methods. All isolates were obtained from female patients (median 33, from 17 to 65 years) with vaginitis, diagnosed at the Centre of Microbiological Research and Autovaccines in Cracow, Poland.

The including criteria were as follows: *C. albicans* or *C. dubliniensis* in a phenotypic screening assay. Species other than *C. albicans* or *C. dubliniensis* were excluded from the analysis in this paper.

Phenotypic assay. Phenotypic analysis based on culturing methods was performed as part of routine clinical practice at the Centre of Microbiological Research and Autovaccines in Cracow, Poland.

Two (2) vaginal swabs were taken from the posterior vaginal vault, using a sterile speculum. The first vaginal swab was used for Gram staining with PREVI® Color Gram stainer (bioMérieux, France).

The second swab was used to perform cultures of aerobic bacteria (TSA with 5% sheep blood; GRASO, Poland), *Gardnerella vaginalis* (Agar Gardnerella; bioMérieux, France), and yeast-like fungi (Sabouraud dextrose agar with chloramphenicol; GRASO, Poland and chromID® Candida; bioMérieux, France). The media were incubated for 24 h or 48 h at 35°C under appropriate conditions. The identification of species was conducted on VITEK®2 (bioMérieux, France).

Vulvovaginal candidiasis (VVC) was diagnosed by positive *Candida* spp. cultures and microscopic observation of yeast-like cells and/or hyphae in the Gram-stained vaginal smears evaluated according to the scale developed by Kuczyńska, modified by Kasprowicz. The scale assessed the presence of leukocytes, clue cells, lactobacilli, other bacteria, and fungi based on the abundance scale ranging from 0–3 in oil-immersion microscope fields (1,000×) (Kasprowicz and Białecka 2012).

Initial species identification of strains was carried out based on the appearance of the colonial morphology on chromogenic medium (chromID®, bioMérieux, France). As a control of chromogenic agar, the reference strains were included: *C. albicans* ATCC® 90028™, *Candida glabrata* ATCC® 2001™, *Candida tropicalis* ATCC® 9968™ (Fig. 1A), *C. albicans* ATCC® 90028™, *C. dubliniensis* NCPF 3949 and *C. africana* MYA 2669™ (Fig. 1B).

Afterward, further identification was performed by an automatic biochemical method using the YST Card, VITEK®2 compact (bioMérieux, France) according to the manufacturer's instruction. The results were approved when the acceptable score was generated using the database system, covering 27 *Candida* genus species (with the exception of *C. africana*). The results were interpreted by the VITEK®2 database at different confidence levels as excellent identification (probability range from 96% to 99%), very good (93–95%), good (89–92%), acceptable (85–88%), low discrimination (< 85%), and unidentified microorganisms. The results of yeast identification were considered acceptable when the confidence level was ≥ 85% probability.

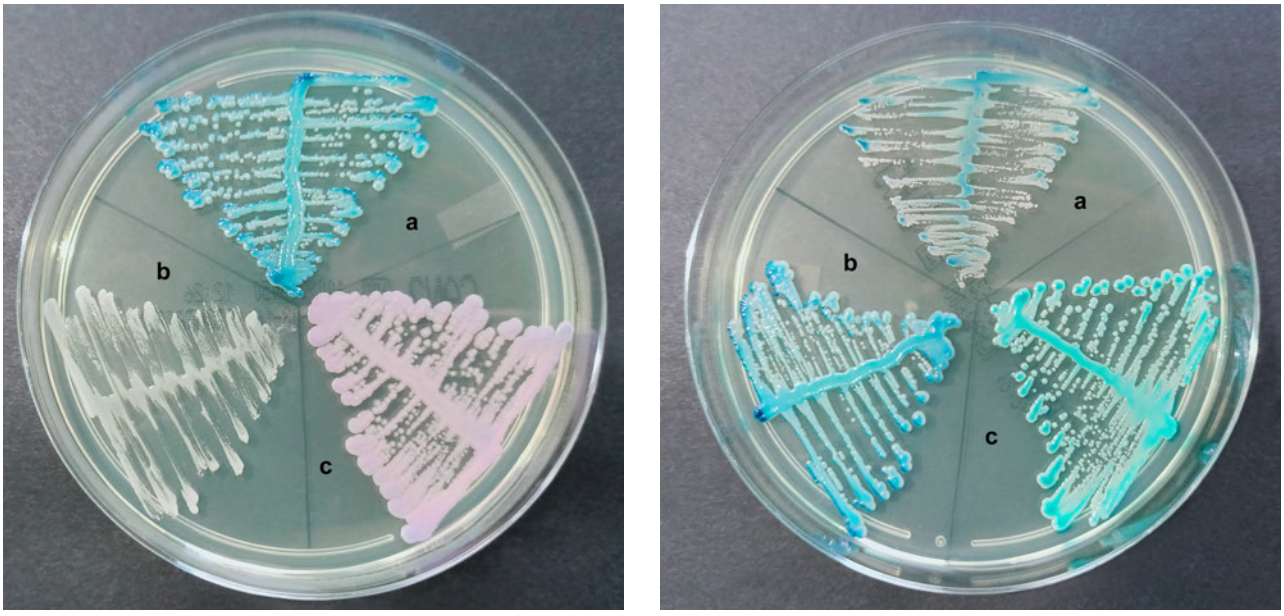

Fig. 1. Morphology of reference strains of yeasts colonies after 48 h incubation in 37°C on chromID® Candida (bioMérieux, France).
A. a) *Candida albicans* ATCC® 90028™ (dark blue colonies), b) *Candida glabrata* ATCC® 2001™ (white colonies),
c) *Candida tropicalis* ATCC® 9968™ (pink colonies).
B. a) *Candida africana* ATCC® MYA 2669™ (small, light blue colonies), b) *Candida albicans* ATCC® 90028™ (dark blue colonies),
c) *Candida dubliniensis* clinical strain (turquoise colonies).

Molecular identification. The molecular tests were conducted at the Department of Pharmaceutical Microbiology of the Jagiellonian University Medical College, Cracow, Poland. Fungal DNA was extracted with the Yeast Mini isolation kit (A&A Biotechnology, Poland), according to the manufacturer's instructions.

The species identification of *Candida* isolates was performed using PCR with CR-F (5'-GCTACCACTTCAGAATCATCATC-3') and CR-R (5'-GCACCTTCAGCTGTAGAGACG-3') primers that targeted the *hwp*1 (hyphal wall protein 1) gene, encoding a mannoprotein specific for the filamentous form of *C. albicans*, as previously described (Romeo and Criseo 2008).

The PCR conditions were as follows: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 15 s, annealing at 63°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min, in a T-Personal Thermal Cycler (Biometra, Germany). The reaction mixture of the final volume 25 µl contained: 5 µl of GoTaq® Flexi Buffer, 0.5 µl of PCR Nucleotide Mix (10 mM each) (Promega, USA), 1 µl of each primer (Genomed, Poland), 2 µl of genomic DNA, 2 µl of $MgCl_2$ (25 mM), 0.125 µl of GoTaq® DNA Polymerase (5 u/µl) (Promega, USA) and Nuclease-Free Water (Promega, USA). The PCR products of the size of 941 bp, 569 bp, and 700 bp for *C. albicans*, *C. dubliniensis*, and *C. africana* respectively, were expected.

The PCR amplicons were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide staining (Sigma-Aldrich Chemie, Germany).

Reference strains included *C. albicans* ATCC® 90028™, *C. dubliniensis* NCPF 3949, and *C. africana* MYA 2669™.

MALDI TOF assay. Simultaneously with the molecular assay, MALDI-TOF MS (Matrix-assisted laser desorption ionization-time of flight mass spectrometry) identification of the selected strains of *Candida* (including isolates identified by VITEK®2 at a low discrimination level) was performed using the VITEK®2 MS Biotyper (bioMérieux, France) at the University Hospital in Cracow, Poland. The identification accuracy was determined by a confidence level of > 80%, according to the manufacturer's recommendation.

***In vitro* antifungal susceptibility assay.** A commercial assay for *in vitro* antifungal susceptibility testing Fungitest™ (BioRad, France), was used to determine the efficiency of miconazole at the concentration of 0.5 and 8 µg/ml, ketoconazole at the concentration of 0.5 and 4 µg/ml, itraconazole at the concentration of 0.5 and 4 µg/ml, and fluconazole at the concentration of 8 and 64 µg/ml. The colorimetric analysis and interpretation of the results were performed according to the manufacturer's recommendations. The reference strain *Candida parapsilosis* ATCC® 90018™ was used as a quality control to establish the reproducibility of the susceptibility testing results.

According to the manufacturer's recommendation of Fungitest™ (BioRad, France), we use the following criteria of drug susceptibility: susceptible (S), intermediate (I) (referring to "susceptible, increased exposure" according to the EUCAST recommendations (EUCAST 2020), and resistant (R).

Ethics approval. Our report is a retrospective *in vitro* study without the participation of human or animal subjects. The study materials were yeast strains collected by routine laboratory practice, not for scientific purposes, and stored in glycerol tubes at –80°C as a biobank collection. All human data associated with strains were anonymized before the researcher gained access to it. Following the guidelines of the Jagiellonian University Medical College Bioethics Committee, Cracow, Poland, this type of research does not meet the criteria of a medical experiment and is not subject to ethics review.

Results

Results of phenotypic analysis. According to a phenotypic analysis based on culture on chromogenic media, all 326 *Candida* strains included in the study were obtained from patients diagnosed with vulvovaginal candidiasis. In addition, the presence of aerobic bacteria in vaginal swabs was confirmed, such as: Gram-positive cocci (*Staphylococcus* spp.*)* in one patient, streptococci (group B *Streptococcus* – GBS) in 55 patients, Gram-negative bacilli (*Escherichia coli* in 13*,* and *Klebsiella* spp. in one patient) and colonization by anaerobic bacteria (*G. vaginalis*) in 73 patients (Table I). All women with co-occurrence of *Candida* spp. with bacteria presented reduced *Lactobacillus* spp.

Results of molecular analysis. Based on the PCR assay targeting the *hwp*1 gene, preceded by MALDI TOF analysis, we confirmed the initial phenotypic identification in 307 (94.17%) samples as *C. albicans*, while 17 (5.22%) strains belonged to the *C. dubliniensis* species (Table II). Moreover, molecular identification demonstrated two co-infections (0.61%) by *C. albicans* and *C. dubliniensis*. No strain of *C. africana* was detected (Fig. 2).

Table I
Prevalence of single VVC and co-occurrence with bacteria.

Type of co-occurrence	No. (%) n = 326
Single VVC	197 (60.43)
VVC + bacteria:	129 (39.57)
Gardnerella vaginalis	60 (18.40)
Streptococcus agalactiae	41 (12.58)
Gardnerella vaginalis + *Streptococcus agalactiae*	11 (3.37)
Escherichia coli	9 (2.76)
Escherichia coli + *Streptococcus agalactiae*	3 (0.92)
Gardnerella vaginalis + *Escherichia coli*	2 (0.61)
Methicillin susceptible *Staphylococcus aureus*	1 (0.31)
Streptococcus pyogenes	1 (0.31)
Klebsiella pneumoniae + *Streptococcus agalactiae*	1 (0.31)

VVC – vulvovaginal candidiasis

Table II
Molecular discrimination among *Candida albicans* complex.

Species	No. (%) n = 326
Candida albicans	307 (94.17)
Candida dubliniensis	17 (5.21)
Candida albicans + *Candida dubliniensis*	2 (0.61)

Comparison of *C. albicans* complex identification results from VITEK®2, MALDI-TOF MS, and PCR assays. Phenotypic analysis based on chromogenic medium (chromID® Candida; bioMérieux, France) identified all clinical isolates as *C. albicans* strains. Despite slight differences in the appearance and colors of the yeast colonies (Fig. 1B), no distinction between *C. albicans* sensu stricto, *C. dubliniensis*, and *C. africana* species was possible according to the manufacturer's recommendations.

The reference method for this study found as "a gold standard", was molecular identification based on the *hwp*1 gene amplification, which confirmed that all

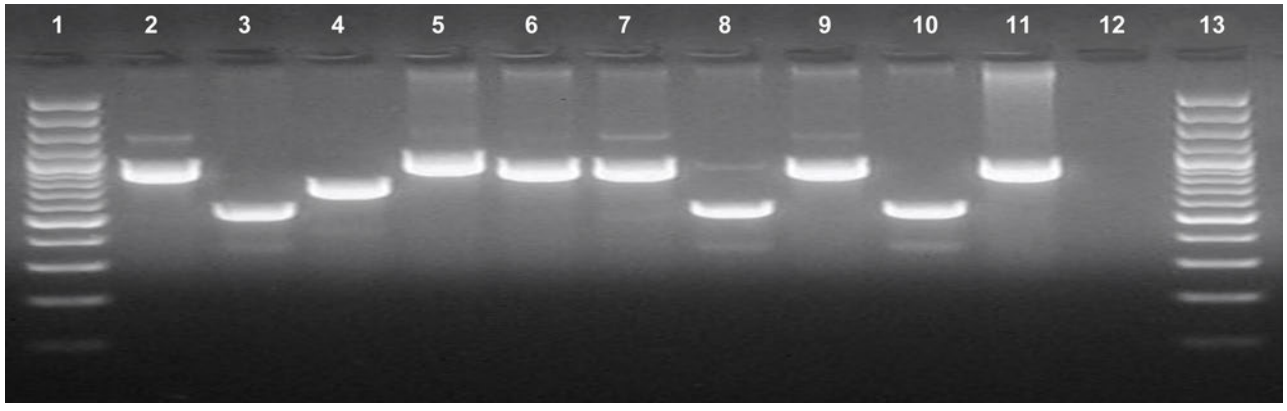

Fig. 2. Representative samples of the *hwp*1 gene PCR assay visualised in electrophoresis.
Line 1 and 13 – molecular weight marker (O'GeneRuler 50 bp DNA Ladder; Thermo Scientific, USA)
Line 2 – positive control of *Candida albicans* ATCC® 90028™ (product size 941 bp). Line 3 – positive control of *Candida dubliniensis* NCPF 3949 (product size 569 bp). Line 4 – positive control of *Candida africana* ATCC® MYA 2669™ (product size 700 bp)
Line 5, 6, 7, 9, 11 – clinical *Candida albicans* strains. Line 8, 10 – clinical *Candida dubliniensis* strains. Line 12 – negative control

Table III
Percentage of compatible results obtained by the methods used to discriminate species among *Candida albicans* complex (VITEK®2 system and MALDI-TOF MS) with the *hwp*1 gene amplification with PCR.

	The compliance with PCR assay No. (%) n = 326	
VITEK 2	319 (97.85)	7 (2.15)
MALDI-TOF MS	323 (99.08)	3 (0.92)

reference strains comprised the *C. albicans* complex: *C. albicans* sensu stricto (ATCC® 90028™), *C. dubliniensis* (NCPF 3949), and *C. africana* MYA 2669™.

The VITEK®2 system (bioMérieux, France) showed agreement with the molecular assay in 97.85% of clinical strains. Most *C. albicans* sensu stricto were identified at an excellent and very good discrimination level. All the incorrect identification results (2.15%) referred to *C. dubliniensis* strains (Table III, IV), which were finally discriminated by PCR assay. The VITEK®2 system did not allow identification of *C. africana*. The reference strain of *C. africana* MYA 2669™ was identified as *C. dubliniensis*/*C. parapsilosis* at the low discrimination level (Table IV).

The protein analysis by MALDI-TOF MS (VITEK®2 MS Biotyper; bioMérieux, France) did not require lengthy biochemical reactions. It was more accurate method than the biochemical analysis with the VITEK®2 system. Based on MALDI-TOF MS, 99.08% of clinical isolates were correctly identified as *C. albicans* or *C. dubliniensis.* Only 0.92% of the identification results differed from the results obtained with PCR (Table III). However, MALDI-TOF MS failed to detect the *C. africana* reference strain, and neither did the VITEK®2 system (Table IV).

Results of antifungal susceptibility testing. Antifungal susceptibility testing covered susceptibility to azoles, which are recommended for VVC. The susceptibility to amphotericin B and 5-flucytosine was found clinically unsuitable for VVC treatment (Workowski et al. 2021).

In total, 100% of *C. albicans* isolates were susceptible to fluconazole and ketoconazole, while 99.35% (305/307) of them were susceptible to miconazole, and 96.74% (297/307) to itraconazole. The ratio of *C. albicans* intermediate to itraconazole (referring to "susceptible, increased exposure" according to the EUCAST recommendations (EUCAST 2020)) amounted to 3,26% (10/307). Only 0.65% (2/307) were intermediate resistant to miconazole (Fig. 3). All *C. dubliniensis* strains were susceptible to all antifungals tested.

Table IV
Discrepancies between species identification among *Candida albicans* complex obtained by the Vitek®2 system, MALDI-TOF MS and the *hwp*1 gene amplification with PCR.

Isolate No.		VITEK 2		MALDI-TOF MS		PCR assay based on the *hwp1* gene amplification	
		Species identification	Confidence level (probability)	Species identification	Confidence level	Species identification	Product size (bp)
CA67	clinical strain	*Candida albicans*/ *Candida dubliniensis*	low (< 85%)	*Candida dubliniensis*	99.90%	*Candida dubliniensis*	569
CA95	clinical strain	*Candida albicans*	excellent (96%)	*Candida albicans*	99.90%	*Candida albicans* + *Candida dubliniensis*	941 + 569
CA98	clinical strain	not identified	–	*Candida dubliniensis*	99.90%	*Candida dubliniensis*	569
CA125	clinical strain	*Candida ciferrii*	acceptable (86%)	*Candida dubliniensis*	99.90%	*Candida dubliniensis*	569
CA13	clinical strain	*Candida albicans*/ *Candida dubliniensis*	low (< 85%)	*Candida dubliniensis*	99.90%	*Candida dubliniensis*	569
CA137	clinical strain	*Candida albicans*	good (91%)	*Candida albicans*	99.90%	*Candida dubliniensis*	569
CA313	clinical strain	*Candida albicans*	very good (95%)	*Candida albicans*	99.90%	*Candida albicans* + *Candida dubliniensis*	941 + 569
Candida albicans ATCC® 90028™	reference strain	*Candida albicans*	excellent (98%)	*Candida albicans*	99.90%	*Candida albicans*	941
Candida dubliniensis NCPF 3949	reference strain	*Candida dubliniensis*	very good (93%)	*Candida dubliniensis*	99.90%	*Candida dubliniensis*	569
Candida africana MYA 2669™	reference strain	*Candida dubliniensis*/ *Candida parapsilosis*	low (< 85%)	*Candida albicans*	99.90%	*Candida africana*	700

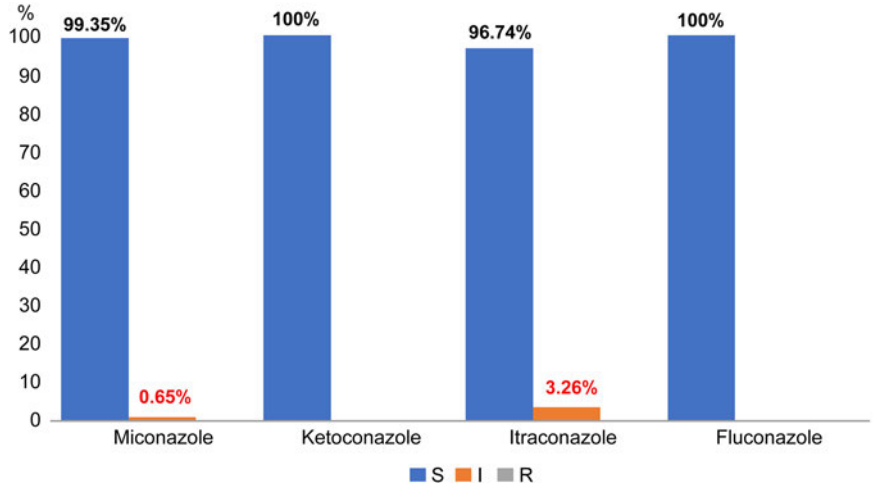

Fig. 3. Susceptibility of 309 *Candida albicans* clinical isolates caused VVC obtained by Fungitest™ (BioRad, France).
S – susceptible, I – intermediate (referring to "susceptible, increased exposure" according to the EUCAST recommendations), R – resistant.

Discussion

Vulvovaginal candidiasis (VVC) remains a threatening problem among women (Sustr et al. 2020). All 326 *Candida* strains included in the study were obtained from women with confirmed VVC. Furthermore, in 128 cases (~39%), the coincidence of yeast and aerobic and/or anaerobic bacteria was revealed by phenotypic methods. We are in line with the other observations that the occurrence of yeasts in the genital tract disrupted the natural bacterial microbiota, especially the number of *Lactobacillus* spp., which is generally considered to constitute a major part of the normal vaginal microbiota and are natural competitor of *Candida* spp. (Zangl et al. 2019).

C. albicans is the most common isolated species among women with VVC; however, there are still problems with discriminating closely related species among *C. albicans* complex; thus, their prevalence and clinical importance remains misestimated. Despite the phenotypic and biochemical differences (e.g., production chlamydospores and ability to assimilate N-acetylglucosamine and glucosamine), the identification of *C. africana* remains challenging with the use of currently existing methods in routine laboratory practice (Tietz et al. 2001; Criseo et al. 2015).

One of the most commonly utilized, rapid, and cost-effective tests are chromogenic media, which are frequently used to detect and identify yeasts of the *Candida* genus. However, culture-based methods may still need to be improved as they are often influenced by various culture conditions (Tietz et al. 2001; Criseo et al. 2015; Scharmann et al. 2020). Despite subtle differences in the *C. albicans* sensu stricto, *C. dubliniensis*, and *C. africana* colonies morphology on the ChromID® medium used in this study, no distinction of these species is possible according to the manufacturer's recommendations (Fig. 1B).

The other method commonly used in routine laboratory practice for yeast identification is the VITEK®2 system. This automated system is based on kinetic analysis detecting metabolic changes and continuous colorimetric-based monitoring of reactions which provides reliable identification of 27 medically relevant species of *Candida* genus, including both *C. albicans* and *C. dubliniensis.* To this day, there is no possibility of *C. africana* identification by VITEK®2, ID YST Card. Identifying newly described species, species of modified systematic names, and rare ones is problematic for most commercial systems because the databases do not include a representative set of profiles, resulting in misidentifying closely related microorganisms.

Due to the limited ability of phenotypic approaches, such as chromogenic media and the VITEK®2 system, to identify rare species and to confirm the identification of *C. africana* isolates, phenotypic tests have to be supplemented with molecular methods.

According to the performed molecular assay based on the *hwp*1 gene amplification, we revealed that the diagnosis of 326 strains initially identified by phenotypic methods as *C. albicans* was confirmed in 94.17% (307/326), while 5.21% (17/326) strains were identified as *C. dubliniensis.* Moreover, molecular identification allowed the detecting of two cases of co-infection with *C. albicans* and *C. dubliniensis*. Although *C. africana* is isolated from vaginal specimens worldwide, we could not isolate any species of this organism from the presumptive vaginal isolates of *C. albicans*.

The meta-analysis provided by Ghareghbolagh et al. (2020), showed that the distribution of *C. albicans* closely related species varies among the world's regions, and the global prevalence of *C. africana* among *C. albicans* complex reached 1.67%. Our results align with those from Turkey, Malaysia, and China, where 195, 98, and 87 *Candida* isolates from vulvovaginitis

cases were detected (Gumral et al. 2011; Yazdanpanah and Khaithir 2014; Hu et al. 2015). Furthermore, a single study from Iran has confirmed the lack of *C. africana* in vaginal specimens, even though the overall prevalence of *C. africana* in this country was one of the highest (~ 3%) (Pakshir et al. 2017). It confirms that variations in the prevalence of this species could be observed among and across continents and even within one country.

Epidemiological data indicated that the incidence of *C. dubliniensis* in vaginal samples has varied within the tested population from 0 to even 29.52% (Jamilian et al. 2007; Shan et al. 2014). Several studies demonstrated the very low prevalence of *C. dubliniensis* in VVC/RVVC patients (Theill et al. 2016; Hazirolan et al. 2017; Mucci et al. 2017). Our results based on molecular discrimination confirmed that 5.22% of the VVC cases were caused by *C. dublinienis* and approximated those reported by Borman et al. (2013) (6%). Furthermore, we revealed two patients (0.61%) who presented with concurrent co-infection by *C. albicans* and *C. dubliniensis*. Although *C. dubliniensis* has been frequently associated with oral infections in HIV-positive patients, it has also demonstrated significant levels of adhesion to vaginal epithelial cells (average adherence 85.6%), suggesting that this species is well adapted, in terms of adhesion capability, to the vaginal environment (Vidotto et al. 2003).

Multiple global studies have shown that *C. albicans* and the related species isolated from VVC patients are usually susceptible to antifungal agents (Theill et al. 2016). The results of the Fungitest™ assay used in this study confirmed these findings. Whereas only single strains of *C. albicans* have shown reduced susceptibility to itraconazole and miconazole, all *C. dubliniensis* strains were susceptible to all antifungals tested. However, *C. dubliniensis* is known to develop rapid resistance to fluconazole, and consequently, patients do not respond to standard doses of fluconazole (Kolekar et al. 2019). Furthermore, global overuse of azole antifungal agents without prescription for the treatment of *Candida* vaginal infection and repeated exposure to antifungal agents can lead to the induction of azole resistance in strains (Naeimi et al. 2018; Mohammadi et al. 2021). In our study, we did not observe any strains resistant to fluconazole. At the same time, resistance to this antifungal was frequently reported among *C. albicans* isolates from VVC patients, with a resistance rate ranging from 14.7% to even 53.7% (Mohammadi et al. 2021). The microbroth dilution method is the reference method for antifungal susceptibility testing of glucose-fermenting yeasts (EUCAST 2020; CLSI 2022); however, the commercial kits (e.g., Fungitest™; BioRad, France) are still in use as a screening test for the detection of antifungal efficacy (Cuenca-Estrella et al. 2005). The microdilution method recommended by EUCAST is demanding and rarely used in routine diagnostics of *C. albicans* strains isolated from women with VVC. Methods such as E-test strips or automatic VITEK®2 YST08 methods are expensive and are not applicable in the routine diagnosis of genital tract infections. In addition, the VITEK®2 system and the available antibiogram charts provide data for fluconazole, only one of the drugs used in VVC treatment. Moreover, the interpretation criteria according to EUCAST for drugs used in the treatment of VVC apply only to fluconazole and itraconazole. There are currently no other recommendations regarding topical antifungal drugs (e.g., miconazole, econazole, clotrimazole).

According to the Centers for Disease Control and Prevention recommendations (CDC), treatment of uncomplicated VVC (which constitutes the majority of VVC) is based on empirical therapy. Culture and antifungals susceptibility testing should be considered only for patients who remain symptomatic after failure of the empirical treatment (Workowski et al. 2021). However, according to the demands reported by the physicians, susceptibility tests are performed. Currently, there are few commercial tests available that can be used to assess the susceptibility testing of drugs applicable in the treatment of VVC. Fungitest™ (BioRad, France) was the only method used to test antifungal susceptibility in this study and is still routinely used in many microbiological laboratories in Poland. However, the concentrations of drugs available in this kit do not meet the current European (EUCAST 2020) and American criteria (CLSI 2022). According to the manufacturer's instructions, the interpretation of drug susceptibility is based only on scientific reports. Moreover, this semi-quantitative method has many advantages: it does not require complex handling and is cost-effective. Furthermore, the agreement with the European Committee on Antibiotic Susceptibility Testing (EUCAST) data for Fungitest™ was high, with correlation indices of 0.95 ($p < 0.01$) (Cuenca-Estrella et al. 2005).

Conclusions

C. albicans sensu stricto remains the major factor of vulvovaginal candidiasis, while *C. dublinienis*, and *C. africana* are a minor species within the *C. albicans* complex (Gharehbolagh et al. 2020). Our results based on molecular discrimination confirmed that the most common species causing vulvovaginal candidiasis in Southern Poland is *C. albicans* sensu stricto (94.17%). However, we argue that the reported incidence of *C. dubliniensis* and *C. africana* suffers from certain limitations, such as a low number of studies from each country and the unavailability of data for the majority of countries. While it has been estimated that *C. albicans*

is responsible for 78.3% of genital tract candidiasis in Poland (Wójkowska-Mach et al. 2021), the regional occurrence of closely related *C. albicans* species such as *C. dubliniensis* and *C. africana* is unknown. As far as we know, this is the first study concerning the prevalence of *C. albicans* and its closely related species isolated from the genital tract in our country.

The global prevalence of *C. albicans* may be overestimated due to a lack of routinely used tests aimed at the discrimination of closely-related species; therefore, *C. dubliniensis* and *C. africana* isolates continue to be misidentified as *C. albicans* (Shokoohi et al. 2021). Due to minor phenotypic differences between closely-related species belonging to the *C. albicans* complex, it is not easy to recognize them properly by routine diagnostic methods (e.g., biochemical tests, chromogenic media).

A PCR assay targeting the *hwp*1 gene was reliable in identifying species among the *C. albicans* complex. Its simplicity, rapidity, and selectivity make this method helpful in investigating *C. albicans* and its closely related species epidemiology.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Stethoscopes or Maybe "Bacterioscopes" – Is hand Hygiene Solely Capable of Preventing Hospital-Associated Infections?

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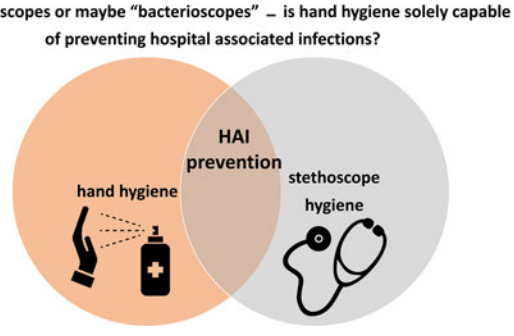
Abstract

The stethoscope remains an indispensable diagnostic tool for medical students. Improper stethoscope hygiene may cause bacterial infections, including hospital-associated infections (HAIs), which challenge the Polish medical system. The study's main objective was to evaluate the hygiene habits declared by medical students. Moreover, microbiological control with the characteristics of potentially pathogenic microorganisms was performed. The study included 66 medical students from the Faculty of Medicine at the Jagiellonian University Medical College in Cracow, Poland. The participants filled in an anonymous questionnaire. Stethoscope contamination was assessed through isolation, identification, testing of antibiotic resistance, and clonality of the isolates bacterial pathogens. The survey showed that only 30.3% of students cleaned their stethoscopes after each patient, and 1.5% never did this. Of the 66 stethoscopes tested, 100% were positive for bacterial growth. *Staphylococcus* spp. was the most frequently isolated contaminant (50.5%). The questionnaire results demonstrated the necessity of the validated procedures for cleaning the stethoscopes. Stethoscopes used by medical students are contaminated with numerous bacterial species, including multidrug-resistant organisms. The clonal structure of the MRSA and MRSE populations acquired from stethoscopes has been demonstrated. Our results confirm the possibility that these medical devices mediate the spread of hazardous pathogens in the hospital environment. Practical exercises are essential to forming the correct hygiene habits involving stethoscopes, which enable practicing and checking the correctness of the established skills.



K e y w o r d s: hospital-associated infections (HAIs), medical student stethoscopes, bacterial contamination, methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE)

Introduction

The World Health Organization (WHO) recognizes the phenomenon of healthcare-associated infections (HAIs) as a global public health problem still at the forefront of the challenges facing healthcare nowadays (Álvarez et al. 2016; Napolitani et al. 2020). These infections pose a risk primarily to hospitalized patients, most often affect people with impaired immunity, and are usually caused by multidrug-resistant organisms

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(MDRO) with a high spread potential (Álvarez et al. 2016; Knecht et al. 2019; Napolitani et al. 2020). Many cross-infections are mainly transmitted by healthcare workers' (HCWs) hands, which actively contribute to transferring pathogens from one patient to another and within the healthcare institutions, thus, contributing to many HAIs (Álvarez et al. 2016; Kalra et al. 2021). Besides HCW hands, critical or invasive devices are also responsible for HAIs (Arora et al. 2020). Moreover, it is worthwhile to mention that non-critical medical devices, such as stethoscopes, white coats, masks, and latex gloves, can significantly contribute to HAIs, too, and have been taken into account in many outbreaks of HAIs (Datta et al. 2018; Messina et al. 2018; Millán-Lou et al. 2022).

Despite the continuous development of medical equipment used in diagnostic methods, the stethoscope poses an inseparable attribute of HCWs, including medical students, in their daily practice, and it is the primary clinical tool for patient care (Álvarez et al. 2016; Datta et al. 2018; Napolitani et al. 2020). The familiar sight of a doctor with a stethoscope significantly impacts the patient's confidence when in contact with the physician (Knecht et al. 2019; Kalra et al. 2021). On the other hand, contaminated stethoscopes often enable the transmission of MDRO (Raksha et al. 2014; Gazibara et al. 2015; Haddad et al. 2019). Scientific studies carried out worldwide confirm a very high degree, on average 85% (range 66–100%) of the HCW stethoscopes contamination with a multitude of various bacteria, including dangerous pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Datta et al. 2018; Kalra et al. 2021).

Medical students are going to become HCWs when they finish their medical studies. The period of their studies is the time for education and developing good habits and practices, including hand and stethoscope hygiene. Therefore, their hygiene habits are only fully developed, established, or sometimes correct once they finish their education (Uneke et al. 2008; Saunders et al. 2013).

Disinfecting procedures are applied to control the decrease in the microbial population in medical devices (CDC 2016). The cleaning and disinfection status of the stethoscope is an essential factor in considering the possibility of environmental influences on the spread and transmission of bacterial pathogens (Álvarez et al. 2016). Although the Centers for Disease Control and Prevention (CDC) recommend cleaning the stethoscope, the guidance is vastly different from for hand hygiene. The CDC recommends cleaning the stethoscope with alcohol only once a week or if it is visibly soiled (CDC 2016). Alternatively, it recommends using disposable stethoscopes (Whittington et al. 2009). In Poland, stethoscope disinfection procedures and practices barely exist or are unknown. At the same time, WHO recommendations in the form of "the five moments for hand hygiene" are commonly known. WHO rules precisely define the frequency and situations in which hand hygiene should be performed (WHO 2004). The CDC and the Food and Drug Administration (FDA) guidelines for hand hygiene are also known (Pittet et al. 2001; CDC 2016). This lack of rules for stethoscope hygiene and well-known recommendations for hand hygiene might be confusing, especially for students.

Given this context, the objective of this study was to evaluate the hygiene habits of medical students, including the examination of disinfection practices, confronted with the cleanliness of the stethoscopes they use. Therefore, the main aim of our study was to investigate the contamination of stethoscopes employed by medical students. It involved isolation, identification, testing of antibiotic resistance, and the clonality of the isolated bacterial pathogens.

Experimental

Materials and Methods

Ethical approval. The Bioethical Committee of Jagiellonian University approved the study (No. KBET/122.6120.335.2016).

Study population and settings. This study was conducted between December 2016 and July 2017. Sixty-six students of 3rd (n = 25), 4th (n = 28), and 5th (n = 13) years of study from the Faculty of Medicine at the Jagiellonian University Medical College (JUMC) in Cracow (Poland) participated in the investigation.

Survey research. The participating students completed an anonymous questionnaire including demographic data and information on self-reported hygiene habits. The Authors developed the modified questionnaire following the protocol by Uneke et al. (2008). The structured questionnaire consisted of 15 questions about the frequency, methods, and approach to disinfecting the stethoscopes. It was distributed among the participants. The students' responses to seven questions were scored using a five-point Likert scale (Likert 1932), and the remaining ones were closed-ended questions, which also involved distractors (Table I, II and III).

Sample collection and processing. The samples were taken from the students' stethoscopes when they had a block of classes in the hospital wards that lasted from two to four weeks. Swabs from the students' stethoscopes were collected in the middle of the clinical course. Two samples from each stethoscope, one from the diaphragm and one from the bell, were taken using a sterile cotton swab (EQUIMED, Poland), moistened in sterile 0.9% saline, placed in the Amies transport

medium (Deltalab, Spain), and delivered to the laboratory at the temperature of about 20–25°C.

The collected swab samples were inoculated on BD BBL™ Columbia Agar with 5% Sheep Blood (Becton Dickinson, USA), Chapman Agar (Biocorp, Poland), Bile Esculin Agar (BEA, Biocorp, Poland), BD BBL™ MacConkey II Agar (Becton Dickinson, USA), and incubated aerobically at 35 ± 2°C for 24 hours.

Bacterial isolates and species identification. The strains were identified by standard microbiological identification techniques (Bulanda 2015). In addition, species identification was carried out with the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) biotyper (Bruker Daltonics GmbH & Co. KG, Germany). Microorganisms recognized as commensal microbiota, according to the definitions proposed by Drakulovic et al. (2001) were excluded from antibiotic resistance studies.

Antimicrobial resistance patterns. Methicillin-resistance for *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE), high-level aminoglycoside resistance (HLAR), and vancomycin resistance for *Enterococcus* species (VRE) were screened respectively according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (EUCAST 2017; 2018). The antibiotic susceptibility for the isolated pathogenic strains was performed by a disk diffusion technique in BD BBL™ Mueller Hinton II Agar (Becton Dickinson, USA) using the following antibiotic discs: clindamycin (2 μg), erythromycin (15 μg), cefoxitin (30 μg), gentamicin (30 μg), streptomycin (300 μg), vancomycin (5 μg), and teicoplanin (30 μg) (Oxoid, UK). Macrolide-lincosamide-streptogramin B resistance (MLS_B) phenotype was tested for MRSA, MRSE, and *Streptococcus* strains with the method described by Steward et al. (2005).

Detection of MRSA and MRSE resistance genes. MRSA and MRSE isolates were chosen for further, detailed analysis because they are classified as some of the most dangerous pathogens, especially in immunocompromised patients (Fenelon et al. 2009; Campos-Murguía et al. 2014), and as the most crucial component of the stethoscope surface biofilms (Johani et al. 2018). Bacterial genomic DNA was isolated using a commercial Genomic Mini kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. The detection of the *mec*A gene by polymerase chain reaction (PCR) confirmed methicillin-resistant isolates detected by the disk diffusion method (Choi et al. 2003). Reference strains were used: *S. aureus* ATCC® 33591™ and *S. epidermidis* ATCC® 35984™ as a positive control for *mec*A, and *S. aureus* ATCC® 25923™ and *S. epidermidis* ATCC® 700296™ as a negative control.

Restriction enzyme analysis with pulsed-field gel electrophoresis (REA-PFGE). Eleven MRSA and fifteen MRSE strains were selected for the genetic similarity analysis. Genotyping was performed using restriction enzyme analysis with pulsed-field gel electrophoresis (REA-PFGE) following genomic DNA extraction and digestion with *Sma*I endonuclease (Fermentas, Lithuania) following the protocol by Murchan et al. (2003). *S. aureus* ATCC® 25923™ was used as the reference strain. The electrophoresis was run on the CHEF DR®II system (Bio-Rad Laboratories, USA). Gel Compar II 6.5 software (Applied Maths, Belgium) was used for cluster analysis using the Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) using 1% tolerance and 1% optimization settings.

Data analysis and interpretation. The data gathered from the questionnaire were analyzed and the microbiological findings from the culture were recorded. Statistical analyses were performed using IBM® SPSS® Statistics 28 for Windows software. Data for qualitative variables are presented as frequencies and percentages. Questions on the Likert scale were presented as medians and quartiles and were compared between the study groups using the Kruskal-Wallis test with Dunn's post-hoc test. The relationship between the distributions of the frequency of responses to the questions asked was evaluated using the chi-square test. The correlation between the studied variables was performed using Spearman's *rho*, two-sided; p-values < 0.05 were considered significant.

Results

Characteristics of the student population and survey analysis. Twenty-five students from the 3rd year, twenty-eight from the 4th year, and thirteen from the 5th year, with mean ages of 22.8 vs. 22.8 vs. 24.0, respectively, participated in the study. Among the 3rd year students, there were more men than women (15/10), while among the 4th and 5th-year students, there were almost the same numbers of women and men (13/15 vs. 6/7).

Based on the results of the survey, it was found that 32% (n = 8) of the 3rd, 35.7% (n = 10) of the 4th, and 15.4% (n = 2) of the 5th year students claimed to have cleaned their stethoscopes after every patient (Table I). Actually, 16% (n = 4), 35.7% (n = 10), and 23.1% (n = 3) of them declared to have cleaned their stethoscopes several times a day (details in Table I). The most frequently used method of stethoscope disinfection was swabbing with hand disinfectant gel. It was the case for 91.7% (n = 22) of 3rd-year students, 100% (n = 28) of 4th-year students, and 84.6% (n = 11) of 5th-year students (Table I).

Students claimed to have predominantly cleaned their stethoscope diaphragms and bells (66.2% vs. 61.5%). As many as 78.4% of the respondents were never instructed on stethoscope hygiene, and 64.6)% had not

Table I
The results of a survey on self-reported stethoscopes hygiene habits given by medical students from 3rd, 4th and 5th years of study at Jagiellonian University Medical College (JUMC) (authors' elaboration based on Uneke et al. 2008).

Question	Answers	Year of study						total		Statistical parameters		
		3rd		4th		5th						
		n	%	n	%	n	%	n	%	χ^2	*df*	*p*
How often do you clean your stethoscope?	Never	1	4.0%	0	0.0%	0	0.0%	1	1.5%	14.97	12	0.317
	Once a year	3	12.0%	0	0.0%	0	0.0%	3	4.5%			
	Once a month	2	8.0%	2	7.1%	4	30.8%	8	12.1%			
	Once a week	3	12.0%	3	10.7%	2	15.4%	8	12.1%			
	Once a day	4	16.0%	3	10.7%	2	15.4%	9	13.6%			
	Several times a day	4	16.0%	10	35.7%	3	23.1%	17	25.8%			
	After each examination	8	32.0%	10	35.7%	2	15.4%	20	30.3%			
	Total	25	100.0%	28	100.0%	13	100.0%	66	100.0%			
Which part of the stethoscope has been cleaned?	Membrane	15	62.5%	20	71.4%	8	61.5%	43	66.2%	0.62	2	0.744
	Bell	15	62.5%	19	67.9%	6	46.2%	40	61.5%	1.78	2	0.432
	Flexible tubing	4	16.7%	8	28.6%	3	23.1%	15	23.1%	1.03	2	0.554
	Metal tubing	3	12.5%	1	3.6%	1	7.7%	5	7.7%	1.45	2	0.510
	Olives	3	12.5%	2	7.1%	1	7.7%	6	9.2%	0.49	2	0.858
Which chemical disinfectant do you use to clean the stethoscope?	Alcohol swabs	0	0.0%	0	0.0%	2	15.4%	2	3.1%	11.66	4	0.022
	Hand disinfectant	22	91.7%	28	100.0%	11	84.6%	61	93.8%			
	Special agent dedicated for medical appliances	2	8.3%	0	0.0%	0	0.0%	2	3.1%			
	Total	24	100.0%	28	100.0%	13	100.0%	65	100.0%			
Who does use your stethoscope?	Only me	7	30.4%	15	55.6%	5	41.7%	27	43.5%	6.51	8	0.538
	A colleague at every class	1	4.3%	0	0.0%	0	0.0%	1	1.6%			
	Often a colleague	1	4.3%	2	7.4%	1	8.3%	4	6.5%			
	Rarely a colleague	13	56.5%	10	37.0%	6	50.0%	29	46.8%			
	Other	1	4.3%	0	0.0%	0	0.0%	1	1.6%			
	Total	23	100.0%	27	100.0%	12	100.0%	62	100.0%			
Did someone show you the proper stethoscope cleaning technique?	Yes	5	20.0%	6	21.4%	3	25.0%	14	21.5%	2.11	4	0.704
	No, but I was looking for such information on my own	5	20.0%	2	7.1%	2	16.7%	9	13.8%			
	No, and I was not interested	15	60.0%	20	71.4%	7	58.3%	42	64.6%			
	Total	25	100.0%	28	100.0%	12	100.0%	65	100.0%			
Which source did you use to learn about correct technique of stethoscope cleaning?	During clinical classes	8	57.1%	6	50.0%	1	16.7%	15	46.9%	8.56	8	0.403
	Internet	2	14.3%	1	8.3%	1	16.7%	4	12.5%			
	Colleagues'	0	0.0%	1	8.3%	2	33.3%	3	9.4%			
	Clinical practices Outside the university	4	28.6%	3	25.0%	2	33.3%	9	28.1%			
	Other	0	0.0%	1	8.3%	0	0.0%	1	3.1%			
	Total	14	100.0%	12	100.0%	6	100.0%	32	100.0%			
Would you like to know more about stethoscope hygiene?	Yes	14	56.0%	14	50.0%	9	69.2%	37	56.1%	2.18	4	0.644
	No	1	4.0%	1	3.6%	1	7.7%	3	4.5%			
	I didn't think about it	10	40.0%	13	46.4%	3	23.1%	26	39.4%			
	Total	25	100.0%	28	100.0%	13	100.0%	66	100.0%			
Did someone show you the proper hand hygiene?	Yes, during clinical classes	23	92.0%	25	89.3%	12	100.0%	60	92.3%	1.53	4	1.000
	Yes, outside the university	1	4.0%	1	3.6%	0	0.0%	2	3.1%			
	No, but I was looking for such information myself	1	4.0%	2	7.1%	0	0.0%	3	4.6%			
	Total	25	100.0%	28	100.0%	12	100.0%	65	100.0%			

Table I
Continued

Question	Answers	Year of study								Statistical parameters		
		3rd		4th		5th		total				
		n	%	n	%	n	%	n	%	χ^2	*df*	*p*
How often do you disinfect your hands?	After each patient	23	92.0%	27	96.4%	13	100.0%	63	95.5%	4.70	4	0.329
	Several times a day	2	8.0%	0	0.0%	0	0.0%	2	3.0%			
	Once a day	0	0.0%	1	3.6%	0	0.0%	1	1.5%			
	Total	25	100.0%	28	100.0%	13	100.0%	66	100.0%			

a – multiple choice question, *df* – degrees of freedom, n – number of participants, *p* – statistical significance, % – number of participants expressed as a percentage, χ^2 – chi–squared test statistic

Table II
The survey results on self-reported stethoscopes hygiene habits given by medical students from 3th, 4th, and 5th years of study at Jagiellonian University Medical College (JUMC) were given on a Likert scale.

Questions	Year of study													*p*
	3rd (n = 25)				4th (n = 28)				5th (n = 13)					
	Average rank	Me	Q_1-Q_3	Min-Max	Average rank	Me	Q_1-Q_3	Min-Max	Average rank	Me	Q_1-Q_3	Min-Max	H(2)	
Cleaning agents for stethoscopes are widely accessible during clinical classes	35.50	2	1–4	1–5	34.30	2.5	1–3	1–5	27.92	2	1–2.5	1–4	1.53	0.466
I would disinfect my stethoscope more often if the cleaning agents were more accessible	33.98	4	3–5	1–5	31.34	4	3–5	1–5	37.23	4	4–5	3–5	0.95	0.622
I would disinfect my stethoscope more often if the supervisors put more emphasis on that issue	31.04	4	3–5	1–5	34.79	4.5	3.25–5	1–5	35.46	4	4–5	2–5	0.77	0.682
It is important for me to be sure that my stethoscope is clean	38.00	5	4–5	1–5	29.54	4	3–5	1–5	33.38	4	3.5–5	1–5	2.97	0.227
It is important for me to keep my hands clean	35.00	5	5–5	5–5	32.66	5	5–5	1–5	32.42	5	5–5	1–5	1.9	0.387
Stethoscope may be a vector for microorganisms	31.70	5	4.5–5	4–5	34.64	5	5–5	1–5	34.50	5	5–5	4–5	0.79	0.673
Disinfectants can cause damage to the stethoscope	27.66	3	2–4	1–5	33.89	3[a]	2.25–4	1–5	43.88	4[a]	3–5	3–5	6.58	0.037
I see my colleagues disinfecting their stethoscopes regularly	34.50	2	1–3	1–4	37.00	2	1.25–4	1–5	24.04	2	1–2	1–3	4.48	0.107

a – medians that divide the letter index differ from one another at a level of $p < 0.05$; Me – median; Min-Max – minimum and maximum value; *p* – statistical significance; Q_1-Q_3 – first and third quartile; specific points on a Likert scale means 1 – I strongly disagree, 2 – I disagree, 3 – Neither agree nor disagree, 4 – I agree, 5 – I strongly agree

searched for such information on their own. The students looked for different sources of knowledge about stethoscope disinfection techniques. For most of them, clinical classes inside and outside the university were the most popular source of proper hygiene practices (46.9% vs. 28.1%). Almost everyone used a chemical hand sanitizer to disinfect his or her stethoscope (93.8%; $p = 0.022$). Almost equal numbers of students used stethoscopes themselves, and at the same time, many of them rarely lent their stethoscopes to friends during clinical classes (43.5% vs. 46.8%). Almost everyone cleaned their hands after examining each patient (95.5%) and declared that almost everyone was taught the correct hand hygiene technique during classes (92.3%).

Most of the students admitted that they would disinfect their stethoscopes more frequently (answer 4 was given more often than the others on the Likert scale) if teachers emphasized stethoscope hygiene (Table II). Students declared that disinfectants were not available in adequate numbers in places where they had classes and contact with patients (answer 2 was given more often than the others on the Likert scale). Most students

Table III
Correlations between answers given in the Likert scale (Likert 1932) and declared frequency of stethoscopes' disinfection declared by medical students of the 3rd, 4th, and 5th year of study at Jagiellonian University Medical College (JUMC).

Answers given on a Likert Scale	Correlation' indicators	How often you clean your stethoscope?			
	year	3rd	4th	5rd	Total
Cleaning agents for stethoscopes are widely accessible during clinical classes	*rho* Spearman's	0.21	0.07	0.08	0.16
	p-value	0.321	0.724	0.786	0.197
I would disinfect my stethoscope more often if the cleaning agents more accessible	*rho* Spearman's	0.34	0.06	–0.23	0.12
	p-value	0.095	0.778	0.454	0.33
I would disinfect my stethoscope more often if the supervisors put more emphasis on that issue	*rho* Spearman's	0.01	–0.18	0.24	0.01
	p-value	0.953	0.355	0.430	0.986
It is important for me to be sure that my stethoscope is clean	*rho* Spearman's	0.41	0.49	0.26	0.36
	p-value	0.041	0.008	0.390	0.003
It is important for me to keep my hands clean	*rho* Spearman's	a	0.24	–0.24	0.06
	p-value		0.210	0.436	0.637
A stethoscope may be a vector for microorganisms	*rho* Spearman's	0.35	0.05	–0.09	0.18
	p-value	0.084	0.814	0.776	0.159
Disinfectants can cause damage to the stethoscope	*rho* Spearman's	0.03	0.24	–0.06	0.04
	p-value	0.878	0.226	0.846	0.733
I see my colleagues disinfecting their stethoscopes regularly	*rho* Spearman's	0.50	0.38	–0.06	0.40
	p-value	0.012	0.046	0.854	<0.001

a – all respondents gave the same answer

declared that they would disinfect the stethoscope more often if disinfectants were available in adequate amounts (answer 4 was more frequent). At the same time, the respondents believed that disinfectants might cause damage to the stethoscopes, which was statistically significant ($p = 0.0037$) when comparing declarations of the 4th and 5th-year students.

Most respondents strongly believed that the stethoscope might be a vector for microorganisms (answer 5 was more frequent) regardless of the year of study. For most of them, it was important to keep their stethoscopes and hands clean (answer 5 was more frequent). The participants also declared that they did not see their friends cleaning their stethoscopes regularly (answer 2 was given more often than the others on the Likert scale; details in Table II). There was a positive correlation between the frequency of stethoscope disinfection and individuals who believed that maintaining good hygiene of the stethoscope is important ($p = 0.003$; details in Table III). The correlation between students' observations concerning disinfecting stethoscopes by other students and the declared frequency of stethoscope disinfection was also positive ($p < 0.001$).

Profile of bacteria recovered from stethoscope surfaces. Out of 66 stethoscopes cultured, all (100%) were found to be contaminated with at least one bacterial species. In total, 277 strains (including nine genera, 19 species) of bacteria were isolated. A bacteriological overview of the microorganisms isolated from the contaminated stethoscopes belonging to medical students with a particular emphasis on the *Staphylococcus* species is summarized in Fig. 1. Concerning other bacterial strains, *Corynebacterium* spp. (n = 3; 1.1%), *Kocuria rhizophila* (n = 3; 1.1%), *Chryseomonas* spp. (n = 1; 0.36%), *Enterococcus faecalis* (n = 1; 0.36%), and *Neisseria mucosa* (n = 1; 0.36%) were isolated. All collected *Micrococcus* spp. (n = 48; 17.3%) were identified as *Micrococcus luteus*. Among the ten species belonging to the genus *Streptococcus*, six *S. equisimilis* (2.17%), three *S. sanguinis* (1.08%) and one *S. mitis* (0.36%) were identified.

All (100%) of the 11 *S. aureus* and 31.9% (n = 15) of the 47 *S. epidermidis* strains showed methicillin resistance. All MRSA and MRSE strains also had the MLS_B phenotype (resistance to erythromycin and resistance to clindamycin). All isolates tested harbored the *mec*A gene. None of the *Streptococcus* strains showed the presence of the MLS_B phenotype. Furthermore, none of the *Enterococcus* strains displayed either the HLAR or VRE phenotypes. Neither HLAR nor VRE was detected in the *Enterococcus* strains.

The diaphragms were more contaminated than bells as they included one hundred and fifty-eight (57%) vs. one hundred and nineteen (43%) bacterial strains (Fig. 2). The stethoscopes of 3rd-year students gave rise to the isolation of one hundred and twelve bacterial

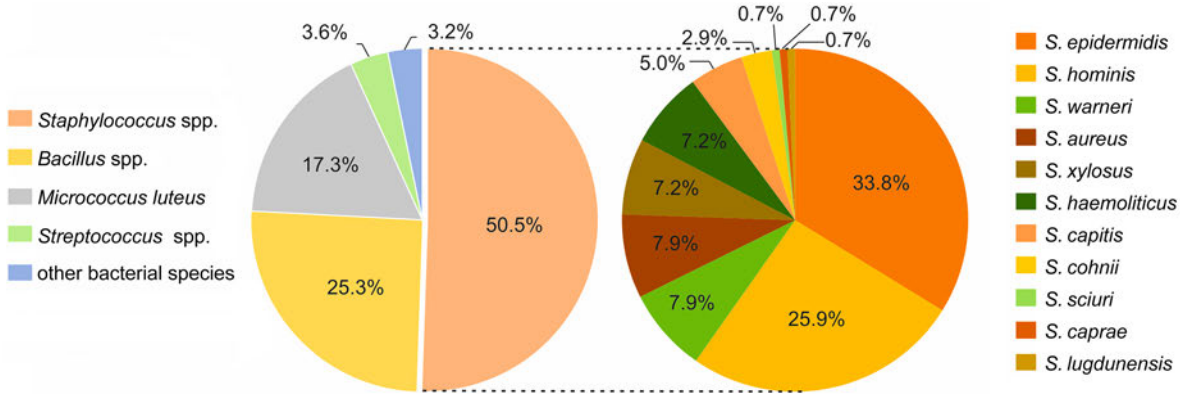


Fig. 1. Bacteriological profile of microorganisms isolated from personal medical students' stethoscopes with particular emphasis on *Staphylococcus* species.

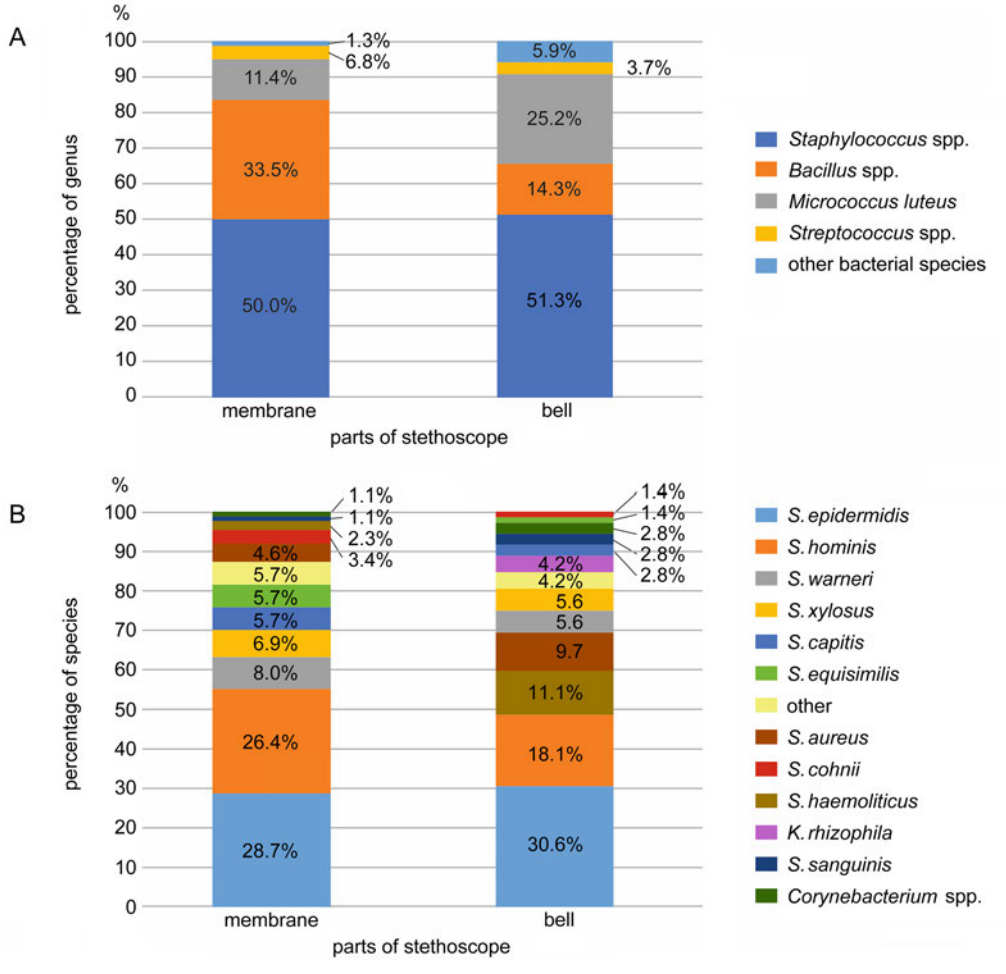
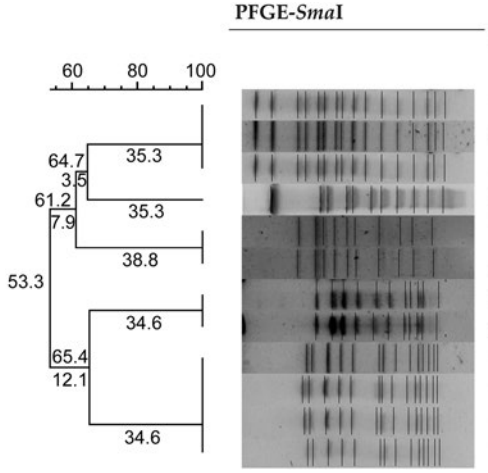


Fig. 2. Bacteria strains isolated from membranes and bells of personal stethoscopes used by medical students from Jagiellonian University Medical College (JUMC). Bacteria are presented as general taxa (A) and listed as species (B).

strains, including sixty-three (56.25%) from diaphragms and forty-nine (43.75%) from bells. Whereas 112 strains were isolated (including 66 strains from diaphragms and 46 from bells) in the group of the 4th year students. The stethoscopes of 5th-year students carried 55 strains (n = 31 from diaphragms and n = 24 from bells).

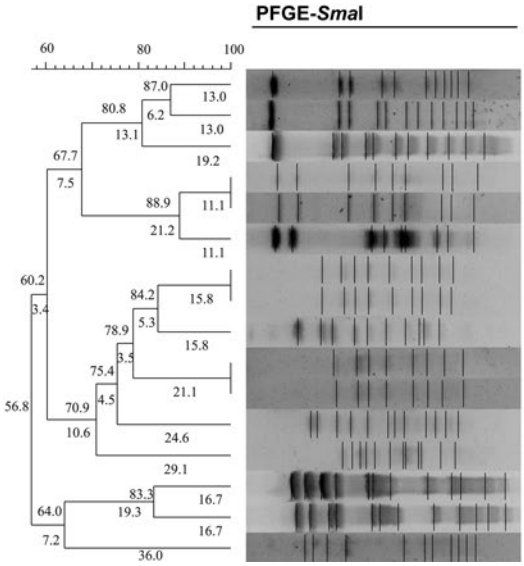
Molecular typing of MRSA and MRSE. MRSA and MRSE strains were subjected to molecular typing. Generally, among 11 MRSA isolates, four different PFGE (A-D) clusters were identified (Fig. 3 and S1). Ten MRSA isolates came from 5th-year students' stethoscopes assigned to one exercise group 11. One strain,



Isolate no.	Students code	Year	Group	Part of the stethoscopes	PFGE	Declared frequency of disinfection
192	11V8	5th	11	bell	B	2
207	11V12	5th	11	bell	B	5
63	11V13	5th	11	bell	B	4
S. aureus ATCC 25923 REFERENCE STRAIN						
197	11V10	5th	11	bell	C	1
202	11V11	5th	11	bell	C	3
209	11V13	5th	11	membrane	D	4
210	11V13	5th	11	membrane	D	4
124	11III2	3th	11	membrane	A	4
188	11V7	5th	11	bell	A	1
189	11V7	5th	11	membrane	A	1
193	11V8	5th	11	bell	A	2

Fig. 3. Macrorestriction analysis of the chromosomal DNA of methicillin-resistant *S. aureus* strains (MRSA) with concomitant macrolide, lincosamide, and streptogramin B (MLS_B) resistance phenotype isolated from the surface of stethoscopes after digestion with the restriction enzyme *Sma*I, plotted using the Gel Compar® II program (Applied Maths).

Legend: The frames indicate three students' stethoscopes on which surfaces both MRSA and MRSE (presented in the Fig. 4) strains were identified. The declared frequency of stethoscope disinfection were as follow: 1 – after each examination, 2 – several times a day, 3 – once a day, 4 – once a week, 5 – once a year.



Isolate no.	Students code	Year	Groups	Parts of the stethoscope	PFGE	Declared frequency of disinfection
213	11III6	3rd	11	bell	D	3
216	11III8	3rd	11	bell	E	2
S. aureus ATCC 25923 REFERENCE STRAIN						
117	11III2	3rd	11	membrane	A	5
214	11III7	3rd	11	membrane	A	1
32	2IV13	4th	2	bell	F	2
61	12III1	3rd	12	membrane	B	3
80	12III10	3rd	12	membrane	B	2
5	2IV2	4th	2	membrane	G	4
196	11V10	5th	11	bell	C	1
200	11V11	5th	11	bell	C	3
30	2IV	4th	2	membrane	H	1
7	13	4th	2	bell	I	2
206	11V12	5th	11	bell	J	5
82	12III10	3rd	12	bell	K	2
211	12III1	3rd	12	membrane	L	3

Fig. 4. Macrorestriction analysis of the chromosomal DNA of methicillin-resistant *S. epidermidis* strains (MRSE) with concomitant macrolide, lincosamide, and streptogramin B (MLS_B) resistance phenotype isolated from the surface of stethoscopes after digestion with the restriction enzyme *Sma*I, plotted using the Gel Compar® II program (Applied Maths).

Legend: The frames indicate three students' stethoscopes on which surfaces both MRSA (presented in Fig. 3) and MRSE strains were identified. Declared frequency of stethoscope disinfection were as follow: 1 – after each examination, 2 – several times a day, 3 – once a day, 4 – once a week, 5 – once a year.

number 124, was isolated from a 3rd-year students' stethoscope assigned to the same exercise group 11. Clone A gathered four strains with identical genetic profiles from 5th and 3rd-year students' stethoscopes assigned to group 11, including two strains (no. 188 and 189) from one student, derived from the bell and diaphragm of the same stethoscope. Three strains of identical pulsotype from 5th-year students' stethoscopes belonging to the same group 11 were classified into clone B. All strains representing clone B were from the bell. Clones C and D were formed by two MRSA strains with identical pulsotypes within the clone, also isolated from the 5th-year and group 11 students' stethoscopes, where the C clone included bell-derived strains and clone D formed strains isolated from the diaphragm.

In the molecular analysis of 15 MRSE strains, three clones (A-C) were distinguished. In addition, ten strains representing unique restriction patterns (singletons) (D-L) were detected (Fig. 4). Clone A consisted of two strains with identical pulsotype derived from the diaphragm of stethoscopes of students belonging to the same 3rd-year and the same group 11. Similarly,

clone B was created by two strains from stethoscopes from students from the same 3rd year and the group 12, also isolated from the diaphragm. Clone C gathered two strains derived from stethoscope bells of 5th-year students classified to group 11. The remaining MRSE strains (D-L) were not related to each other.

We observed that the stethoscopes of three students were contaminated with MRSA and MRSE strains simultaneously (Fig. 3 and 4).

Discussion

The hands, and in particular the fingers of physicians, are a source of bacteria that can be transmitted to the patient during a medical examination, so compliance with hand hygiene guidelines is an essential aspect of HAI prevention (Napolitani et al. 2020). However, as scientists from around the world prove, the source of infections with bacteria, including HAIs, can also be medical stethoscopes, which, when used many times a day, come into direct contact with the patients' skin (Tschopp et al. 2016; Knecht et al. 2019). Since these instruments are frequently used on multiple patients, they can quickly become a source of transmission of many microorganisms, including the most dangerous pathogens, for example, MRSA (Torres-Ballesteros et al. 2017; Datta et al. 2018). Contaminated hands of HCWs can also be the source of stethoscope contamination. It is a possible way of transmitting bacterial strains because compliance rates of HCWs with hand hygiene principles before the COVID-19 pandemic were known to be around 50% (Vasudevan et al. 2019). The vast majority of JUMC students pay appropriate attention to proper hand hygiene. However, the risk associated with contaminated stethoscopes cannot be overlooked. Recent studies show that many stethoscopes are contaminated with numerous pathogenic bacteria (Campos-Murguía et al. 2014; Haddad et al. 2019). Various authors estimate the degree of stethoscope colonization to be very high: 85–87.3% (Álvarez et al. 2016), 66–100% (Datta et al. 2018), and 30–97% (Fafliora et al. 2014). Our study confirms this phenomenon by demonstrating that 100% of students' stethoscopes gave rise to a positive bacterial culture, including commensal microbiota and potentially pathogenic microorganisms. These findings align with the other studies, which recorded that 100% of the 62 stethoscopes tested showed bacterial growth (Bansal et al. 2019). Analysis of our survey results demonstrated that only 30.3% of the JUMC students cleaned their stethoscopes properly. Moreover, some students declared that they had never cleaned the stethoscope. Other studies showed that 33.33–36% of students never cleaned their stethoscopes (Bukharie et al. 2004; Raksha et al. 2014;). As a comparison, in Serbia, the survey data showed that 40% of 4th-year students cleaned their stethoscopes at least once a day compared with 30% for 6th year students (Gazibara et al. 2015). In Poland, only 15.18% of the students disinfect the stethoscope before examining the patient, but 9.82% do not do it at all (Kawalec et al. 2014).

Our survey results indicate that students from different years of study most often used hand sanitizer containing ethyl alcohol to disinfect stethoscopes. Research carried out by Grandiere-Perez et al. (2015) confirmed that disinfection of stethoscopes by ethanol-based hand sanitizer (gel or foam) was effective also at reducing MRSA colonies. Parmar et al. (2004) found that 66% ethyl alcohol was an effective disinfectant for stethoscope cleaning. In our study, students believed that disinfectants could damage the stethoscope. What is also interesting, students gave contradicting answers stating that they would disinfect the stethoscopes more often if sanitizers were easily accessible. At the same time, they claimed that they had broad access to the disinfectant agents. Neither of these responses was statistically significant. Our study showed that MRSA and MRSE strains survived the disinfection. Bacteria that survive disinfection can quickly become resistant to subsequent disinfection attempts; therefore, it is recommended to strictly observe the operating time of disinfectants, which is indicated on the packaging (Knecht et al. 2019).

Generally, the analysis of the survey results revealed that the students needed to have adequate knowledge about the hygiene of stethoscopes. Such a conclusion was presented earlier in the study by Gazibara et al. (2015). It is disturbing that many JUMC students felt that teachers and tutors supervising their classes in medical facilities did not give them the knowledge and proper cleaning techniques to apply stethoscope hygiene. Elsewhere, studies showed that students and other HCWs did not follow the standard protocol set to prevent infections by using stethoscopes (Arora et al. 2020). Altogether, these results prove a need for essential knowledge about stethoscope hygiene. In their colleagues' opinion, observation of each other's hygiene practices among the students showed that they do not clean their stethoscopes frequently enough. We asked students to answer this question because observation practices are often used in hospitals as one of the HAI surveillance tools (Vasudevan et al. 2019). This type of supervision may positively affect students' stethoscopes and hand hygiene.

On the other hand, it is comforting that the students in our study declared their willingness to learn proper techniques for disinfecting stethoscopes, and they feel at ease when they know that their stethoscopes are clean. Therefore, medical students should be educated and motivated to practice not only hand hygiene but also current and correct disinfecting procedures

for stethoscopes as important parts in preventing HAIs. Other researchers also believe that educating students about stethoscope hygiene is crucial to developing good practices in the future and essential to reduce the number of HAIs (Gaisser et al. 2021).

For our research, we had chosen the bell and the diaphragm as they come in direct contact with the fingers of HCWs and are applied directly to the patient's body to receive vibrations from a selected skin surface area and transmit them to the inside of the waveguide. According to the literature data, bacterial colonization of the diaphragm is much more frequent than the colonization of the bell (Dutra et al. 2013; Campos-Murguía et al. 2014; Queiroz Júnior et al. 2021). It is also confirmed by our results from all tested stethoscopes, regardless of the year of a student's education. Generally, it is known that, in Polish conditions, the bell is used less frequently than the diaphragm, which is in line with our results. As declared by the students, the frequency of cleaning individual elements of the stethoscopes also varied. Both in our study and the one by Gazibara et al. (2015), students cleaned the diaphragm more often (48.5% vs. 84% vs. 89.9%) than the bell (33.3% vs. 42% vs. 51%). Similarly, in the Indian study conducted by Datta et al. (2018), 60% of medical interns cleaned the diaphragm more frequently, while 40% cleaned both the diaphragm and the bell.

Our study did not include additional protection measures, such as disposable stethoscope diaphragm barriers. These kinds of solutions are still infrequent in Polish hospitals. Although many authors indicate them as having a more favorable protective effect than stethoscope disinfection (Vasudevan et al. 2020; Kalra et al. 2021; Peacock et al 2023), these studies are generally relatively new.

During our study (pre-COVID-19 era), there was a lack of information about the existence of disposable stethoscope diaphragm barriers in Poland. Considering the neglectful approach to disinfection and the protection of medical equipment in Poland, the emphasis on the disinfection of stethoscopes is more than justified. As disposable aseptic diaphragm barriers were shown to work as efficient solutions to reduce the transmission of pathogens via stethoscopes, it seems reasonable to change the attitude and practice concerning the hygiene of stethoscopes. Adequate procedures, the attitude of training, and practice of stethoscope hygiene should become an essential topic of discussion with people responsible for healthcare organizations in Poland and worldwide. There is a need to purchase special disinfectants for medical equipment and disposable stethoscope diaphragm barriers in Polish hospitals. Furthermore, there is a need to provide adequate knowledge, procedures, and practice of stethoscope hygiene to medical students during their studies.

According to literature data, the profile of bacteria contaminating the surfaces of stethoscopes represents an enormous species diversity with a predominance of Gram-positive organisms representing a regular part of the human skin and mucous membranes microbiota (Álvarez et al. 2016; Thapa and Sapkota 2017; Johani et al. 2018; Knecht et al. 2019). It is confirmed by the findings in our study, which showed that the dominant contaminant species were staphylococci (50.5%), headed by representatives of the CoNS group (46.5%), including 10.7% of MRSE isolates. As a comparison, observations carried out in Tunisia confirm the contamination of stethoscopes also mainly by the genus *Staphylococcus*, but to a lower degree (30.7%, including CoNS 17.9% and methicillin-resistant CoNS 5.1%) (Haddad et al. 2019). The isolation of *S. epidermidis* in our and other studies indicates that stethoscopes are easily contaminated with microbiota from the skin of patients who undergo physical examinations. Unfortunately, it is disturbing that we isolated strains of *S. epidermidis* resistant to antibiotics (MRSE) from the surface of the stethoscopes.

In our study, *S. aureus* strains accounted for 7.9% of all analyzed bacteria, and were methicillin-resistant (MRSA strains). It is worth noting that, for severe infections, the risk of death is approximately twofold higher with MRSA than with methicillin-susceptible *S. aureus* (MSSA) (Fenelon et al. 2009). Furthermore, MRSA strains can survive up to nine days on stethoscopes (Sahiledengle 2019). Other studies showed that contamination of stethoscopes with *S. aureus* was higher, but the percentage of strains resistant to beta-lactam antibiotics was lower than in our study. In a study by Uneke et al. (2008), *S. aureus* amounted to 41.6%. According to Queiroz Júnior et al. (2021), 80% to 100% of stethoscopes in use are contaminated with *S. aureus*, of which 20–40% are resistant to methicillin. As a comparison, in the study by Gazibara et al. (2015), *S. aureus* was most frequently isolated at 62.64%, of which 21.05% were MRSA isolates. In studies by Datta et al. (2018), *S. aureus* isolates accounted for 15% of all bacteria isolated, and MRSA strains accounted for 25%. Perhaps it is related to the type of disinfection agent and the response to exposure to the disinfectant (CDC 2016). In contrast, no *S. aureus* strain was detected in a study by a research team in Saudi Arabia (Bukharie et al. 2004).

In our study, the isolation of bacteria considered to be permanent or transient skin microbiota resulted in the genera *Bacillus* (25.3%), *Micrococcus* (17.3%), and *Streptococcus* (3.6%), which altogether amounted to 46.2% of the detected bacteria. For most people, these bacteria are not etiologic agents of infections. However, for children, immunosuppressed patients, or patients undergoing invasive medical procedures during hospitalization, micrococci, and streptococci can cause severe

invasive infections (Laupland et al. 2019; Martín Guerra et al. 2019; Neff et al. 2020; Zhu et al. 2021). Contrary to our findings, other studies showed a much lower percentage of contamination with bacilli, micrococci, and streptococci on the surfaces of stethoscopes. Other authors showed stethoscope contamination with *Bacillus* spp. at the rate of 2.6% to 16.48% (Raksha et al. 2014; Haddad et al. 2019). Also, Raksha et al. (2014) showed contamination of stethoscopes with *Micrococcus* spp. (4.40%). However, Haddad et el. (2019), did not detect micrococci. Others also showed that *Streptococcus* spp. contaminated 9% of stethoscopes or not at all (Johani et al. 2018; Haddad et al. 2019). In our study, *E. faecalis* was isolated only once. Contrary to our study, others showed a much higher contamination with enterococcal strains. For example, among bacteria isolated from stethoscopes of medical students in Nigeria, *E. faecalis* strains accounted for 16.1% and were identified as the HLAR phenotype (Uneke et al. 2008). Johani et al. (2018) revealed a relatively high percentage of vancomycin-resistant *Enterococcus* (VRE) at 7%. Numerous studies have shown that antibiotic-resistant strains of common healthcare pathogens (e.g., *Enterococcus*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli*) were as sensitive to disinfectants as antibiotic-sensitive strains. It may explain the lack of detection of Gram-negative rods on the surfaces of the stethoscopes in our study. According to Arora et al. (2020), 12.7% of Gram-negative bacteria and 14.3% of enterococci were isolated from stethoscopes.

The aim of PFGE analysis was to assess the clonality and persistence of MRSA and MRSE isolates among JUMC students' stethoscopes. The PFGE study of the tested MRSA and MRSE strains indicated a selection of resistant isolates of an epidemic nature and their transmission between students. Our study focused solely on evaluating the persistence of MRSA and MRSE strains on students' stethoscopes. The obtained results are difficult to discuss due to the minimal number of studies on this subject or even the lack of studies on molecular analyzes of MRSA and MRSE isolates from stethoscopes used by medical students. We only found studies proving the involvement of stethoscopes in the spread of MDRO and HAIs involving Gram-negative bacteria (Crespo et al. 2004; Messina et al. 2018; Lee et al. 2021; Millán-Lou et al. 2022).

The main limitation of our study was the lack of nasal swabs (carrier assessment) from stethoscope owners and environmental samples. In the future, it will be important to extend the research that can prove the link between the possible transmission of strains contaminating stethoscopes of medical students and the hospital environment and newly hospitalized patients.

Training proper hygiene habits should be improved during classes in each year of studies. It is known that consolidating proper hygiene habits requires a correct example from teachers, knowledge of procedures, repeating activities, and the possibility of verifying the acquired skills. If students develop good hygiene habits during their studies, there is a high probability that they will apply them properly while practicing their profession.

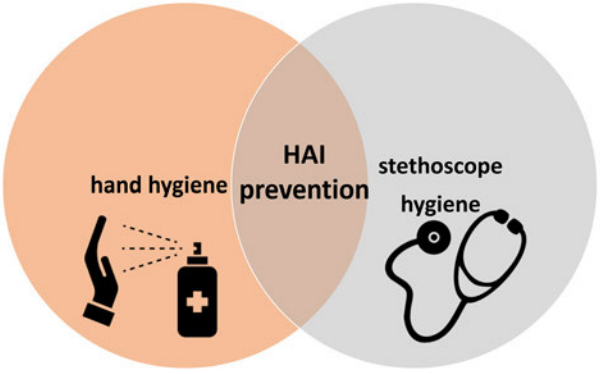


Fig. 5. The importance of stethoscope hygiene as an essential factor in preventing hospital-associated infections (HAIs) [The infographic prepared according to the author's own idea].

Our study, together with many others, has asserted the need to elevate the priority of stethoscope hygiene in the daily practice of HCWs and to update the disinfection guidelines in care facilities. Therefore, medical students should be educated about hand and stethoscope hygiene as a collaborative effort to prevent HAIs (Fig. 5).

Conclusions

Based on the results of the questionnaires, the necessity of the validated procedures for cleaning the stethoscopes was demonstrated. The survey results show the supervisor's key role in shaping students' proper hygiene habits. This study highlights the fact that the stethoscopes of medical students are contaminated with numerous bacteria, including multidrug-resistant organisms. The clonal structure of the MRSA and MRSE populations derived from stethoscopes has been demonstrated. Our results confirm the possibility that these medical devices mediate the spread of hazardous pathogens in the hospital and non-hospital environments. Practical exercises are essential to forming the correct hygiene habits involving stethoscopes, which enable practicing and checking the correctness of the established skills.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Changes in the Protein Profile in Staphylococcal Strains from Patients Infected with the SARS-CoV-2 Virus

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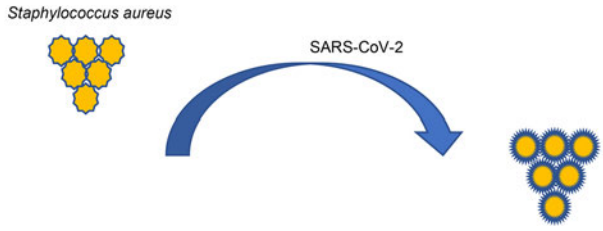
Abstract

Staphylococcus aureus strains are particularly often isolated from patients with SARS-CoV-2 infection. The aim of the current research was to determine whether the SARS-CoV-2 virus infection affects the protein profile of *S. aureus*. Bacteria were isolated from the forty swabs collected from the patients in the hospitals of the Pomeranian region. MALDI-TOF MS spectra were obtained using a Microflex LT instrument. Twenty-nine peaks were identified. The peak (2,430) is described here for the first time and was unique for the isolates



from patients infected with the SARS-CoV-2 virus. These results support the hypothesis of bacterial adaptation to the conditions caused by viral infection.

K e y w o r d s: *Staphylococcus aureus*, SARS-CoV-2, source of isolation, MALDI-TOF

Coronavirus disease 2019 (COVID-19) has affected a huge number of patients; however, the mortality rate often results from bacterial co-infection. The phenomenon of co-infection itself is widely known, but only some publications describe this mechanism in relation to SARS-CoV-2 (Mirzaei et al. 2020). As the gut bacterial diversity COVID-19 patients is reduced, bacterial co-infections might be a critical element that promotes severities of COVID-19 and mortality rates (Hoque et al. 2021). It has also been shown that the commensal microbiota can regulate and be regulated by invading viruses, causing stimulatory or suppressive effects (Bernardo et al. 2002; Franco et al. 2011; Fetsch et al. 2014). In the viral infections of the respiratory tract, bacterial co-infections are very common and vary from 3.0% to 68.0% (Hoque et al. 2021). It is speculated that the mechanism of co-infection includes virus-induced airway damage, cell loss, goblet cell hyperplasia, altered mucus secretion, reduced ciliary beat frequency, function and clearance, reduced oxygen exchange, and damage to the immune system (Hoque et al. 2021).

Due to the high incidence of infections, high infection rate with a severe course, and the severity of post-infection complications, particular interest should be paid to the co-infection with *S. aureus* as it is the most commonly identified pathogen in patients with SARS-CoV-2 (Clancy and Nguyen 2019). These bacteria may significantly impact the outcome of SARS-CoV-2 infections. Additionally, infections of the lower respiratory tract caused by multidrug-resistant strains

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of *S. aureus* may lead to substantial morbidity and mortality (Clancy and Nguyen 2019). Moreover, these bacteria may colonize healthy people's respiratory tract and cause infections of varying severity. These infections range from local to non-invasive, from relatively mild skin infections to severe, life-threatening sepsis, infective endocarditis, and necrotizing pneumonia (Lowy 1998, van Belkum 2009).

The risk of staphylococcal infections, which causes considerable mortality, increases in patients with viral infections (Morens et al. 2008). *S. aureus* is particularly often isolated from patients with SARS-CoV-2 infections, especially in fatal cases (Lai et al. 2020). The reasons for this phenomenon have yet to be fully understood, but the mechanism may be multifactorial. It can be assumed that the virulent strains of *S. aureus* are selected during viral infections or that there are adaptive changes in potentially pathogenic staphylococci. As bacterial cell populations are frequently heterogenous, we may observe variations in pathogenicity.

Genetic typing methods have been used to recognize more virulent strains and follow the spread of such strains. In the epidemiology of staphylococcal infection, *spa* typing is widely accepted. *Spa* typing amplifies fragment X of the *spa* gene responsible for the production of species-specific *S. aureus* protein A, which is further sequenced (Shopsin et al. 1999; Hallin et al. 2009; Hasman et al. 2010). Fragment X of the *spa* gene is responsible for the attachment of protein A to the cell wall and contains a variable number of short repeat sequences (SSRs), each 21–24 nucleotides long (Shopsin et al. 1999). Their formation is associated with spontaneous point mutations, duplications, or deletions of repetitions in staphylococcal evolution (Frénay et al. 1996; Shopsin et al. 1999). Replicates obtained from sequencing are sequentially numbered and processed using the Ridom Spa Type program (http://spaserver.ridom.de). The result is a numeric code that identifies the *spa* types along with sequence and region changes (Harmsen et al. 2003).

The molecular techniques in microbiology in the last decade have been supplemented with a MALDI-TOF technique. This method allows for identifying microorganisms at the species level by analysis of total protein mass spectra and comparison to reference spectra (Sauget et al. 2017). Considering the above, protein profiling is a promising method in bacterial typing. In contrast to molecular techniques, MALDI-TOF MS requires minimal sample preparation, and is quick and inexpensive. This technique has successfully typed Gram-negative and Gram-positive bacteria (Sauget et al. 2017). It has been observed that modification of protein expression is reflected in the peak intensity. On the other hand, it is impossible to distinguish if the loss of the signal results from a lack of gene encoding the particular protein or a lack of gene expression. In contrast, a slight change in protein's *m*/*z* value (peak shift) can be used as a reliable biomarker since it usually results from slight protein changes (Böhme et al. 2012; Josten et al. 2013; Østergaard and Møller 2018). Our previous research showed the increased expression of proteins crucial for the pathogenesis of bacteria during concomitant viral infection (Jarzembowski et al. 2013). The aim of our current research was to determine whether the SARS-CoV-2 virus infection affects the protein profile of *S. aureus*, and to find a biomarker for the risk assessment of bacterial co-infection development.

Forty swabs, including 17 wound swabs from diabetes patients, 12 nasal swabs from patients hospitalized due to SARS-CoV-2 infection, and 11 nasal swabs from patients without current viral infection, were collected from the patients of the hospitals of the Pomeranian region (Table I). The samples were inoculated on Columbia Blood Agar (Graso, Poland) containing 5% (vol/vol) horse blood at 35–37°C for 18–24 h. Identification of *S. aureus* strains was performed using the advanced colorimetric method VITEK® GP, VITEK 2® Compact (bioMerieux, Poland). The isolates from SARS-CoV-2 positive patients were compared by protein profile with the isolates from patients without SARS-CoV-2 and symptoms of respiratory tract infection (negative reference). The *spa* typing method was used to distinguish between the selection and adaptation of strains.

Strain protein profiling. A colony material sample was transferred to the target steel plate (Bruker Daltonik GmbH, Germany) without a protein extraction procedure (direct method). Samples were overlaid with

Table I
Clinical characteristics of patients.

	Age (median)	Oxygen saturation (median)	CRP (median)	Diabetes (n of patients)	Hypertension (n of patients)	n
Patients hospitalized due to SARS-CoV-2 infection	71	90	61,4	6	8	12
Patients with wound infection (diabetes, SARS-CoV-2 negative)	64	N/A	N/A	17	N/A	17
S. aureus carriers (diabetes)	58	N/A	N/A	11	N/A	11

1 µl of HCCA matrix (alpha-cyano-4-hydroxycinnamic acid) solution. MALDI-TOF MS spectra were obtained using a Microflex LT smart system with Bruker's proprietary smartbeam™ solid-state laser technology at 200 Hz repetition rate. The system was controlled by the flexControl software Version 3.4 (Bruker Daltonik GmbH, Germany) and calibrated using a sample of Bruker bacterial test strain (BTS) containing *Escherichia coli* DH5. Spectra for individual specimens were compiled as an averaged result from 100 shots for *m*/*z* values in the range of 2,000–10,000. Spectra for the two replicates were compared to determine the within-isolate peak reproducibility, then summed to minimize random effects. Data analysis of protein profile was conducted by mas software (Strohalm et al. 2008). Classification and Regression Tree (C&RT) and ANOVA comparison of protein peaks in groups of strains was performed using the Statistica software package (ver. 13.0).

***Spa* typing**. *Spa* typing was applied to evaluate the distribution and selection of the staphylococcal strains in SARS-CoV-2 infected patients. The polymorphic fragment X of the *spa* gene encoding protein A was amplified by PCR reaction using the primers proposed by Frénay et al. (1996). 5 µl of bacterial DNA (5 ng/µl) was mixed with 5 µl of reaction buffer (100 mM TRIS-HCl pH = 8, 500 mM KCl, 15 mM $MgCl_2$, 0.8% detergent; MBI Fermentas, Lithuania), 5 µl of dNTP mixture (dATP, dCTP, dGTP, dTTP) at a concentration of 2 mM each, 1 µl of each of the SPA-1 and SPA-2 primers (100 µM each concentration), 2 U Taq DNA polymerase (MBI Fermentas, Lithuania) and made up to a volume of 50 µl with water (Sigma, USA). Amplification was performed in a GeneAmp PCR System 2400 automatic thermocycler (PerkinElmer, USA) according to the scheme (Harmsen et al. 2003). Amplification results were read on a 4% agarose gel (Sigma, USA) stained with ethidium bromide (ICN Biomedicals Inc., Austria). For this purpose, 2 µl of the loading buffer (60% glycerol, 0.09% bromophenol blue, 0.09% xylene, 60 mM EDTA; MBI Fermentas, Lithuania) was added to 10 µl of the reaction mixture, and the whole was applied to the covered in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA; Sigma, USA) wells in an agarose gel (2% agarose in TAE buffer supplemented with ethidium bromide; Sigma, USA). The electrophoresis was performed for 90 minutes (at a current of 45 mA) using a STABNAP 300 power supply (Kucharczyk TE, Poland). Then the gel was photographed on a UV transilluminator (Sigma, USA). The amplified *spa* gene fragment's size was compared with the molecular weight marker position (GeneRuler 50 bp DNA Ladder, MBI Fermentas, Lithuania). PCR product sequencing was performed using an ABI 377 apparatus (Applied Biosystems, USA). The resulting short repeated sequences (SSRs) were numbered and compiled using the Ridom SpaType program available at http://spaserver.ridom.de. The results were given as a numeric code identifying the *spa* types.

In our study, we identified 29 peaks ranging from 2,151 to 9,632 *m*/*z*. Out of them, 12 peaks were already described in the literature as characteristic of *S. aureus* (Table II). Five of the peaks were the most common (prevalence > 87,5%), including 5,035, 6,820, and peaks 3,445, 4,307, and 6,892, which were detected in all isolates (prevalence 100%). What is important, one of the peaks (2,430) was described here for the first time and was unique for the isolates from patients infected with the SARS-CoV-2 virus.

The protein peaks expressed high diversity in prevalence among strains (5–100%) and relative amount (11–100%). Within all groups of isolates, we noticed the high diversity of *spa* types. In isolates from SARS-CoV-2 positive patients, six different *spa* types were found, and all of them had a unique peak of 2,430 *m*/*z*. A peak of 2,151 *m*/*z*, described for the first time in this study, was found in the t019 and t253 *spa* types, while the peak of 2,896 in the *spa* type t230. C&RT data analysis of the prevalence and intensity of protein peaks resulted in the identification of seven groups of strains (Fig. 1). Isolates from the wound were found in all of them, while isolates from SARS-CoV-2 positive patients were only in two protein groups.

Among proteins designated as a split in C&RT, the abundance of protein with *m*/*z* 3,410 was the highest among *S. aureus* isolates from the nose swab, as the abundance of protein 2,652 – was the highest in isolates from wounds. In the strains from nose swabs from SARS-CoV-2 positive patients, the abundance of proteins 3,445 and 5,306 was lower than in other groups (Fig. 2).

The protein profile of bacteria is used for identifying species by the matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Such identification of organisms is based on the generation of mass spectra obtained from a colony, which are compared to the spectra of known species in a reference library (Clark et al. 2013). However, until now, few studies have described using MALDI-TOF MS as a method of discrimination between different strains of *S. aureus* (Bernardo et al. 2002; Jackson et al. 2005; Wolters et al. 2011; Lasch et al. 2014). In our study, we have analysed the relative signal intensity as the amplitude of peaks in MALDI-TOF. Even though spectra are known to show variability due to several factors (Jackson et al. 2005; Wiśniewska et al. 2014; Mirzaei et al. 2020), we have identified 12 protein peaks already described in the literature. These results correspond to the previous reports and support the opinion on the described protein significance. Similarly to Østergaard and Møller (2018), we have found that peaks 6,892 and 3,445 were present in all strains, pointing to their essentiality for the species. However, one should consider

Table II
Characteristics of the protein peaks identified in the study. The *m*/*z* value refers to the mass/charge ratio.

m/*z*	Literature	Relative peak intensity	*m*/*z*	Literature	Relative peak intensity	*m*/*z*	Literature	Relative peak intensity
2,151	This study	N = 7 Med 14	3,212	This study	N = 17 Med 16	5,528	Bernardo et al. 2002; Østergaard et al. 2010; Böhme et al. 2012; Josten et al. 2013; Jackson et al. 2020	N = 30 Med 43.5
2,430	This study	N = 6 Med 11.5	3,410	This study	N = 30 Med 25	5,554	Sauget et al. 2017	N = 6 Med 23
2,515	This study	N = 14 Med 11.5	3,445	Bernardo et al. 2002; Østergaard et al. 2010; Böhme et al. 2012; Josten et al. 2013	N = 40 Med 49	6,032	Østergaard et al. 2010	N = 3 Med 23
2,652	This study	N = 23 Med 14	3,785	This study	N = 9 Med 11	6,357	This study	N = 29 Med 13
2,750	This study	N = 8 Med 15.5	4,307	This study	N = 40 Med 28	6,426	Østergaard et al. 2010	N = 10 Med 11
2,770	Sauget et al. 2017	N = 24 Med 15	4,458	Østergaard et al. 2010;	N = 6 Med 13	6,575	Jackson et al. 2005; Østergaard et al. 2010; Böhme et al. 2012; Josten et al. 2013	N = 17 Med 12
2,869	This study	N = 23 Med 13	4,594	Østergaard et al. 2010;	N = 3 Med 11	6,820	Sauget et al. 2017	N = 38 Med 54
2,895	This study	N = 2 Med 15	**4,819**	Østergaard et al. 2010; Sauget et al. 2017	Med 30.5 N = 28	**6,892**	Østergaard et al. 2010; Böhme et al. 2012	Med 100 N = 40
3,005	Jackson et al. 2005; Østergaard et al. 2010; Böhme et al. 2012; Josten et al. 2013	N = 15 Med 26	5,035	Bernardo et al. 2002; Jackson et al. 2005; Østergaard et al. 2010; Böhme et al. 2012; Josten et al. 2013	N = 36 Med 36.6	**9,632**	Østergaard et al. 2010; Sauget et al. 2017	N = 26 Med 28
3,177	This study	N = 12 Med 13,5	5,306	Sauget et al. 2017	N = 19 Med 17			

that comparison could be limited by variations in spectra resulting from differences in material distribution on the target plate and as a result of different abundance of ionised protein (Østergaard and Møller 2018).

Apart from already described peptides, we have identified the protein unique for SARS-CoV-2 isolates. According to the most common opinion, viral infection facilitates bacterial adherence by modification of epithelial cell properties (Mirzaei et al. 2020). However, this study confirms the previous finding that some proteins, likely adhesins, may have a different expression when the strains are isolated from patients with SARS-CoV-2 infection. For example, changes in bacterial properties have been described in the CMV and enterococcal co-infection (Jarzembowski et al. 2013).

C&RT data analysis showed that the protein profile is related to the prevalence of the strains as commensals in the host or etiological agents of the infection. Furthermore, the significant difference between the intensity of peaks 3,410 and 3,445 between the nose and wound isolates may suggest that the above protein contributes to the infection development and could be a candidate for the marker in the estimation of co-infection risk. In the current study, we failed to find a clear correlation between a *spa* type and the protein profile. The high diversity of *spa* types in the group of different origin makes the hypothesis of selection of strains during the SARS-CoV-2 infection not likely. Considering the above, the results obtained here support the hypothesis of bacterial adaptation do new conditions caused by viral infection.

The analysis of polymorphism in the X region of the protein A gene (*spa* typing) has proven to be an effective typing tool to distinguish strains within a heterogeneous

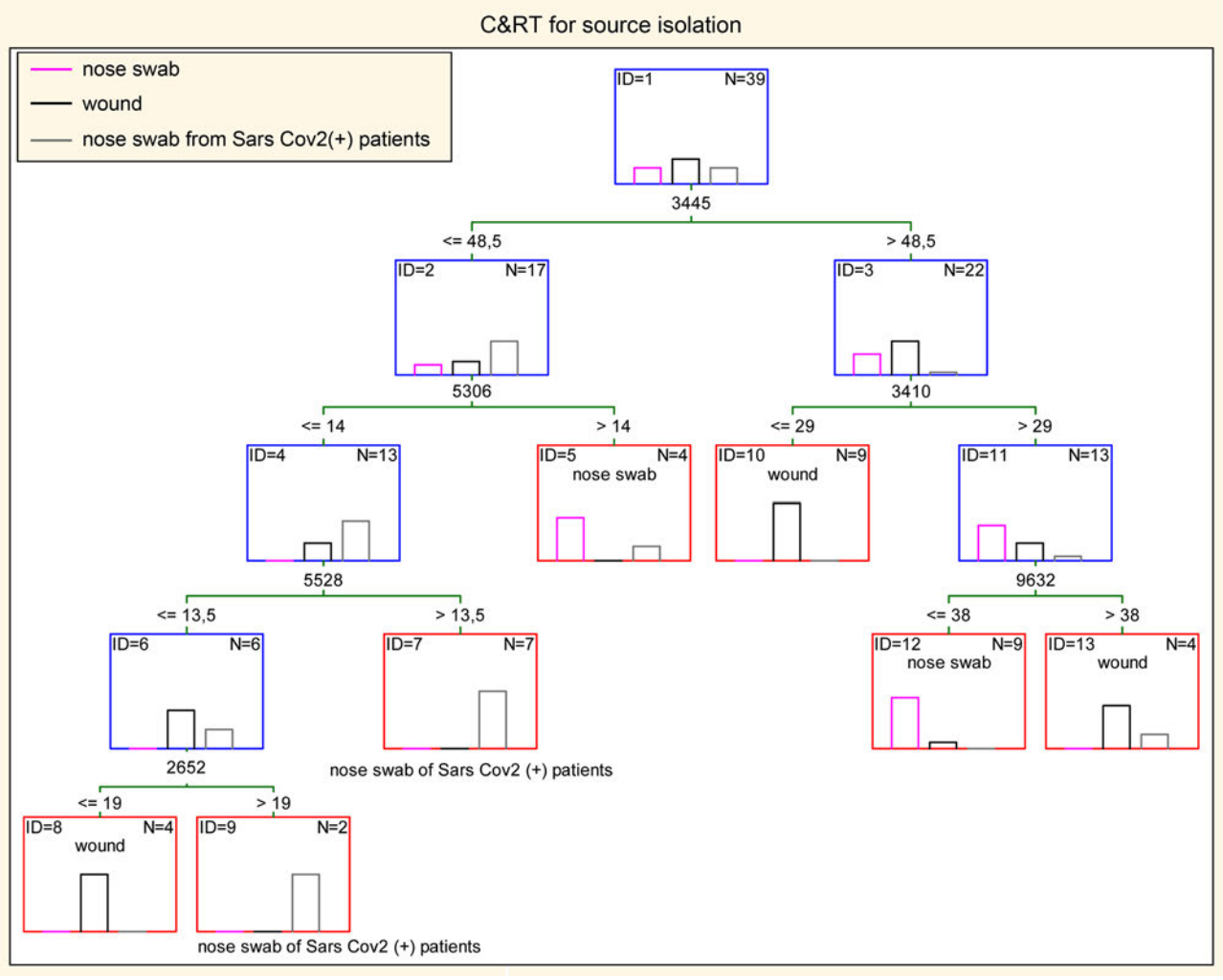

Fig. 1. Classification and Regression Trees of the strains studied by the protein presence and relative amount of particular protein. Red boxes represent the identified categories, while vertical bars are the source of strains.

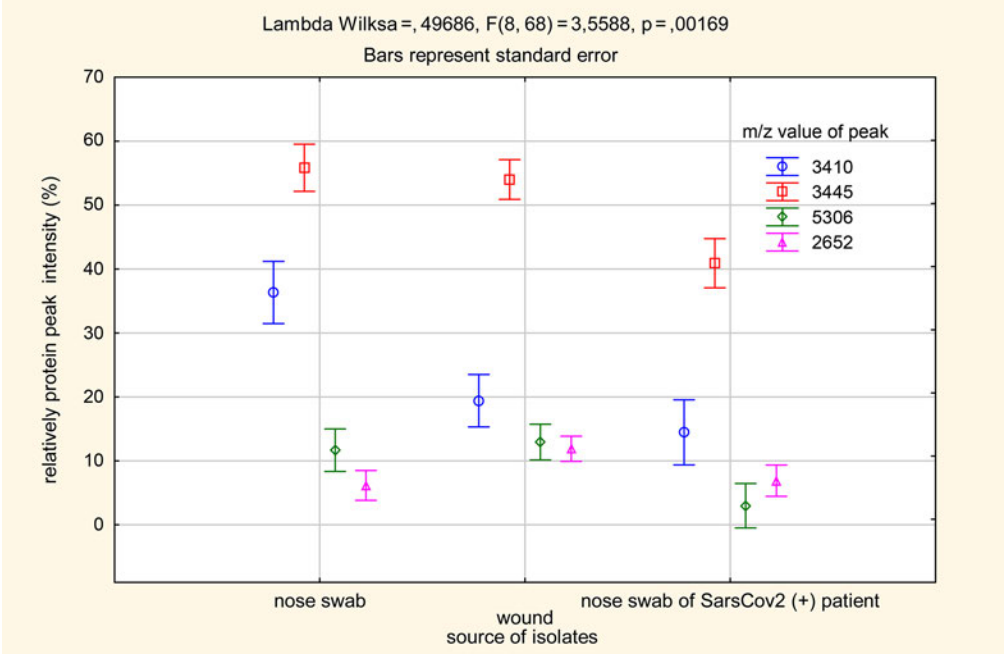

Fig. 2. ANOVA comparison of relative protein peak intensity in isolates from different groups of patients.

species such as *S. aureus*. With *spa* typing, a significant number of different types were obtained. Some correlations between *spa* types and sources of isolation could be found. The *spa* type t127 related to MLST CC1 was frequently isolated from bloodstream isolates in the Gdansk area (Wiśniewska et al. 2014). In Polish laboratories, the most prevalent was t127/ST-1 among MSSA (Methicillin Sensitive *Staphylococcus aureus*) strains (Grundmann et al. 2010). *Spa* type t127 was present among hospital and community patients, but spa t159 – was only among hospital isolates in Malaysia (Ghasemzadeh-Moghaddam et al. 2011). MSSA *spa* type t127 was isolated from the nasal cavity of handless in China, but MRSA (Methicillin Resistant *Staphylococcus aureus*) of *spa* type t127 was found in holdings of breeding pigs in Italy (Franco et al. 2011). An enterotoxin type A producing *S. aureus* of *spa* type t127 was described in human cases and different ice-cream types (Fetsch et al. 2014). Most of the PVL-positive MSSA isolates were obtained from wound infections in Nigeria and classified in clonal complexes CC1 (t127) and CC121 (t159) (Shittu et al. 2011). PVL-producing MSSA affiliated with CC121 are common worldwide (Wiese-Posselt et al. 2007). Kwapisz et al. (2020) found that the predominant clonal complex in dental isolates from infections was CC45, with the most common *spa* type being spaCC t015. CC45 are widely distributed among both the strains colonizing nares and bloodstream infections in Europe (Deasy et al. 2019; Roe et al. 2020), and recent results suggest that the nasal isolates carry the potential to cause an invasive disease (Bonnet et al. 2018). However, no reports of severe infections caused by oral CC45 strains have been published. Bonnet et al. (2018) observed a predominance of spaCC t015 among *S. aureus* strains associated with infective endocarditis, and Deasy et al. (2019) pointed to this *spa* type as an emerging etiological factor of bloodstream infections.

The fact that only local isolates were collected and analysed could be a potential weakness of our study. On the other hand, the strength of the present study is that the MALDI-TOF peaks reported in the published papers were confirmed in our isolates, as well as that some unique proteins were identified in isolates from co-infected patients.

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Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Ethical statement

Ethics approval: Ethics approval: Independent Bioethics Committee for Scientific Research at Medical University of Gdańsk, CNKBBN 525/2021

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Author contributions

All authors contributed to the final version of the manuscript: T. Jarzembowski, L. Piechowicz designed the study and prepared the manuscript draft; M. Bronk and Ł. Naumiuk conducted MALDI-TOF analysis; A. Pałubicka supervised isolation and identification of the strains.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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