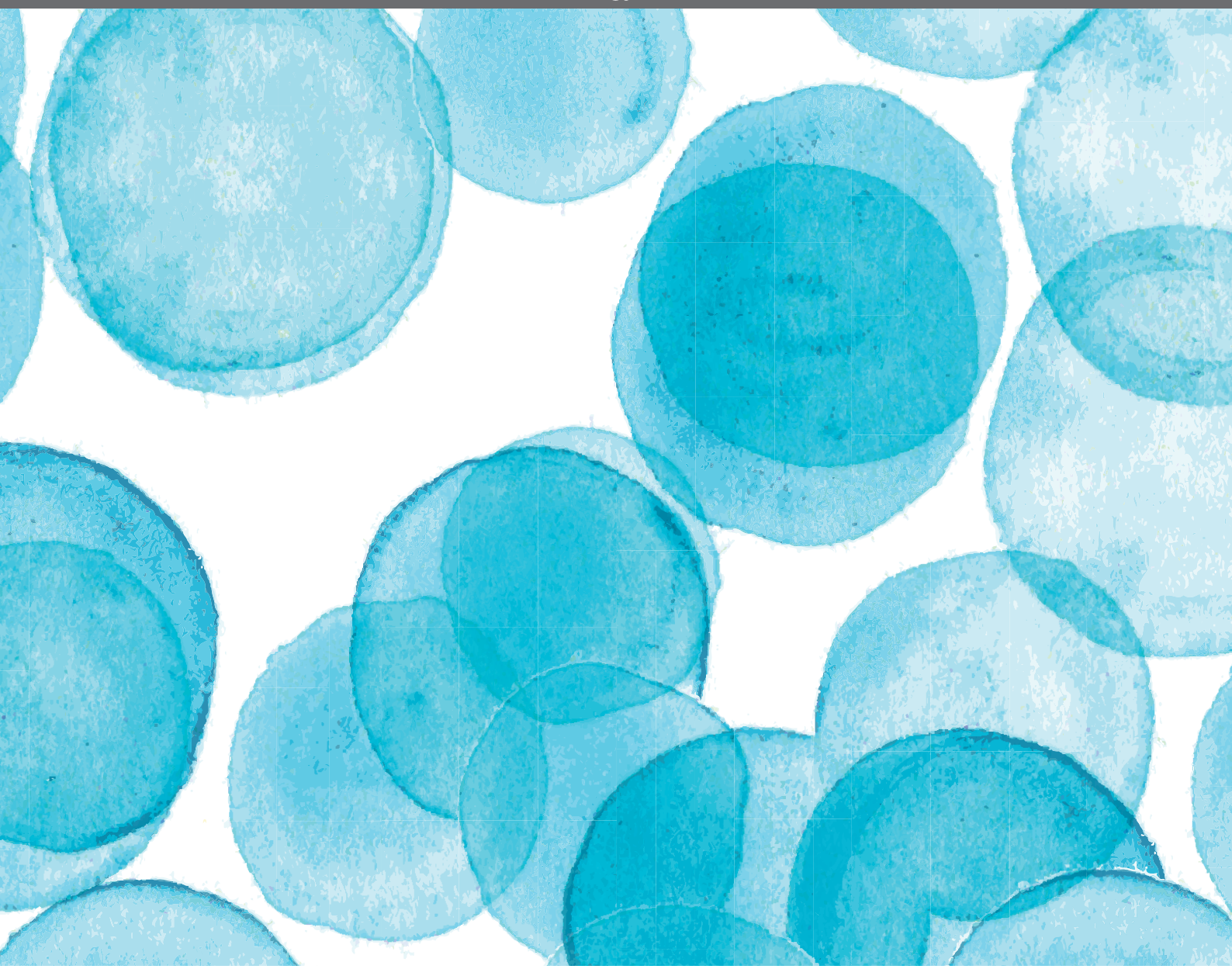
INSIGHTS IN FOOD MICROBIOLOGY: 2021

EDITED BY: Laurent Dufossé and Dario De Medici
PUBLISHED IN: Frontiers in Microbiology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-83250-871-8
DOI 10.3389/978-2-83250-871-8

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

INSIGHTS IN FOOD MICROBIOLOGY: 2021

Topic Editors:
Laurent Dufossé, Université de la Réunion, France
Dario De Medici, National Institute of Health (ISS), Italy

Citation: Dufossé, L., De Medici, D., eds. (2022). Insights in Food Microbiology: 2021. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-871-8

Table of Contents

frontiers | Frontiers in Microbiology

TYPE Editorial
PUBLISHED 08 November 2022
DOI 10.3389/fmicb.2022.1068066

OPEN ACCESS

EDITED BY
Hongshun Yang,
National University of Singapore, Singapore

REVIEWED BY
Steve Flint,
Massey University, New Zealand

*CORRESPONDENCE
Laurent Dufossé
laurent.dufosse@univ-reunion.fr

SPECIALTY SECTION
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

RECEIVED 12 October 2022
ACCEPTED 26 October 2022
PUBLISHED 08 November 2022

CITATION
Dufossé L and De Medici D (2022) Editorial: Insights in food microbiology: 2021. *Front. Microbiol.* 13:1068066. doi: 10.3389/fmicb.2022.1068066

Editorial: Insights in food microbiology: 2021

Laurent Dufossé[1]* and Dario De Medici[2]

[1]ESIROI agroalimentaire, Université de la Réunion, Saint-Denis, France, [2]Microbiological Foodborne Hazard Unit, National Institute of Health (ISS), Rome, Italy

KEYWORDS

food, microbiology, safety, human health, new trends

Editorial on the Research Topic
Insights in food microbiology: 2021

This Research Topic is part of the Insights in Frontiers in Microbiology series launched in 2021. As we are entering the third decade of the twenty-first century, and, especially in the last years, the achievements made by scientists in the field of Microbiology have been exceptional, leading to major advancements. Frontiers has organized a series of Research Topics to highlight the latest advancements in science in order to be at the forefront of science in different fields of research. This specific Editorial initiative was focused on new insights, novel developments, current challenges, latest discoveries, recent advances, and future perspectives in the field.

The Research Topic solicited brief, forward-looking contributions from the Editorial board members that describe the state of the art, outlining recent developments and major achieved accomplishments, future challenges and how to address those challenges to move the field forward. Reviews, Mini-Reviews, Perspectives, and Opinions summarizing the current state and future directions of the field were particularly welcome in this Research Topic. This Research Topic aimed to inspire, inform, and provide direction and guidance to researchers in the field.

We are pleased to note that our Research Topic has attracted contributions from many highly regarded researchers deeply involved for many years in Food Microbiology around the world, including from Austria, Brazil, Canada, China, Egypt, Finland, India, Italy, Poland, Portugal, and USA. We received 15 submissions, 13 of which were accepted (10 original research articles, two reviews, one mini-review) for publication after rigorous peer-reviews, with a total of 90 authors.

As usual with food microbiology, articles deal with good and beneficial aspects of microorganisms in food, when others list and investigate detrimental effects of unwanted microorganisms in food. Among the list of microorganisms under scrutiny, we can list the followings (non-exhaustive list): *Salmonella*, *Shigella*, Shiga toxins producing *Escherichia coli (STEC)*, *Yersinia enterocolitica*, *Listeria monocytogenes*, and *Staphylococcus aureus*.

The fermentation of foodstuffs is one of the foundations for the past, current and future developments of humanity on the planet Earth. The literature review by Skowron et al. first lists all the advantages of fermented products (extension of the shelf life of food, inhibition of the growth of pathogenic microorganisms, improvement of the

organoleptic properties, enhancement of product digestibility…). Fermentation of food can also be a source of risk if carried out under uncontrolled conditions (presence of unwanted, pathogenic microorganisms, enterotoxigenic or enterohemorrhagic bacteria). Even after millennia of practice, much remains to be done to ensure that all countries in the world provide healthy fermented products to their populations.

As a continuation of the previous work, Ghatani et al. investigated soft chhurpi, a traditionally Hymalayan Yak fermented milk, prepared by the indigenous community of Sikkim Himalayas. The two main strains found in this fermented product, *Enterococcus durans* and *Enterococcus lactis* present probiotic aspects, such as hypocholesterolemic activity and tolerance to bile salts and acid pH. It is essential for humanity to continue to isolate microbial strains with pro-health effect, from sea level to high mountain altitudes, from oceans to primary forests.

Even if current food research trends are pushing toward insect proteins, cellular meat or meat made up of 100% plant proteins, animal proteins are still widely consumed, and everyone is now aware of the intricacy between animal health and human health (see also the One-Health concept). Monte et al. conducted a large study on the biodiversity of multidrug-resistant *Salmonella* Heidelberg strains isolated from the poultry production chain across Brazil, one the largest poultry producer in the world. Authors emphasized the need for continuous mitigation programs to monitor the dissemination of this high-priority pathogen, combining molecular techniques such as WGS and CRISPR-based genotyping.

Pigs are another large source of animal proteins in the world. Large-scale animal production, often synonymous with many animals on a small surface area, induces pathologies treated with antibiotics. The use, abuse, and inappropriate use of antibiotics in livestock farms can lead to the antimicrobial resistance (AMR) in different microorganisms. Koskinen et al. defined the AMR of 1,016 pathogenic porcine *Yersinia enterocolitica* 4/O:3 strains originating from the United Kingdom, Belgium, Germany, Italy, Russia, Spain, Finland, Latvia, and Estonia. Among the conclusions of these authors, the health situation varies from country to country. More antibiotics for animal health are imported and used by the country and more the AMR increases. Parenteral medications should be preferred to orally administered mass medications, and the prudent use of antimicrobials is essential to control AMR at the farm level.

Microorganisms that are pathogenic to humans can be spread in animal husbandry but can also be present in the food processing chain. Sometimes food factories are huge and a contamination on a slicing line can cause thousands of food poisoning cases. Spampinato et al. surveyed the microbial load and the chemical–physical features of cooked hams, in modified atmosphere packaging (MAP), from five Italian producers which were monitored for a period of 12 days after the opening of the packages (i.e., the secondary shelf life). A whole set of techniques (sensorial properties, volatile metabolites, microbiota monitored by 16S ribosomal RNA gene profiling, and culture-dependent techniques…) allowed the authors to make some recommendations to the food industry. The current period tends to prohibit the use of nitrites in charcuterie and this type of study is therefore of major importance.

Raw milk, consumed as is, or used to prepare cheeses or other dairy products without any heat treatment, is also a microbiologically sensitive food. Oliveira et al. explored the prevalence and genetic diversity of *Staphylococcus aureus* and staphylococcal enterotoxins (SEs) in raw milk from the main dairy region of mainland Portugal. Recommendation is done to develop a broader SEs screening in food safety control as the majority of enterotoxigenic isolates were found to contain genes encoding SEs (SEG, SEH, and SEI) not routinely screened.

Another well-known bacterium, *Listeria monocytogenes* is difficult to control along the whole food production chain. Only long-term studies are indicated to understand how some strains are able to survive and spread as others "disappear." Gattuso et al. reported the complete sequences of 132 clinical strains, sequences that were used to define the evolutionary relatedness among subtypes of *L. monocytogenes* isolated in Italy from 2010 to 2016. Authors stressed that phylogenic studies, based on *Listeria monocytogenes* whole-genome sequence data, using the core genome multilocus sequence type, are able to identify the emergence of highly persistent pathogenic variants, contributing to the improvement of the human hazard characterization of *L. monocytogenes*.

Listing microbial threats to animals and humans is an important task. Finding ways to better control or defeat them is also of crucial importance.

Bacteriophages are beginning to be used in some parts of the world to fight bacterial infections in humans, especially when no active antibiotics are available. Rogovski et al. propose to use the bacteriophages as bacterial control tools and environmental safety indicators in a food chain context. The ecology of the food chain is a process that is very sensitive to the environment, the microorganisms present and their succession over time. In the past, mistakes have been made, for example in trying to have zero microbial life in processes, thus creating a virgin field for contaminants. Many additional studies will be required in the coming years in order to fully appreciate the benefits of using bacteriophages in the food chain.

The elimination of (pathogenic) microorganisms by disinfectants in food processing plants is desirable and mandatory. However, it is confronted with the appearance of increasing resistance. Gundolf et al. used ionic liquids (ILs), considered as a new class of promising antimicrobials, which have been reported to be effective against resistant strains as they interact with bacterial cells in multiple ways. Structure–activity relationships, side-chain effects, cationic head groups, impact on multidrug efflux pumps were among the characteristics under study.

Food microbiology is also a question of (i) detection of microbial pathogens in a large variety of foods and (ii) development of accurate predictive models for growth, survival, or death of these microbial pathogens, in a large variety of foods, too. The detection of foodborne pathogens is increasingly based on molecular techniques but older techniques, well-established techniques are still relevant and interesting. As an example, McMahon et al. demonstrated that microbial antagonism may occur in food-enrichment culture, resulting in inhibition of Shiga toxin-producing *Escherichia coli* (STEC) and *Shigella* species. The impressive production of antimicrobial compounds in cell-free extracts from 200 bacterial strains and 332 food-enrichment broths was assessed in this paper. Considering all the results presented, the recovery of some foodborne pathogens, such as *Shigella sonnei* is an important challenge for food microbiologists and technicians.

Predicting the number of food microorganisms through modeling is a fast-growing research area. Models presented are numerous and adequation with some foods were demonstrated. Li et al. developed a Dimensional Analysis Model (DAM) they applied for *Pseudomonas* in Niuganba, a traditional Chinese fermented dry-cured beef. The study showed that the DAM model was a simple, unified and effective model to predict the number of microorganisms and storage time.

At the end of the presentation of the Research Topic content, we are back to fermentation of food, to the use of fermentation in order to produce useful food and feed ingredients. For thousands of years, humans have fermented various agricultural products to produce alcohol. This is spread all around the planet.

In their experiments, about a strong aromatic liquor, also known as strong aromatic Baijiu in China, Tong et al. described the diversity, functionality, and influence of *Bacillaceae* in the process of this beverage. Multi-microbe mixing and cooperative fermentation process are the key points.

Also initiated in Asia, a few thousand years ago, *Monascus* pigments are spreading in all the world, year after year, and the use of such pigments in food will soon increase with the development of mycotoxin-free, i.e., citrinin-free filamentous fungi strains. Abdel-Raheam et al., instead of using steamed-rice, as usually done, chose to produce pigments on a food waste, generated during potato chips manufacturing. The fungal pigments biosynthesized were then incorporated as coloring agents for ice lollies, with high acceptability from consumers.

This Editorial summarizes the articles published in this Research Topic. We hope that this Research Topic of articles will contribute to the advancement of research in Food Microbiology. Feeding the world's inhabitants without depleting the resources of the planet is a major goal for humanity.

Finally, we want to thank all the authors who contributed their original work to our Research Topic and the reviewers for their valuable comments. We would like to express our sincere gratitude to the Editorial office of Frontiers in Microbiology for their excellent support and for providing us with this opportunity to successfully conduct this Research Topic.

Author contributions

LD drafted the manuscript. LD and DD revised the draft. All authors made a direct and intellectual contribution to the work and approved the final version for publication.

Acknowledgments

LD deeply thanks the Conseil Régional de La Réunion and the Conseil Régional de Bretagne for continuous support of research activities dedicated to food microbiology and food biotechnology. We would like to thank the authors and reviewers for their valuable contributions and constructive criticisms to this Research Topic. Deep thanks to the whole Frontiers team, efficient as usual.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

frontiers in Microbiology

MINI REVIEW
published: 30 November 2021
doi: 10.3389/fmicb.2021.793135

Uses of Bacteriophages as Bacterial Control Tools and Environmental Safety Indicators

Paula Rogovski[1], Rafael Dorighello Cadamuro[1], Raphael da Silva[1], Estêvão Brasiliense de Souza[1], Charline Bonatto[2,3], Aline Viancelli[4], William Michelon[4], Elmahdy M. Elmahdy[5], Helen Treichel[3], David Rodríguez-Lázaro[6,7] and Gislaine Fongaro[1]**

[1] Laboratory of Applied Virology, Department of Microbiology, Immunology and Parasitology, Federal University of Santa Catarina, Florianópolis, Brazil, [2] Department of Chemical and Food Engineering, Federal University of Santa Catarina, Florianópolis, Brazil, [3] Laboratory of Microbiology and Bioprocesses, Federal University of Fronteira Sul (UFFS), Erechim, Brazil, [4] Contested University (PMPECSA), Concórdia, Brazil, [5] Laboratory of Environmental Virology, Environmental Research Division, Department of Water Pollution Research, National Research Centre, Giza, Egypt, [6] Division of Microbiology, Department of Biotechnology and Food Science, Universidad de Burgos, Burgos, Spain, [7] Centre for Emerging Pathogens and Global Health, Universidad de Burgos, Burgos, Spain

OPEN ACCESS

Edited by:
Laurent Dufossé, Université de la Réunion, France

Reviewed by:
Diogo Silva, University of Brighton, United Kingdom
Hak-Kim Chan, The University of Sydney, Australia

***Correspondence:**
David Rodríguez-Lázaro
drlazaro@ubu.es
Gislaine Fongaro
gislaine.fongaro@ufsc.br

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *11 October 2021*
Accepted: *11 November 2021*
Published: *30 November 2021*

Citation:
Rogovski P, Cadamuro RD, da Silva R, de Souza EB, Bonatto C, Viancelli A, Michelon W, Elmahdy EM, Treichel H, Rodríguez-Lázaro D and Fongaro G (2021) Uses of Bacteriophages as Bacterial Control Tools and Environmental Safety Indicators. Front. Microbiol. 12:793135. doi: 10.3389/fmicb.2021.793135

Bacteriophages are bacterial-specific viruses and the most abundant biological form on Earth. Each bacterial species possesses one or multiple bacteriophages and the specificity of infection makes them a promising alternative for bacterial control and environmental safety, as a biotechnological tool against pathogenic bacteria, including those resistant to antibiotics. This application can be either directly into foods and food-related environments as biocontrol agents of biofilm formation. In addition, bacteriophages are used for microbial source-tracking and as fecal indicators. The present review will focus on the uses of bacteriophages like bacterial control tools, environmental safety indicators as well as on their contribution to bacterial control in human, animal, and environmental health.

Keywords: bacteriophages, food safety, biocontrol, foodborne pathogens, antimicrobial resistance

INTRODUCTION

Bacteriophages, also known as phages, are prokaryotes viruses, being the most abundant life form, present in all environments and the predominant entities in the sea (Boehme, 1993; Suttle, 2005). Several studies have demonstrated a 1:5 relative abundance between bacteria and bacteriophage (Fuhrman, 1999; Balter, 2000; Rohwer, 2003). They were discovered independently by Twort (1915), who isolated them from *Staphylococcus* spp., and from patients with dysentery. D'Herelle (1926) described bacteriophage as a virus that has the capability to parasitize bacteria (Twort, 1915; Delbruck, 1942). Bacteriophages vary greatly in morphology and replicative characteristics, containing either RNA or DNA, being these parameters currently used by the International Committee on Taxonomy of Viruses (ICTV) for bacteriophage classification

(King et al., 2012; **Table 1**). However, the identification of bacteriophages is difficult since there are no universally conserved markers, unlike e.g., the bacterial 16S rRNA gene (Paul et al., 2002), with only minor parts of bacteriophage genomes being used to determine family specific makers, such as the viral capsid g20 of T4 (Fuller et al., 1998; Marston and Sallee, 2003; Sullivan et al., 2008).

Bacteriophages can present different life cycles: lytic, lysogenic, and chronic (**Figure 1A**). Lytic bactériophages, such as T4 and MS2, insert their genetic material inside the bacteria, forcing the cell to produce a large amount of bacteriophage copies. After replication the membrane is then ruptured, releasing the new bacteriophages. Lysogenic bacteriophages (such as T1) possesses an alternative sub-cycle, in which the virus may integrate its DNA in the bacterial genome, becoming non-infectious and replicating together with the bacterial chromosome; the bacteriophage then becomes a prophage, producing new bacteriophage particles under appropriate conditions. Finally, chronic bacteriophages (such as M13) preserve their genome in the bacterial cell, in which the release from the host occurs gradually with less damage to the cell, preserving it longer (Clokie et al., 2011; Cann, 2016; Janczuk et al., 2016). There is an intimate relation between bacteriophages and bacterial cell functions acquisition (Forterre, 1999; Filée et al., 2002, 2003). Bacteriophages can serve as points for genomic rearrangements due to their mosaic nature, with lysogenic bacteriophages even protecting bacteria from lytic infection in certain conditions (Brüssow et al., 2004; Tree et al., 2014; Penadés et al., 2015). While bacterial hosts can benefit from the presence of bacteriophages (as they can express important regulators for adaptation to specific niches by the addition of bacteriophage genes in the cell's genome) bacteriophages can be involved in the transfer of virulence genes, producing proteins participating in invasion, immune evasion, and toxins related to toxin-mediated diseases (Brüssow et al., 2004; Boyd, 2012; Tree et al., 2014; Penadés et al., 2015).

The specificity of bacteriophage infection allows their application in several areas such as biotechnology, ecology, health and environment (bacterial control), and as environmental monitoring agents (Armon and Kott, 1996; Leclerc et al., 2000; Arredondo-Hernandez et al., 2017; McMinn et al., 2017; Friedman and Lai, 2018; Vandamme and Mortelmans, 2019).

In this review, a vast amount of scientific literature has been reviewed on the application of phage-based products, discussing the benefits and limitations of the use of bacteriophages as bacterial control tools in the health, food, and environmental fields.

BACTERIOPHAGES APPLICATION

Fighting Bacterial Infections

Bacterial infections are a major public health concern worldwide, representing an enormous economical and medical burden with a fatal outcome in a significant proportion of those affected. Dysentery caused by *Shigella* spp., *Salmonella* spp., *Proteus* spp. *Staphylococcus*, *Escherichia coli*, and *Pseudomonas*, usually associated with contamination of food and water, is a serious health problem affecting millions of people annually in the world, with shigellosis, a disease caused by *Shigella* spp., resulting in approximately 600,000 deaths each year (Walker et al., 1990; World Health Organization [WHO], 2017).

Lytic bacteriophages are the main tools for phage therapy, for their capacity to invade the bacterial cell and kill it. Lysogenic bacteriophages could also have an application, the transduction mechanisms could allow the use of bacteriophages as genetic tools to increase bacterial susceptibility to antibiotics; however, this approach has not been widely studied (Lu and Collins, 2009; Edgar et al., 2012). The use of a bacteriophage cocktail for the treatment and prophylaxis of intestinal infections caused by *Shigella* resulted in the patient recovery in 9 days, while antibiotic chemotherapy revealed only a clinical improvement after 29 days (Kutateladze and Adamia, 2008). Similarly, many other bacterial infections can be alternatively treated with bacteriophages, including chronic otitis, chronic infections of wounds, cystic fibrosis-associated pulmonary infections, osteomyelitis, mastitis, chronic infections of the urinary tract, gastrointestinal infections, dental caries, and endodontic infections (Harada et al., 2018; Abedon, 2019).

There is evidence that bacteriophages can be effectively used against bacterial infections, including those that have proved to be resistant to treatments with antibiotics (Abedon, 2019). *Staphylococcus aureus*, for example, is reported to be resistant to methicillin (MRSA), vancomycin (VRSA), and vancomycin-intermediate sensitivity (VISA) (Fadlallah et al., 2015). Some studies have shown that bacteriophage therapy for the treatment of infections caused by such bacteria has been successful. Fadlallah et al. (2015) reported the treatment of corneal abscess caused by VISA using the bacteriophage *S. aureus* SATA-8505 (ATCC PTA-9476).

Although treatment with bacteriophages seems a promising advantage compared to conventional antibiotics and disinfectants, a major drawback of this approach is the need for identification of the specificity range against the pathogenic bacteria prior to starting the bacteriophage treatment and the lack of protocols for testing bacterial susceptibility *in vitro* (Kutateladze and Adamia, 2010). As with antibiotics, if incomplete bacterial elimination by bacteriophages occurs, this could result in the pathogen reemergence (Carlton et al., 2005; Bigwood et al., 2008). A probable explanation could be that bacteria might show a temporal resistance, or that the bacteriophage infection results in high levels of reduction but not a complete elimination of bacteria (Hoskisson and Smith, 2007; Tokman et al., 2016; Moye et al., 2018).

However, contrasting findings of the bacteriophages cocktails effectiveness (compared to "conventional" treatments such as antibodies) were also achieved, with limitations and advantages in the use of cocktails to treat bacterial infections being extensively reviewed (Altamirano and Barr, 2019; Furfaro et al., 2018; Principi et al., 2019). In study conducted by Jault et al. (2019), a cocktail contend 12 bacteriophages was compared to antibody treatment in patients with skin infections, in a randomized control trial. At the end of the study, the conventional treatment with a 1% sulfadiazine silver emulsion

TABLE 1 | Taxonomy, morphological, and molecular characteristics of bacteriophage groups.

Family	Genus	Nucleic acid	Morphology	Main host
Leviviridae	*Levivirus*	ssRNA	Icosahedral	*E. coli*
Cystoviridae	*Cystovirus*	dsRNA	Icosahedral	*Pseudomonas* spp.
Microviridae	*Phix174microvirus*	ssDNA	Icosahedral	*E. coli*
Inoviridae	*Fibrovirus*	ssDNA	Filamentous or rod	*Vibrio* spp.
Podoviridae	*P22virus*	dsDNA	Tailed	*S. typhimurium*
Plasmaviridae	*Plasmavirus*	dsDNA	Spherical to pleomorphic	*Mycoplasma* spp.

cream was still more effective than the cocktail. However, more randomized, placebo-controlled trials must be done in order to have some consensus in dealing with the use of bacteriophages to treat infections.

There are some limitations in the use of bacteriophages for treating human infections. Due to the ability of certain bacteriophages to integrate their genome into the host's genome, care must be taken when selecting isolated bacteriophages. Some bacteriophages have potential for gene transfer; for instance, the bacterial acquisition of antibiotic resistance genes (ARGs) occurs by transduction, with bacteriophages acting as mobile genetic elements (MGE). Consequently, bacteriophages have been studied as possible vehicles of ARGs, not only as a source, but also as propagators in the environment (Gunathilaka et al., 2017). Bacteriophages containing ARGs are present in a wide range of environments; however, some environmental niches have a greater abundance, such as freshwater or marine environments (Lekunberri et al., 2017a,b; Calero-Cáceres and Luis, 2019). Bacteriophages can be also found in hospital wastewater, yet human-associated viromes rarely charge ARGs (**Figure 1B**; Enault et al., 2017; Lekunberri et al., 2017a,b).

Control Tools for Food and Environmental Bacterial Contamination

Foodborne pathogens are a major food safety threat, in 2010 an estimated 2 billion individuals contracted foodborne illnesses, resulting in 1 million deaths around the world (Kirk et al., 2015). Food safety is regarded by the World Health Organization [WHO] (2015) as a major obstacle in human development, especially in developing countries that lack infrastructure and proper environmental health practices to counter the issue. The application of bacteriophages has been proposed as an alternative tool to disinfect food and food-related environments (Pang et al., 2017). The advantage of this method is that bacteriophages kill their bacterial hosts without changing food organoleptic properties (Loc-Carrillo and Abedon, 2011; Perera et al., 2015). Also, bacteriophage low-cost large scale production, self-replicating nature, and low toxicity provide a cheap and safe disinfecting agent for low-income communities, being employed in the former Soviet Union for over 100 years (Skurnik et al., 2007; Abedon et al., 2011; Wójcik et al., 2020).

The United States Department of Agriculture (USDA) approved some products based on bacteriophages as food sanitizers, such as ListShield^{TM}, Listex P-100^{TM}, SalmoFresh^{TM}, and Salmonelex^{TM} (Hagens and Loessner, 2010). The use of a bacteriophage cocktail to inactivate foodborne bacteria like *S. enteritidis* and *S. typhimurium* on the chicken breast has also been proposed (Duc et al., 2018). These bacteriophages have been isolated from environmental sources such as wastewater, sewage, water or food (Pereira et al., 2016). Bacteriophages may also be applied for biofilm control on the food industry, such structures form on surfaces that have been improperly sanitized (Jessen and Lammert, 2003). Outbreaks of bacterial pathogens associated to biofilms in food chain have been related to the presence of *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Salmonella* spp., *Staphylococcus* spp. and *E. coli* O157:H7 (Aarnisalo et al., 2007). In this context, bacteriophages have been suggested as a green strategy for biofilm control, as they may provide a natural, highly specific, non-toxic, feasible approach for biofilm formation control (Grant et al., 2017). Biofilm control using bacteriophages has also been used to prevent dental caries, where the bacteriophages were first isolated from saliva samples and also in biofilm-mediated illness like endodontic disease, which is caused by dental pulp infection (Stevens et al., 2009; Dalmasso et al., 2015). However, it is important to highlight that each bacterial serovar could show different degrees of susceptibility to different bacteriophages (Grant et al., 2017). In addition, it is important to highlight that biofilm control by bacteriophages is mediated by the chemical composition, environmental factors, growth stage and bacteriophage concentration. Additionally, bacteriophage-biofilm interactions depend on the susceptibility of the biofilm cells and availability of receptor sites, where bacteriophage production of polysaccharide-degrading enzymes combined with effective cell lysis may rapidly destroy the biofilm (Simões et al., 2010).

Bacteriophages also show significant potential in the animal production chain such as fish, poultry, shrimps, oysters, sheep, pork and also as additives in food products such as poultry meat and eggs (Moye et al., 2018). They can prevent foodborne pathogens such as *Campylobacter* spp., *E. coli*, *L. monocytogenes*, *Salmonella enterica*, and *Shigella* spp., that are the top five foodborne public health threatening pathogens (Figueiredo and Almeida, 2017; Harada et al., 2018; Kim et al., 2020).

Bacteriophages have shown very effective to control *L. monocytogenes* by the commercial product based on bacteriophages LISTEXP^{TM}100 reported a better efficacy against *L. monocytogenes* than nisin and sodium lactate in ready-to-eat (RTE) sliced pork ham (Figueiredo and Almeida, 2017). Chibeu et al. (2013) used a Listeria-specific bacteriophage

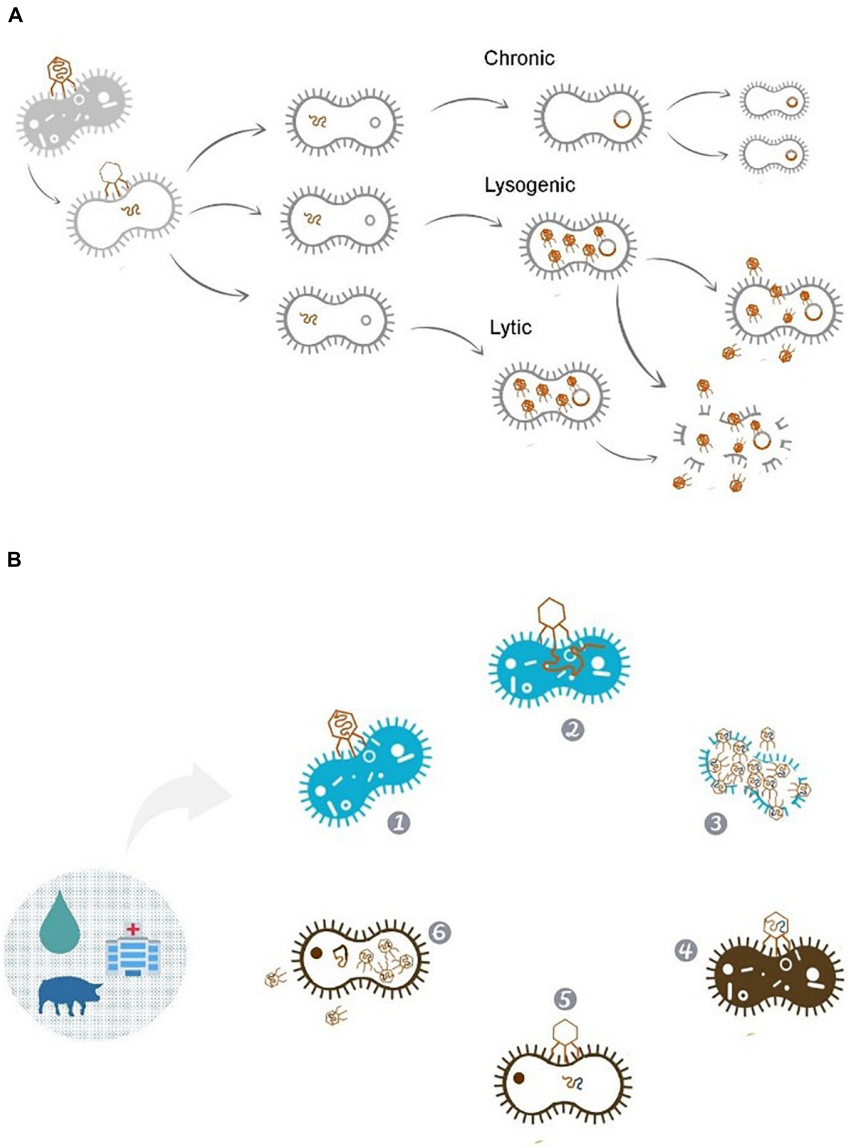


FIGURE 1 | (A) Bacteriophage life cycle. Bacteriophages can replicate by lytic, lysogenic or remain in a chronic state. In the lytic cycle of replication the viruses are released from the host after completing their replication. Lysogenic cycle involves the inclusion of genetic material into the genome of host cells, this way phages can contribute to the transferring of tetracycline resistance plasmids to bacterial cells (Pratama and Van Elsas, 2017). This is evidenced considering bacteriophages genomes, which can represent up to 13 and 5% of the *Escherichia coli* O157:H7 and *Salmonella* Newport genomes, respectively (Bobay et al., 2013). **(B)** Phage's journey through different cell lines in different environments: (1) Bacteriophage adhering to the resistant bacterial host. (2) Insertion of the phage genetic material. (3) New phages carrying resistance genes derived from the infected bacteria, being released into the environment. (4) Adhesion of the new phage on non-resistant bacteria. (5) Passage of resistance genes inserted into the phages genome to the bacteria. (6) After the genes insertion, the bacteria can enjoy the resistance genes acquired while new bacteriophages can be released keeping the journey of resistance genes on the environment.

on the surface of deli meats; a single bacteriophage strain was effective in reducing the numbers of *Listeria* cells (ATCC 19115). The evaluation of LISTEX^{TM}P100 as a bacteria controller measured the bacteriophage inactivation using black tea extract and ferrous sulfate and isolation of regrowth bacteria and their control. The result was the reduction of 1.5–2.1 $\log_{10}$ CFU/cm^2 on RTE meat samples by application of 100 μl LISTEXP^{TM}100 covering 10 cm^2 area during 28 days, resulting on 10^7 PFU/cm^2 final concentration. A cocktail of bacteriophages can be a more effective approach against a unique species of bacteria, ensuring that resistant bacteria are not selected. The application of the cocktail ListShield^{TM} including six *L. monocytogenes* specific bacteriophages efficiently reduces this pathogen in cheese, smoked salmon, apple slices, and frozen entrees (reduction of 91, 82, 90, and 99%, respectively), without changing the food organoleptic properties (Perera et al., 2015). Similarly, reductions of up to 5 logs of *L. monocytogenes* were observed in various solid foods, such as smoked salmon, iceberg lettuce

leaves, sliced cabbage, hot dogs, mixed seafood, turkey meat, and mozzarella cheese brine (Guenther et al., 2009). In fact, the use of a lytic bacteriophage on soft cheese was able to reduce 2 logs of the *Listeria* contamination while maintaining the natural microbial community of the cheese, reinforcing the host specificity of bacteriophages, and in this case the bacteriophage A511 (Guenther and Loessner, 2011).

The number of commercial solutions containing bacteriophages is increasing worldwide, being an emerging industry and field of research (Sulakvelidze, 2013; Vikram et al., 2020). Different examples of bacteriophage applications on food industry are already available: a three-bacteriophage cocktail (containing EC6, EC9, and EC11) was able to reduce *E. coli* contamination; *E. coli* ATCC 25922 and *E. coli* O127:H6 in Ultra High Temperature (UHT) milk at 25°C and under refrigeration temperatures (5–9°C) (McLean et al., 2013). The cocktail EcoShield™ was able to reduce 2 logs of *E. coli* O157:H7, 30 min after administration on leafy greens under packaging storage (Boyacioglu et al., 2013). Magnone et al. (2013) verified the disinfection of *E. coli*, *Salmonella* and *Shigella* from broccoli, cantaloupe and strawberries, with the use of commercial bacteriophage cocktails (EcoShield™, SalmoFresh™, and ShigActive™) being as effective or even more than chlorine wash. *Salmonella* is a major threat for the food industry and the most common zoonotic foodborne pathogen isolated from livestock (Jajere, 2019). The bacteriophage FO1-E2 was able to reduce the levels of *Salmonella* contamination on milk and mixed seafood for 24 h, remaining undetectable at 8 and 15°C (Guenther et al., 2012). Similarly, bacteriophage wksl3 was also able to decrease by 3 logs *Salmonella* contaminations on chicken skin (Kang et al., 2013). Likewise, some bacteriophage cocktails for *Salmonella* control are also available. The commercial formulation SalmoFresh™ was able to reduce 2–3 logs of *Salmonella* on lettuce and sprouts, showing greater reduction (2.7–3.8 logs) when associated with chlorinated water (Zhang et al., 2013). An outstanding biocontrol activity was seen with the use of a bacteriophage cocktail composed by LPSTLL, LPST94, and LPST153, being able to reduce >5.23 log viable *Salmonella* counts on biofilm grow in microtiter plates and steel chips, for 72 h. The same bacteriophages combination was also able to completely reduce the *Salmonella* inoculum on chicken breast and milk (Islam et al., 2019). **Table 2** summarizes the bacteriophages commercially available and the conditions for its applicability on treatment against different bacterial agents.

Indicators of the Presence of Foodborne Pathogens

Bacteriophages have been suggested as an alarm system in food and environmental microbiology and epidemiology since they generally fit the indicator criteria of pollution. Bacteriophages can be used as fecal indicators or microbial water quality bioindicators as an early warning of contamination by sewage, and as an efficiency marker of water or wastewater treatment (Yahya et al., 2015). This can be attributed to the bacteriophage response to the presence of pollutants, they are characteristic to adsorb to solid particles in the environment, and also due to some limitations of traditional indicators for public health such as fecal coliforms, *E. coli* and enterococci (Armon and Kott, 1993; Ashbolt et al., 2001; Jofre et al., 2016; McMinn et al., 2017).

Somatic coliphages are more persistent than traditional indicators, being also more resistant to sludge treatments, particularly when adsorbed to surfaces (Martín-Díaz et al., 2020). Many authors highlight the use of bacteriophages as indicators not only for enteric pathogenic bacteria, but also for enteric viruses such as human noroviruses, adenoviruses, and rotaviruses (Guelin, 1948; Dutka, 1990; Cornax et al., 1991; Kott, 1992; Armon and Kott, 1996; Leclerc et al., 2000; Arredondo-Hernandez et al., 2017; McMinn et al., 2017). This characteristic is due to the wide stability of phages in waste, water, soils and residues, with F-specific phages and somatic coliphages being the most used for monitoring water quality (Grabow, 2001; Sinton et al., 2002).

One of the challenges with bacteriophage application is related to bacteriophage-host interaction, which could vary depending on exposition temperature, where greater bacterial reductions are associated with higher temperatures (Tomat et al., 2013). The use of bacteriophages on wastewater treatment systems is based on their lytic capacity, which is a useful tool for the removal of human and animal pathogenic bacteria from wastewater or applied as an indicator for the presence of bacteria in wastewater treatment systems (Stefanakis et al., 2019). MS2 bacteriophages have been proposed to be suitable as operational monitoring indicators as established by guidelines of Australia, due to resistance to variation of pH and temperature (Amarasiri et al., 2017). Other applications of bacteriophages in the improvement of environmental quality are based on their survival in the environment, and soil percolation to control pathogenic bacteria in underground water (Ye et al., 2019). However, there are still some challenges for the use of bacteriophages in wastewater treatment: a high bacteriophage dosing must be used for a successful application and the use of polyvalent bacteriophages with a wider host variety could result in the reduction of beneficial bacteria. The bacterial analysis of the system is a basic requirement for bacteriophage application, as the bacterial population can change in the wastewater treatment plant (Jassim et al., 2016).

CHALLENGES, CONCERNS AND TRENDS IN THE USE OF BACTERIOPHAGES FOR ENVIRONMENTAL HEALTH PURPOSES

Although a worldwide acceptance of bacteriophages as environmental agents is not yet achieved, bacteriophage-based technologies in the environmental field are still being developed. Besides being employed as monitoring agents, or by directly controlling pathogens, bacteriophages have demonstrated promising results in agricultural microbiome modulation, increasing crop production by infecting crop detrimental bacteria in leaves and soil (Jones et al., 2012; Ye et al., 2019).

TABLE 2 | Current commercial products containing bacteriophages and conditions for use.

Host	Bacteriophage	Dose	Treatment time	Matrix	Reduction log	References
Listeria monocytogenes	LISTEX™ P100	10^7 PFU/cm^2	30 min, 1, 2, 3, 7, 10, 14, 20, and 28 days	Roast beef and cooked turkey	2 $\log_{10}$ CFU/cm^2	Chibeu et al. (2013)
	FWLLm1	2.5×10^7 PFU/cm^2	24 h	Ready-to-eat chicken breast roll	2.5 $\log_{10}$ CFU/cm^2	Bigot et al. (2011)
	P100/A511	3×10^8 PFU/g	6 days	Hot dogs (sausages), cooked and sliced turkey breast meat (cold cuts), smoked salmon, mixed seafood (cooked and chilled cocktail of shrimp, mussels, and calamari), chocolate milk (pasteurized, 3.5% fat), mozzarella cheese brine (unsalted pasteurized whey from plastic bag containers containing fresh mozzarella cheese), iceberg lettuce (leaves), and cabbage (sliced fresh leaves)	1–3 $\log_{10}$ CFU/cm^2	Guenther et al. (2009)
	ListShield™	10^9 PFU/mL	0, 2, 5, and 7 days	Fresh-cut melons and apples	3.5 $\log_{10}$ CFU/cm^2	Leverentz et al. (2001)
Salmonella nteritidis	SJ2	10^8 PFU/mL	24 h	Raw and pasteurized milk cheeses	1–2 $\log_{10}$ CFU/cm^2	Modi et al. (2001)
	PHL 4	10^{10} PFU/mL	24 h	Poultry carcass	3 $\log_{10}$ CFU/mL	Higgins et al. (2005)
Salmonella typhimurium	Felix-O1	5.25×10^6 PFU	24 h	Chicken frankfurters	2 $\log_{10}$ CFU/g	Whichard et al. (2003)
	SalmoFresh	10^8 PFU/mL	5 h	Ready-to-eat chicken products	2 $\log_{10}$ CFU/mL	
S. enteritidis and *S. typhimurium*	wksl3	2.2×10^8 PFU/mL	1, 2, 3, 5, and 7 days	Chicken skin	3 $\log_{10}$ CFU/mL	Kang et al. (2013)
	SalmoFREE	10^8 PFU/mL	36 days	*In vivo*–chicken production	3 $\log_{10}$ CFU	Clavijo et al. (2019)
	Salmonelex™	10^9 PFU/mL	24 h	Ground beef and ground pork	1.1 and 0.9 $\log_{10}$ CFU/g	Yeh et al. (2017)

Plant-soil microbiome modulation by bacteriophages was even related to an increase in ammonium concentration, likely through lysis of certain bacteria and overall community shifting (Braga et al., 2020). The use of bacteriophages on plant soil was referred to as a safer and more reliable antibacterial agent than antibiotics, in which the exaggerated use of these chemicals was related to the development of ARGs and inhibition of soil phosphatase activity (Liu et al., 2009; Zhang et al., 2017; Sun et al., 2019).

Similar to soil applications, bacteriophages appears to have a low environmental impact in fish farming plants compared to "traditional" methods such as antibodies, as it is necessary a continuous application since seawater is considered a reservoir of antibiotic resistance bacteria (Almeida et al., 2009; Alves et al., 2014; Hatosy and Martyiny, 2015). Even though bacteriophages can be considered as highly flexible and cheap tools, some drawbacks concerning the safety and overall effectiveness of the phage product may hinder their implementation as a widely accepted technology (Payne and Jansen, 2003). Bacteriophages can increase bacteria pathogenicity and fitness by transferring toxin and environmental resistance encoding genes to nearby bacteria, essentially creating genetic hazards in the area of application (Colomer-Lluch et al., 2011; Feiner et al., 2015). Besides bacteriophage-induced resistance, the bacteria may also become resistant to the virus activity through spontaneous mutations or through adaptive immunity *via* the CRISPR system (Labrie et al., 2010). Another possible major drawback in bacteriophage application is the potential disruption of the local microbiome, consequently favoring the development of harmful bacteria or health problems associated to a microbiome disbalance. Bacteriophage application has been tied to microbiome dysbiosis in humans, and can be related to the subsequent development of intestinal and mental diseases (Norman et al., 2015; Tetz et al., 2018). Microbiome disruption was also related to the development of diseases in both livestock and plants, therefore an improper bacteriophage-based product (i.e., bacteriophages that may infect healthy microbiome) may also potentially harm animal and plant farming production (Meaden and Koskella, 2013; Zeineldin et al., 2018; Lei, 2020).

In sight that bacteriophages may persist in food production plants due the virus high stability, potentially creating a genetic hazard in such facilities, the adoption of strategies for the use and manipulation of bacteriophages are required to counter bacteria resistance and achieve successful pathogen control (Hungaro et al., 2013; Chaturongakul and Ounjai, 2014; Fister et al., 2016, 2019). In this regard, practices that reduce the probability of bacteriophage resistance occurrence must be preferred, such as a two-stage self-cycling or a cellstat process (García et al., 2019). Bacteriophage cocktails have been also been employed as a way to counter bacterial bacteriophage resistance, in this strategy the bacteria would be unable to adapt (or have their viability greatly reduced) to the different infective dynamics of each virus, however, knowledge about the cocktail pharmacodynamic is required to achieve a multi-targeting system against the same bacterial strain (Abedon et al., 2021).

Aside from ARGs screening and mapped host targeting, the phage product must be suitable to the external factors present in the area of application, being resistant to the pH, temperature, UV radiation, salinity and ionic profile of the environment (Jończyk et al., 2011; Zaczek-Moczydłowska et al., 2020). In addition, the criteria for bacteriophage use in food and the environment, such as minimum exposure time, minimum effective dosage and characterization of animal local application must be established to achieve the expected therapy result while avoiding potentials drawbacks such as the presence of inhibitory compounds like antibodies, whey proteins or bacteriocins (Abedon, 2012; Vongkamjan et al., 2013; Ly-Chatain, 2014).

Special regards covering the bacteriophage properties are also advisable for an optimal and highly scalable confection of the final viral product, being of special relevance in extensive environmental applications. Bacteriophage production is directly related to the characteristics of the bacterial host (e.g., metabolic activity, growth rate, stage in cell life cycle, and abundance of bacteriophage receptors on cell surface), and the bacteriophage attributes (e.g., lysis time, burst size, and adsorption rate) (Agboluaje and Sauvageau, 2018). In addition, the initial multiplicity of infection (MOI), pH, aeration rate, presence of ions or cofactors, agitation and medium composition may also influence the outcome of infections, thus affecting bacteriophage production (Agboluaje and Sauvageau, 2018). Therefore, a full characterization of the virus and host synergy is highly advisable for easy escalation of the phage product (García et al., 2019).

With advances in molecular biology the engineering of bacteriophage particles allows a selected virus (favorited due desirable characteristics to the target therapy, such as host range and replicative potential) to be further enhanced through genetic modifications, removing undesirable viral properties that could hinder the application of the bacteriophage product as a safe and reliable object (Górski et al., 2018). Genetic engineering of phage products was able to remove toxin encoding genes and increment the virus stability in low pH environments, enhancing the functionality and removing safety hazards of the final viral product without requiring the selection of new bacteriophage strains (Nobrega et al., 2016; Park et al., 2017).

Although bacteriophages present certain safety drawbacks, largely due to negligence of mapping the product properties, bacteriophages are still considered safer than chemical treatments in environmental and food processing plants treatments applications (Meaden and Koskella, 2013; Zaczek et al., 2014). Bacteriophages stand as cheap and highly flexible structures, being able to be selected and edited for different approaches (Farr et al., 2014; Sunderland et al., 2017). Most of the research on bacteriophages has highlighted the potential for *in vitro* applications, and the number of scientific publications has increased in the last decades due to the potential use of bacteriophages in a broad spectrum of applications. In health sciences, bacteriophages are a promising approach in the fight against antibiotic-resistant bacteria, and, in the food chain, they could be a safe alternative for the control of foodborne pathogens. However, to guarantee effectiveness, a detailed understanding of the interaction between bacteriophages and the hosts is needed, considering restrictive criteria for their use to minimize their negative impact on food and food-related environments.

AUTHOR CONTRIBUTIONS

PR directs the first version of the manuscript. GF and DR-L revised the first version of the manuscript and wrote the final version of the manuscript. The rest of the authors gave fundamental contributions to the first version of the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

Aarnisalo, K., Lundén, J., Korkeala, H., and Wirtanen, G. (2007). Susceptibility of *Listeria monocytogenes* strains to disinfectants and chlorinated alkaline cleaners at cold temperatures. *LWT Food Sci. Technol.* 40, 1041–1048. doi: 10.1016/j.lwt.2006.07.009

Abedon, S. T. (2012). Bacterial 'immunity' against bacteriophages. *Bacteriophage* 2, 50–54. doi: 10.4161/bact.18609

Abedon, S. T. (2019). Use of phage therapy to treat long-standing, persistent, or chronic bacterial infections. *Adv. Drug Deliv. Rev.* 145, 18–39. doi: 10.1016/j.addr.2018.06.018

Abedon, S. T., Danis-Wlodarczyk, K. M., and Wozniak, D. J. (2021). Phage cocktail development for bacteriophage therapy: toward improving spectrum of activity breadth and depth. *Pharmaceuticals* 14:1019. doi: 10.3390/ph14101019

Abedon, S. T., Kuhl, S. J., Blasdel, B. G., and Kutter, E. M. (2011). Phage treatment of human infections. *Bacteriophage* 1, 66–85. doi: 10.4161/bact.1.2.15845

Agboluaje, M., and Sauvageau, D. (2018). Bacteriophage production in bioreactors. *Methods Mol. Biol.* 1693, 173–193. doi: 10.1007/978-1-4939-7395-8_15

Almeida, A., Cunha, Â, Gomes, N. C., Alves, E., Costa, L., and Faustino, M. A. (2009). Phage therapy and photodynamic therapy: low environmental impact approaches to inactivate microorganisms in fish farming plants. *Mar. Drugs* 7, 268–313. doi: 10.3390/md7030268

Altamirano, F. L. G., and Barr, J. J. (2019). Phage therapy in the postantibiotic era. *Clin. Microbiol. Rev.* 32:e066-18. doi: 10.1128/CMR.00066-18

Alves, M. S., Pereira, A., Araújo, S. M., Castro, B. B., Correia, A. C. M., and Henriques, I. (2014). Seawater is a reservoir of multi-resistant *Escherichia coli*, including strains hosting plasmid-mediated quinolones resistance and

extended-spectrum beta-lactamases genes. *Front. Microbiol.* 5:426. doi: 10.3389/fmicb.2014.00426

Amarasiri, M., Kitajima, M., Nguyen, T. H., and Okabe, S. (2017). Bacteriophage removal efficiency as a validation and operational monitoring tool for virus reduction in wastewater reclamation: review. *Water Res.* 121, 258–269. doi: 10.1016/j.watres.2017.05.035

Armon, R., and Kott, Y. (1993). A simple, rapid and sensitive presence/absence detection test for bacteriophage in drinking water. *J. Appl. Bacteriol.* 74, 490–496. doi: 10.1111/j.1365-2672.1993.tb05159.x

Armon, R., and Kott, Y. (1996). Bacteriophages as indicators of pollution. *Crit. Rev. Environ. Sci. Technol.* 26, 299–335. doi: 10.1080/10643389609388494

Arredondo-Hernandez, L. J. R., Diaz-Avalos, C., Lopez-Vidal, Y., Castillo-Rojas, G., and Mazari-Hiriart, M. (2017). FRNA bacteriophages as viral indicators of faecal contamination in Mexican tropical aquatic systems. *PLoS One* 12:e0170399. doi: 10.1371/journal.pone.0170399

Ashbolt, N. J., Grabow, W. O. K., and Snozzi, M. (2001). "Indicators of microbial water quality," in *Water Quality: Guidelines, Standards and Health*, eds L. Fewtrell and J. Bartram (London: IWA Publishing), 289–315.

Balter, M. (2000). Evolution on Life's fringes. *Science* 289, 1866–1867. doi: 10.1126/science.289.5486.1866

Bigot, B., Lee, W., Mcintyre, L., Wilson, T., Hudson, J. A., Billington, C., et al. (2011). Control of *Listeria monocytogenes* growth in a ready-to-eat poultry product using a bacteriophage. *Food Microbiol.* 28, 1448–1452. doi: 10.1016/j.fm.2011.07.001

Bigwood, T., Hudson, J. A., Billington, C., Carey-Smith, G. V., and Heinemann, J. A. (2008). Phage inactivation of foodborne pathogens on cooked and raw meat. *Food Microbiol.* 25, 400–406. doi: 10.1016/j.fm.2007.11.003

Bobay, L. M., Rocha, E. P. C., and Touchon, M. (2013). The adaptation of temperate bacteriophages to their host genomes. *Mol. Biol. Evol.* 30, 737–751. doi: 10.1093/molbev/mss279

Boehme, J. (1993). Viruses, bacterioplankton, and Phytoplankton in the Southeastern Gulf of Mexico: distribution and contribution to Oceanic DNA pools. *Mar. Ecol. Prog. Ser.* 97, 1–10. doi: 10.3354/meps097001

Boyacioglu, O., Sharma, M., Sulakvelidze, A., and Goktepe, I. (2013). Biocontrol of *Escherichia coli* O157. *Bacteriophage* 3:e24620. doi: 10.4161/bact.24620

Boyd, E. F. (2012). "Bacteriophage-encoded bacterial virulence factors and phage-pathogenicity island interactions," in *Advances in Virus Research*, 1st Edn, eds M. Łobocka and W. T. Szybalski (Amsterdam: Elsevier), 91–118. doi: 10.1016/B978-0-12-394621-8.00014-5

Braga, L. P. P., Spor, A., Kot, W., Breuil, M.-C., Hansen, L. H., Setubal, J. C., et al. (2020). Impact of phages on soil bacterial communities and nitrogen availability under different assembly scenarios. *Microbiome* 8, 45–72. doi: 10.1186/s40168-020-00822-z

Brüssow, H., Canchaya, C., and Hardt, W. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol. Mol. Biol. Rev.* 68, 560–602. doi: 10.1128/MMBR.68.3.560-602.2004

Calero-Cáceres, W., and Luis, J. (2019). Antibiotic resistance genes in bacteriophages from diverse marine habitats. *Sci. Total Environ.* 654, 452–455. doi: 10.1016/j.scitotenv.2018.11.166

Cann, A. J. (2016). *Principles of Molecular Virology*, 6th Edn, Cambridge, MA: Academic Press.

Carlton, R. M., Noordman, W. H., Biswas, B., de Meester, E. D., and Loessner, M. J. (2005). Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regul. Toxicol. Pharmacol.* 43, 301–312. doi: 10.1016/j.yrtph.2005.08.005

Chaturongakul, S., and Ounjai, P. (2014). Phage-host interplay: examples from tailed phages and gram-negative bacterial pathogens. *Front. Microbiol.* 5:442. doi: 10.3389/fmicb.2014.00442

Chibeu, A., Agius, L., Gao, A., Sabour, P. M., Kropinski, A. M., Balamurugan, S., et al. (2013). Efficacy of bacteriophage LISTEX P100 combined with chemical antimicrobials in reducing *Listeria monocytogenes* in cooked turkey and roast beef. *Int. J. Food Microbiol.* 167, 208–214. doi: 10.1016/j.ijfoodmicro.2013.08.018

Clavijo, V., Baquero, D., Hernandez, S., Farfan, J. C., Arias, J., Arévalo, A., et al. (2019). Phage cocktail SalmoFREE reduces *Salmonella* on a commercial broiler farm. *Poult. Sci.* 98, 5054–5063. doi: 10.3382/ps/pez251

Clokie, M. R. J., Millard, A. D., Letarov, A. V., and Heaphy, S. (2011). Phages in nature. *Bacteriophage* 1, 31–45. doi: 10.4161/bact.1.1.14942

Colomer-Lluch, M., Jofre, J., and Muniesa, M. (2011). Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS One* 6:e17549. doi: 10.1371/journal.pone.0017549

Cornax, R., Morilgligo, M. A., Balebona, M. C., Castro, D., and Borrego, J. J. (1991). Significance of several bacteriophage groups as indicators of sewage pollution in marine waters. *Water Res.* 25, 673–678. doi: 10.1016/0043-1354(91)90042-O

Dalmasso, M., De Haas, E., Neve, H., Strain, R., Cousin, F. J., Stockdale, S. R., et al. (2015). Isolation of a novel phage with activity against *Streptococcus mutans* biofilms. *PLoS One* 10:e0138651. doi: 10.1371/journal.pone.0138651

Delbruck, M. (1942). "Bacterial viruses (Bacteriophages)," in *Advances in Enzymology - and Related Areas of Molecular Biology*, Vol. 2, eds F. F. Nord and C. H. Werkman (Hoboken, NJ: Wiley), 1–30. doi: 10.1002/9780470122471.ch1

D'Herelle, F. (1926). The bacteriophage and its behaviour. *Nature* 118, 183–185. doi: 10.1038/118183a0

Duc, H. M., Son, H. M., Honjoh, K., and Miyamoto, T. (2018). Isolation and application of bacteriophages to reduce *Salmonella* contamination in raw chicken meat. *LWT Food Sci. Technol.* 91, 353–360. doi: 10.1016/j.lwt.2018.01.072

Dutka, B. J. (1990). The presence of bacterial virus in groundwater and treated drinking water microbiological tests. *Environ. Pollut.* 63, 293–298. doi: 10.1016/0269-7491(90)90136-Z

Edgar, R., Friedman, N., Molshanski-Mor, S., and Qimron, U. (2012). Reversing bacterial resistance to antibiotics by phage-mediated f dominant sensitive genes. *Appl. Environ. Microbiol.* 78, 744–751. doi: 10.1128/AEM.05741-11

Enault, F., Briet, A., Bouteille, L., Roux, S., Sullivan, M. B., M-A, Petit, et al. (2017). Phages rarely encode antibiotic resistance genes: a cautionary tale for virome analyses. *ISME J.* 11, 237–247. doi: 10.1038/ismej.2016.90

Fadlallah, A., Chelala, E., and Legeais, J. M. (2015). Corneal infection therapy with topical bacteriophage administration. *Open Ophthalmol. J.* 9, 167–168. doi: 10.2174/1874364101509010167

Farr, R., Choi, D. S., and Lee, S.-W. (2014). Phage-based nanomaterials for biomedical applications. *Acta Biomater.* 10, 1741–1750. doi: 10.1016/j.actbio.2013.06.037

Feiner, R., Argov, T., Rabinovich, L., Sigal, N., Borovok, I., and Herskovits, A. A. A. (2015). new perspective on lysogeny: prophages as active regulatory switches of bacteria. *Nat. Rev. Microbiol.* 13, 641–650. doi: 10.1038/nrmicro3527

Figueiredo, A. C. L., and Almeida, R. C. C. (2017). Antibacterial efficacy of nisin, bacteriophage P100 and sodium lactate against *Listeria monocytogenes* in ready-to-eat sliced pork ham. *Braz. J. Microbiol.* 48, 724–729. doi: 10.1016/j.bjm.2017.02.010

Filée, J., Forterre, P., and Laurent, J. (2003). The role played by viruses in the evolution of their hosts: a view based on informational protein phylogenies. *Res. Microbiol.* 154, 237–243. doi: 10.1016/S0923-2508(03)00066-4

Filée, J., Forterre, P., Sen-lin, T., and Laurent, J. (2002). Evolution of DNA polymerase families: evidences for multiple gene. *J. Mol. Evol.* 54, 763–773. doi: 10.1007/s00239-001-0078-x

Fister, S., Fuchs, S., Stessl, B., Schodera, D., Wagner, M., and Rossmanitha, P. (2016). Screening and characterisation of bacteriophage P100 insensitive *Listeria monocytogenes* isolates in Austrian dairy plants. *Food Control.* 59, 108–117. doi: 10.1016/j.foodcont.2015.05.026

Fister, S., Mester, P., Witte, A. K., Sommer, J., Schodera, D., Rossmanith, P., et al. (2019). Part of the problem or the solution? Indiscriminate use of bacteriophages in the food industry can reduce their potential and impair growth-based detection methods. *Trends Food Sci. Technol.* 90, 170–174. doi: 10.1016/j.tifs.2019.02.031

Forterre, P. (1999). Displacement of cellular proteins by functional analogues from plasmids or viruses could explain puzzling phylogenies of many DNA informational proteins. *Mol. Microbiol.* 33, 457–465. doi: 10.1046/j.1365-2958.1999.01497.x

Friedman, A., and Lai, X. (2018). Combination therapy for cancer with oncolytic virus and checkpoint inhibitor: a mathematical model. *PLoS One* 13:e0192449. doi: 10.1371/journal.pone.0192449

Fuhrman, J. A. (1999). Marine viruses and their biogeochemical and ecological effects. *Nature* 399, 541–548. doi: 10.1038/21119

Fuller, N. J., Wilson, W. H., Joint, I. R., and Mann, N. H. (1998). Occurrence of a sequence in marine cyanophages similar to that of T4 g20 and its application to

PCR-based detection and quantification techniques. *Appl. Environ. Microbiol.* 64, 2051–2060. doi: 10.1128/AEM.64.6.2051-2060.1998

Furfaro, L. L., Payne, M. S., and Chang, B. J. (2018). Bacteriophage therapy: clinical trials and regulatory hurdles. *Front. Cell. Infect. Microbiol.* 8:376. doi: 10.3389/fcimb.2018.00376

García, R., Latz, S., Romero, J., Higuera, G., García, K., and Bastías, R. (2019). Bacteriophage production models: an overview. *Front. Microbiol.* 10:1187. doi: 10.3389/fmicb.2019.01187

Górski, A., Międzybrodzki, R., Łobocka, M., Głowacka-rutkowska, A., Bednarek, A., Borysowski, J., et al. (2018). Phage therapy: what have we learned? *Viruses* 10:288. doi: 10.3390/v10060288

Grabow, W. O. K. (2001). Bacteriophages: update on application as models for viruses in water. *Water* 27, 251–268. doi: 10.4314/wsa.v27i2.4999

Grant, A. Q., Parveen, S., Schwarz, J., Hashem, F., and Vimini, B. (2017). Reduction of *Salmonella* in ground chicken using a bacteriophage. *Poult. Sci.* 96, 2845–2852. doi: 10.3382/ps/pex062

Guelin, A. (1948). Etude quantitative des bactériophages de la mer. *Ann. l'Institut Pasteur* 74:104.

Guenther, S., Herzig, O., Fieseler, L., Klumpp, J., and Loessner, M. J. (2012). Biocontrol of *Salmonella Typhimurium* in RTE foods with the virulent bacteriophage FO1-E2. *Int. J. Food Microbiol.* 154, 66–72. doi: 10.1016/j.ijfoodmicro.2011.12.023

Guenther, S., Huwyler, D., Richard, S., and Loessner, M. J. (2009). Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. *Appl. Environ. Microbiol.* 75, 93–100. doi: 10.1128/AEM.01711-08

Guenther, S., and Loessner, M. J. (2011). Bacteriophage biocontrol of *Listeria monocytogenes* on soft ripened white mold and red-smear cheeses. *Bacteriophage* 1, 94–100. doi: 10.4161/bact.1.2.15662

Gunathilaka, G. U., Tahlan, V., Mafiz, A. I., Polur, M., and Zhang, Y. (2017). International journal of antimicrobial agents phages in urban wastewater have the potential to disseminate antibiotic resistance. *Int. J. Antimicrob. Agents* 50, 678–683. doi: 10.1016/j.ijantimicag.2017.08.013

Hagens, S., and Loessner, M. J. (2010). Bacteriophage for biocontrol of foodborne pathogens: calculations and considerations. *Curr. Pharm. Biotechnol.* 11, 58–68. doi: 10.2174/138920110790725429

Harada, L. K., Silva, E. C., Campos, W. F., Del Fiol, F. S., Vila, M., Dąbrowska, K., et al. (2018). Biotechnological applications of bacteriophages: state of the art. *Microbiol. Res.* 212–213, 38–58. doi: 10.1016/j.micres.2018.04.007

Hatosy, S. M., and Martyiny, A. C. (2015). The ocean as a global reservoir of antibiotic resistance genes. *Appl. Environ. Microbiol.* 81, 7593–7599. doi: 10.1128/AEM.00736-15

Higgins, J. P., Higgins, S. E., Guenther, K. L., Huff, W., Donoghue, A. M., Donoghue, D. J., et al. (2005). Use of a specific bacteriophage treatment to reduce *Salmonella* in poultry products. *Poult. Sci.* 84, 1141–1145. doi: 10.1093/ps/84.7.1141

Hoskisson, P. A., and Smith, M. C. M. (2007). Hypervariation and phase variation in the bacteriophage 'resistome'. *Curr. Opin. Microbiol.* 10, 396–400. doi: 10.1016/j.mib.2007.04.003

Hungaro, H. M., Mendonêa, R. C. S., Gouvça, D. M., Vanetti, M. C. D., and Pintoc, C. L. O. (2013). Use of bacteriophages to reduce *Salmonella* in chicken skin in comparison with chemical agents. *Food Res. Int.* 52, 75–81. doi: 10.1016/j.foodres.2013.02.032

Islam, M. S., Zhou, Y., Liang, L., Nime, I., Liu, K., Yan, T., et al. (2019). Application of a phage cocktail for control of *Salmonella* in foods and reducing biofilms. *Viruses* 11:841. doi: 10.3390/v11090841

Jajere, S. M. (2019). A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. *Vet. World* 12, 2231–2916. doi: 10.14202/vetworld.2019.504-521

Janczuk, M., Niedzió, J., and Szot-Karpi, K. (2016). Bacteriophages in electrochemistry: a review. *J. Electroanal. Chem.* 779, 207–219. doi: 10.1016/j.jelechem.2016.05.019

Jassim, S. A. A., Limoges, R. G., and El-Cheikh, H. (2016). Bacteriophage biocontrol in wastewater treatment. *World J. Microbiol. Biotechnol.* 32, 1–10. doi: 10.1007/s11274-016-2028-1

Jault, P., Leclerc, T., Jennes, S., Pirnay, J. P., Que, Y., Resch, G., et al. (2019). Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by *Pseudomonas aeruginosa* (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *Lancet Infect. Dis.* 19, 35–45. doi: 10.1016/S1473-3099(18)30482-1

Jessen, B., and Lammert, L. (2003). Biofilm and disinfection in meat processing plants. *Int. Biodeter. Biodegrad.* 51, 265–269. doi: 10.1016/S0964-8305(03)00046-5

Jofre, J., Lucena, F., Blanch, A. R., and Muniesa, M. (2016). Coliphages as model organisms in the characterization and management of water resources. *Water* 8:199. doi: 10.3390/w8050199

Jończyk, E., Kłak, M., Międzybrodzki, R., and Górski, A. (2011). The influence of external factors on bacteriophages—review. *Folia Microbiol.* 56, 191–200. doi: 10.1007/s12223-011-0039-8

Jones, J. B., Vallad, G. E., Iriarte, F. B., Obradović, A., Wernsing, M. H., Jackson, L. E., et al. (2012). Considerations for using bacteriophages for plant disease control. *Bacteriophage* 2, 689–709. doi: 10.4161/bact.23857

Kang, H., Kim, J., Jung, T., and Woo, G.-J. (2013). wksl3, a new biocontrol agent for *Salmonella enterica* Serovars Enteritidis and *Typhimurium* in foods: characterization, application, sequence analysis, and oral acute toxicity study. *Appl. Environ. Microbiol.* 79, 1956–1968. doi: 10.1128/AEM.02793-12

Kim, H. J., Giri, S. S., Kim, S. G., Kim, S. W., Kwon, J., Lee, S. B., et al. (2020). Isolation and characterization of two bacteriophages and their preventive effects against pathogenic vibrio coralliilyticus causing mortality of pacific oyster (Crassostrea Gigas) larvae. *Microorganisms* 8:926. doi: 10.3390/microorganisms8060926

King, A. M. Q., Elliot, L., Michael, J. A., and Eric, B. C. (2012). *Virus Taxonomy*. Amsterdam: Elsevier.

Kirk, M. D., Pires, S. M., Black, R. E., Caipo, M., Crump, J. A., Devleesschauwer, B., et al. (2015). World health organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases. *PLoS Med.* 12:e1001940. doi: 10.1371/journal.pmed.1001940

Kott, Y. (1992). Wastewater upgrading as measured by coliphage. *Environ. Toxicol. Water Qual. Ann. Int. J.* 7, 61–69. doi: 10.1002/tox.2530070106

Kutateladze, M., and Adamia, R. (2008). Phage therapy experience at the Eliava Institute. *Med. Mal. Infect.* 38, 426–430. doi: 10.1016/j.medmal.2008.06.023

Kutateladze, M., and Adamia, R. (2010). Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends Biotechnol.* 28, 591–595. doi: 10.1016/j.tibtech.2010.08.001

Labrie, S. J., Samson, J. E., and Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* 8, 317–327. doi: 10.1038/nrmicro2315

Leclerc, H., Edberg, S., and Pierzo, V. (2000). Bacteriophages as indicators of enteric viruses and public health risk in groundwaters. *J. Appl. Microbiol.* 88, 5–21. doi: 10.1046/j.1365-2672.2000.00949.x

Lei, L. (2020). Phyllosphere dysbiosis. *Nat. Plants* 6, 434–434. doi: 10.1038/s41477-020-0674-7

Lekunberri, I., Subirats, J., and Borrego, C. M. (2017a). Exploring the contribution of bacteriophages to antibiotic resistance. *Environ. Pollut.* 220, 981–984. doi: 10.1016/j.envpol.2016.11.059

Lekunberri, I., Villagrasa, M., Luis, J., and Borrego, C. M. (2017b). Contribution of bacteriophage and plasmid DNA to the mobilization of antibiotic resistance genes in a river receiving treated wastewater discharges. *Sci. Total Environ.* 601–602, 206–209. doi: 10.1016/j.scitotenv.2017.05.174

Leverentz, B., Conway, W. S., Alavidze, Z., Janisiewicz, W. J., Fuchs, Y., Camp, M. J., et al. (2001). Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: a model study. *J. Food Prot.* 64, 1116–1121. doi: 10.4315/0362-028X-64.8.1116

Liu, F., Ying, G.-G., Tao, R., Zhao, J.-L., Yang, J.-F., and Zhao, L.-F. (2009). Effects of six selected antibiotics on plant growth and soil microbial and enzymatic activities. *Environ. Pollut.* 157, 1636–1642. doi: 10.1016/j.envpol.2008.12.021

Loc-Carrillo, C., and Abedon, S. T. (2011). Pros and cons of phage therapy. *Bacteriophage* 2, 111–114. doi: 10.4161/bact.1.2.14590

Lu, T. K., and Collins, J. J. (2009). Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4629–4634. doi: 10.1073/pnas.0800442106

Ly-Chatain, M. H. (2014). The factors affecting effectiveness of treatment in phages therapy. *Front. Microbiol.* 5:51. doi: 10.3389/fmicb.2014.00051

Magnone, J. P., Marek, P. J., and Sulakvelidze, A. (2013). Additive approach for inactivation of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* spp. on contaminated fresh fruits and vegetables using bacteriophage cocktail and

produce wash. *J. Food Prot.* 76, 1336–1341. doi: 10.4315/0362-028X.JFP-12-517

Marston, M. F., and Sallee, J. L. (2003). Genetic diversity and temporal variation in the cyanophage community infecting marine *Synechococcus* species in Rhode Island's coastal waters. *Appl. Environ. Microbiol.* 69, 4639–4647. doi: 10.1128/AEM.69.8.4639-4647.2003

Martín-Díaz, J., Lucena, F., Blanch, A. R., and Jofre, J. (2020). Review: indicator bacteriophages in sludge, biosolids, sediments and soils. *Environ. Res.* 182:109133. doi: 10.1016/j.envres.2020.109133

McLean, S. K., Dunn, L. A., and Palombo, E. A. (2013). Phage inhibition of *Escherichia coli* in ultrahigh-temperature-treated and raw milk. *Foodb. Pathog. Dis.* 10, 956–962. doi: 10.1089/fpd.2012.1473

McMinn, B. R., Ashbolt, N. J., and Korajkic, A. (2017). Bacteriophages as indicators of faecal pollution and enteric virus removal. *Lett. Appl. Microbiol.* 65, 11–26. doi: 10.1111/lam.12736

Meaden, S., and Koskella, B. (2013). Exploring the risks of phage application in the environment. *Front. Microbiol.* 4:358. doi: 10.3389/fmicb.2013.00358

Modi, R., Hirvi, Y., and Hill, A. (2001). Effect of phage on survival of *Salmonella* enteritidis during manufacture and storage of cheddar cheese made from raw and pasteurized milk. *J. Food Prot.* 64, 927–933. doi: 10.4315/0362-028X-64.7.927

Moye, Z. D., Woolston, J., and Sulakvelidze, A. (2018). Bacteriophage applications for food production and processing. *Viruses* 10:205. doi: 10.3390/v10040205

Nobrega, F. L., Costa, A. R., Santos, J. F., Siliakus, M. F., Van Lent, J. W. M., Kengen, S. W. M., et al. (2016). Genetically manipulated phages with improved pH resistance for oral administration in veterinary medicine. *Sci. Rep.* 6:39235. doi: 10.1038/srep39235

Norman, J. M., Handley, S. A., Baldridge, M. T., Droit, L., Liu, C. Y., Keller, B. C., et al. (2015). Disease-specific alterations in the enteric virome in inflammatory bowel disease. *Cell* 160, 447–460. doi: 10.1016/j.cell.2015.01.002

Pang, H., Lambertini, E., Buchanan, R. L., Schaffner, D. W., and Pradhan, A. K. (2017). Quantitative microbial risk assessment for *Escherichia coli* O157: H7 in fresh-cut lettuce. *J. Food Prot.* 80, 302–311. doi: 10.4315/0362-028X.JFP-16-246

Park, J. Y., Moon, B. Y., Park, J. W., Thornton, J. A., Park, Y. H., and Seo, K. S. (2017). Genetic engineering of a temperate phage-based delivery system for CRISPR/Cas9 antimicrobials against *Staphylococcus aureus*. *Sci. Rep.* 7:44929. doi: 10.1038/srep44929

Paul, J. H., Sullivan, M. B., Segall, A. M., and Rohwer, F. (2002). Marine phage genomics. *Comp. Biochem. Physiol. Part B* 133, 463–476. doi: 10.1016/S1096-4959(02)00168-9

Payne, R. J. H., and Jansen, V. A. A. (2003). Pharmacokinetic principles of bacteriophage therapy. *Clin. Pharm.* 42, 315–325. doi: 10.2165/00003088-200342040-00002

Penadés, J. R., Chen, J., Quiles-Puchalt, N., Carpena, N., and Novick, R. P. (2015). Bacteriophage-mediated spread of bacterial virulence genes. *Curr. Opin. Microbiol.* 23, 171–178. doi: 10.1016/j.mib.2014.11.019

Pereira, C., Moreirinha, C., Lewicka, M., Almeida, P., Clemente, C., Cunha, A., et al. (2016). Bacteriophages with potential to inactivate *Salmonella Typhimurium*: use of single phage suspensions and phage cocktails. *Virus Res.* 220, 179–192. doi: 10.1016/j.virusres.2016.04.020

Perera, M. N., Abuladze, T., Li, M., Woolston, J., and Sulakvelidze, A. (2015). Bacteriophage cocktail significantly reduces or eliminates *Listeria monocytogenes* contamination on lettuce, apples, cheese, smoked salmon and frozen foods. *Food Microbiol.* 52, 42–48. doi: 10.1016/j.fm.2015.06.006

Pratama, A. A., and Van Elsas, J. D. (2017). A novel inducible prophage from the mycosphere inhabitant *Paraburkholderia terrae* BS437. *Sci. Rep.* 7:9156. doi: 10.1038/s41598-017-09317-8

Principi, N., Silvestri, E., and Esposito, S. (2019). Advantages and limitations of bacteriophages for the treatment of bacterial infections. *Front. Pharmacol.* 10:513. doi: 10.3389/fphar.2019.00513

Rohwer, F. (2003). Global phage diversity. *Cell* 113:141. doi: 10.1016/S0092-8674(03)00276-9

Simões, M., Simões, L. C., and Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT Food Sci. Technol.* 43, 573–583. doi: 10.1016/j.lwt.2009.12.008

Sinton, L. W., Hall, C. H., Lynch, P. A., and Davies-Colley, R. J. (2002). Sunlight inactivation of 615 fecal indicator bacteria and bacteriophages from waste stabilization pond effluent in fresh and 616 saline waters. *Appl. Environ. Microbiol.* 68, 1122–1131. doi: 10.1128/AEM.68.3.1122-1131.2002

Skurnik, M., Pajunen, M., and Kiljunen, S. (2007). Biotechnological challenges of phage therapy. *Biotechnol. Lett.* 29, 995–1003. doi: 10.1007/s10529-007-9346-1

Stefanakis, A. I., Bardiau, M., Trajano, D., Couceiro, F., Williams, J. B., Taylora, H., et al. (2019). Presence of bacteria and bacteriophages in full-scale trickling filters and an aerated constructed wetland. *Sci. Total Environ.* 659, 1135–1145. doi: 10.1016/j.scitotenv.2018.12.415

Stevens, R. H., Porras, O. D., and Delisle, A. L. (2009). Bacteriophages induced from lysogenic root canal isolates of *Enterococcus faecalis*. *Oral Microbiol. Immunol.* 24, 278–284. doi: 10.1111/j.1399-302X.2009.00506.x

Sulakvelidze, A. (2013). Using lytic bacteriophages to eliminate or significantly reduce contamination of food by foodborne bacterial pathogens. *J. Sci. Food Agric.* 93, 3137–3146. doi: 10.1002/jsfa.6222

Sullivan, M. B., Coleman, M. L., Quinlivan, V., Rosenkrantz, J. E., DeFrancesco, A. S., Tan, J., et al. (2008). Portal protein diversity and phage ecology. *Environ. Microbiol.* 10, 2810–2823. doi: 10.1111/j.1462-2920.2008.01702.x

Sun, M., Ye, M., Zhang, Z., Zhang, S., Zhao, Y., Deng, S., et al. (2019). Biochar combined with polyvalent phage therapy to mitigate antibiotic resistance pathogenic bacteria vertical transfer risk in an undisturbed soil column system. *J. Hazard. Mater.* 365, 1–8. doi: 10.1016/j.jhazmat.2018.10.093

Sunderland, K. S., Yang, M., and Mao, C. (2017). Phage-enabled nanomedicine: from probes to therapeutics in precision medicine. *Angew. Chem. Intern. Edn.* 56, 1964–1992. doi: 10.1002/anie.201606181

Suttle, C. A. (2005). Virus in the sea. *Nature* 437, 356–361. doi: 10.1038/nature04160

Tetz, G., Brown, S. M., Hao, Y., and Tetz, V. (2018). Parkinson's disease and bacteriophages as its overlooked contributors. *Sci. Rep.* 8:45. doi: 10.1038/s41598-018-29173-4

Tokman, J. I., Kent, D. J., Wiedmann, M., and Denes, T. (2016). Temperature significantly affects the plaquing and adsorption efficiencies of listeria phages. *Front. Microbiol.* 7:631. doi: 10.3389/fmicb.2016.00631

Tomat, D., Migliore, L., Aquili, V., Quiberoni, A., and Balagué, C. (2013). Phage biocontrol of enteropathogenic and shiga toxin-producing *Escherichia coli* in meat products. *Front. Cell Infect. Microbiol.* 3:20. doi: 10.3389/fcimb.2013.00020

Tree, J. J., Granneman, S., Mcateer, S. P., Tollervey, D., and Gally, D. L. (2014). Identification of bacteriophage-encoded anti-sRNAs in pathogenic *Escherichia coli*. *Mol. Cell* 55, 199–213. doi: 10.1016/j.molcel.2014.05.006

Twort, F. W. (1915). An investigation of the nature of ultra-microscopic viruses. *Lancet* 186, 1241–1243. doi: 10.1016/S0140-6736(01)20383-3

Vandamme, E. J., and Mortelmans, K. (2019). A century of bacteriophage research and applications: impacts on biotechnology, health, ecology and the economy. *J. Chem. Technol. Biotechnol.* 94, 323–342. doi: 10.1002/jctb.5810

Vikram, A., Woolston, J., and Sulakvelidze, A. (2020). Phage biocontrol applications in food production and processing. *Curr. Issues Mol. Biol.* 40, 267–302. doi: 10.21775/9781913652517.08

Vongkamjan, K., Roof, S., Stasiewicz, M. J., and Wiedmann, M. (2013). Persistent *Listeria monocytogenes* subtypes isolated from a smoked fish processing facility included both phage susceptible and resistant isolates. *Food Microbiol.* 35, 38–48. doi: 10.1016/j.fm.2013.02.012

Walker, S. J., Archer, P., and Banks, J. G. (1990). Growth of *Listeria monocytogenes* at refrigeration temperatures. *J. Appl. Bacteriol.* 68, 157–162. doi: 10.1111/j.1365-2672.1990.tb02561.x

Whichard, J. M., Sriranganathan, N., and Pierson, F. W. (2003). Suppression of *Salmonella* growth by wild-type and large-plaque variants of bacteriophage felix O1 in liquid. *J. Food Prot.* 66, 220–225. doi: 10.4315/0362-028X-66.2.220

Wójcik, E. A., Stańczyk, M., Wojtasik, A., Kowalska, J. D., Nowakowska, M., Łukasiak, M., et al. (2020). Comprehensive evaluation of the safety and efficacy of BAFASAL® bacteriophage preparation for the reduction of *Salmonella* in the food chain. *Viruses* 12:742. doi: 10.3390/v12070742

World Health Organization [WHO] (2015). WHO Estimates of the Global Burden of Foodborne Diseases: Foodborne Diseases Burden Epidemiology Reference Group 2007-2015. Available online at: https://www.who.int/publications/i/item/9789241565165 (accessed October, 2021).

World Health Organization [WHO] (2017). *Diarrhoeal Disease*. Available online at: https://www.who.int/en/news-room/fact-sheets/detail/diarrhoeal-disease (accessed October, 2021).

Yahya, M., Hmaied, F., Jebri, S., Jofre, J., and Hamdi, M. (2015). Bacteriophages as indicators of human and animal faecal contamination in raw and treated wastewaters from Tunisia. *J. Appl. Microbiol.* 118, 1217–1225. doi: 10.1111/jam.12774

Ye, M., Sun, M., Huang, D., Zhang, Z., Zhang, H., Zhang, S., et al. (2019). A review of bacteriophage therapy for pathogenic bacteria inactivation in the soil environment. *Environ. Intern.* 129, 488–496. doi: 10.1016/j.envint.2019.05.062

Yeh, Y., Purushothaman, P., Gupta, N., Ragnone, M., Verma, S. C., de Mello, A. S., et al. (2017). Bacteriophage application on red meats and poultry: effects on *Salmonella* population in final ground products. *Meat Sci.* 127, 30–34. doi: 10.1016/j.meatsci.2017.01.001

Zaczek, M., Weber-Dąbrowska, B., and Górski, A. (2014). Phages in the global fruit and vegetable industry. *J. Appl. Microbiol.* 118, 537–556. doi: 10.1111/jam.12700

Zaczek-Moczydłowska, M. A., Young, G. K., Trudgett, J., Plahe, C., Fleming, C. C., Campbell, K., et al. (2020). Phage cocktail containing Podoviridae and Myoviridae bacteriophages inhibits the growth of *Pectobacterium* spp. under in vitro and in vivo conditions. *PLoS One* 15:230842. doi: 10.1371/journal.pone.0230842

Zeineldin, M., Aldridge, B., and Lowe, J. (2018). Dysbiosis of the fecal microbiota in feedlot cattle with hemorrhagic diarrhea. *Microb. Pathog.* 115, 123–130. doi: 10.1016/j.micpath.2017.12.059

Zhang, H., Li, X., Yang, Q., Sun, L., Yang, X., Zhou, M., et al. (2017). Plant growth, antibiotic uptake, and prevalence of antibiotic resistance in an endophytic system of pakchoi under antibiotic exposure. *Intern. J. Environ. Res. Public Health* 14:1336. doi: 10.3390/ijerph14111336

Zhang, H., Wang, R., and Bao, H. (2013). Phage inactivation of foodborne *Shigella* on ready-to-eat spiced chicken. *Poult. Sci.* 92, 211–217. doi: 10.3382/ps.2011-02037

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

REVIEW
published: 31 January 2022
doi: 10.3389/fmicb.2021.811788

Occurrence, Diversity, and Character of *Bacillaceae* in the Solid Fermentation Process of Strong Aromatic Liquors

Wenhua Tong[1,2,3], Ping He[1], Ying Yang[1], Zongwei Qiao[3], Dan Huang[1], Huibo Luo[1] and Xinjun Feng[2]**

[1] College of Bioengineering, Sichuan University of Science and Engineering, Yibin, China, [2] CAS Key Laboratory of Biobased Materials, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences (CAS), Qingdao, China, [3] Wuliangye Yibin Co. Ltd., Yibin, China

Strong aromatic liquors, also known as strong aromatic *Baijiu* (SAB) in China, are manufactured by solid fermentation, with a multi-microbe mixing and cooperative fermentation process that uses *Daqu* as a brewing starter. *Bacillaceae* have a specific action in food fermentation, such as soybean and wine, and more recent studies have found *Bacillaceae* play important roles in the SAB making industry. This review describes the diversity, functionality, and influence of *Bacillaceae* in *Daqu*, pit mud, *Zaopei*, *Huangshui* within making processes of SAB. Furthermore, aromatic flavor components from the *Bacillaceae* metabolism of SAB are discussed in this review. Ultimately, the resulting improvements and deeper understanding will benefit practical efforts to apply representatives of *Bacillaceae* in improving the quality of SAB as well as biological control of the micro-ecological environment of brewing.

Keywords: strong aromatic *Baijiu*, *Bacillaceae*, diversity, function, flavor

OPEN ACCESS

Edited by:
Laurent Dufossé,
Université de la Réunion, France

Reviewed by:
Zhuolin Yi,
Chengdu Institute of Biology, Chinese Academy of Sciences (CAS), China
Chongde Wu,
Sichuan University, China

****Correspondence:***
Wenhua Tong
tongwh@suse.edu.cn
Xinjun Feng
fengxj@qibebt.ac.cn

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *09 November 2021*
Accepted: *07 December 2021*
Published: *31 January 2022*

Citation:
Tong W, He P, Yang Y, Qiao Z, Huang D, Luo H and Feng X (2022) Occurrence, Diversity, and Character of Bacillaceae in the Solid Fermentation Process of Strong Aromatic Liquors. Front. Microbiol. 12:811788. doi: 10.3389/fmicb.2021.811788

INTRODUCTION

Baijiu is a traditional distilling liquor made by solid fermentation that has been made for generations, stretching back over a 100 years. According to the different flavors, *Baijiu* can be divided into three most basic types (strong aromatic-flavor, sauce-flavor, light flavor) and nine derived types from above the three flavors (rice-flavor, jian-flavor, fuyu-flavor, te-flavor, feng-flavor, dong-flavor, chi-flavor, sesame-flavor, laobaigan-flavor) (Zheng and Han, 2016). SAB is world famous for its special flavor, occupying about 70% *Baijiu* market in China, Wuliangye is the representative brand of SAB, and the annual production of SAB exceeded 10 million tons (Liu et al., 2017b; Xu et al., 2017). SAB is mainly made from multiple grains (sorghum, corn, rice, wheat, glutinous rice, and so on), with *Daqu* as a primary saccharification starter, and a solid state fermentation process was carried out in a fermented mud pit. Ultimately, alcohol and various aromatic materials are obtained by distilling (**Figure 1**). The production of SAB was carried out in 60 days or so, and complex metabolic reactions were detected during the long-term SAB fermentation (Hu et al., 2021b). Subsequently, the physical and chemical characteristics of *Zaopei* (mixture of steamed grains, rice husks, fermented grains, etc.) changed constantly, as a result,

abundant compounds were produced (Guan et al., 2020), which were closely associated with the style of SAB. Owing to the natural fermentation style, numerous microbes are involved in the SAB making process (bacteria and fungi) (Ma et al., 2016; Wang et al., 2017; Zhang et al., 2017; Liu et al., 2018b; Qian et al., 2021), which mainly originated from *Daqu*, pit mud, the environment of the distillery and so on. The final SAB liquor structure is produced by the coaction of these microbes (Hu X. et al., 2016). Until now, studies on the microbes of SAB brewing focused on the microbial community structure and relevant flavor substances. Zou et al. (2018) discussed the diversity and function of the microbial community in SAB at the macro level. Several fungi and bacteria were are discussed in this work, however, a detailed introduction of the role for specific species of microbes throughout the SAB ecosystem is lacking, which is vital for brewing quality control. As important food-associated microorganisms, especially in SAB production, the function and roles of *Bacillaceae* were elaborated in the major fermentation phase, thus research in this field will benefit from SAB production by bioaugmentation (or other regulation strategies) of *Bacillaceae* in *Daqu*, pit mud, *Zaopei,* and *Haungshui* (a brown liquid byproduct was formed during SAB brewing, and deposited in the pit bottom) (Ding et al., 2015).

Bacillaceae is a gram-positive bacteria that plays an important role in the SAB making industry (Zheng et al., 2011; Ding et al., 2014; Wang et al., 2014; He et al., 2019b). Most of the *Bacillaceae* isolated from *Daqu*, pit mud, *Zaopei,* and *Haungshui* can produce various aromatic flavor components or flavor precursor, such as acetoin, 2,3-butanediol, four-carbon compound, pyrazines, and so on (Liu et al., 2017c; Zhang et al., 2021a). This feature could also be observed in the making process of various fermented foods. It is well known that the principal fragrance component is ethyl caproate in SAB which is associated with caproic acid bacteria (Luo et al., 2020; Cheng T. et al., 2021). Caproic acid bacteria is a group of microorganisms that can metabolize caproic acid, which mainly consists of *Bacillus megaterium*, *Bacillus fusiformis*, *Bacillus licheniformis,* and *Clostridium*, etc. (Zhao et al., 2012; Hu et al., 2015). Wang et al. (2015b) identified eight *Bacillus* from *Daqu* by employing the traditional method. The results showed that they belonged to five specials, *Bacillus megaterium*, *Paenibacillus macerans*, *Bacillus pumilus*, *Bacillus atrophaeus,* and *Bacillus licheniformis*, respectively. Liu et al. (2017c) identified three dominant *Bacillus* from a century mud pit of Luzhou Laojiao Liquor distillery, which showed *Lysinibacillus sphaerieus*, *Brevibacillus brevis,* and *Paenibacillus larvae subsup. Pulvifaciens*. The distribution *of Bacillaceae* have significant differences among *Daqu*, pit mud, *Zaopei,* and *Huangshui* in different geographical environments and seasons. A previous study suggested that the temperature variation and humidity may be important factors affecting the microbe community (Fu et al., 2011; Yin et al., 2014), as there are differences in these factors in various SAB distilleries (**Table 1**).

As the dominant microorganism in the SAB making process, the review described the diversity of *Bacillaceae,* the function of *Bacillaceae* in *Daqu,* pit mud, *Zaopei,* and *Huangshui*. The effect on the flavor of SAB that contributes to *Bacillaceae* is also discussed. Through the analysis of this article, we hope to provide ideas for exploring the functional microorganisms in the SAB making industry, and accelerate a deeper understanding of the brewing mechanism of SAB, helping to improve the quality of SAB, and facilitating the better application of *Bacillaceae* in the food fermentation industry.

FUNCTION OF *Bacillaceae* IN SAB BREWING PROCESS

Bacillaceae in *Daqu*

The volatile flavor characteristics of SAB are influenced by the microflora of raw materials and the environment. In the production of SAB, pit mud is the foundation of fermentation (Zheng et al., 2013; Zheng and Han, 2016), *Daqu* is the motive power of fermentation (Gou et al., 2015; Deng et al., 2020), and brewing technology is the core of fermentation. Numerous studies have shown that *Bacillus* contributes greatly to the flavor of SAB in *Daqu*, the secondary metabolites of *Bacillus* could interact with other microorganisms, and benefited from maintaining relative stability of the microecosystem of *Daqu* (He et al., 2019a; He G. et al., 2020). *Daqu*, as the saccharification starter of SAB, includes lots of microbes and enzymes, and *Bacillaceae* play a key role in the production of *Daqu* (Li et al., 2014; Hu et al., 2020; Xiang et al., 2020). For example, as the functional microbes in *Daqu*, *Bacillus licheniformis* could produce multiple enzymes, such as amylase, proteases, and lipases in *Daqu* (Yan et al., 2013b), besides, *Bacillus licheniformis* could also reduce the content of higher alcohols in SAB (too high to spoilt SAB taste, easy to cause headaches, easy to drunk) (Shi et al., 2012). *Bacillus* spp. could hydrolyze proteins in fermented foodstuffs, which benefits the flavor and flavor precursor formation in *Daqu* (Beaumont, 2002). Meanwhile, *Bacillus* spp. can also exhibit the capacity of inhibiting the growth of *Streptomyces,* and reduce geosmin to avoid soil odor in *Daqu* (Zhi et al., 2016). Zhai et al. (2020) analyzed. *Daqu* samples in the main product area of SAB (Yibin city) by high throughput sequencing, the result showed that microorganisms consist of *Bacillus* sp., lactic acid bacteria, *Pedicoccus sp.*, *Weissella* sp., *Leuconostoc* sp., *Thermoactinomyces* sp. and *Acetobacter* sp.). Research has shown that *Bacillus subtilis*, *Bacillus horneckiae, Bacillus megaterium*, *Bacillus licheniformis,* and *Brevibacillus* make up common species of *Bacillaceae* in *Daqu* (Zhou et al., 2010; Xiang et al., 2020).

The functions of *Bacillaceae* within *Daqu* in SAB brewing were as follows: firstly, various hydrolases were secreted into *Daqu*, and the utilization ratio of raw material was elevated (Wang et al., 2017; Wu et al., 2020); secondly, flavor substances were produced, for example, *Bacillus* spp. could produce abundant organic acids in *Daqu* (e.g., malic, lactic, acetic, citric, succinic, propionic and butyric acids), and the organic acids were the main flavoring components of SAB and the precursors of ester production (Yan et al., 2013a), meanwhile, the study also found that *Bacillus* spp. could produce aromatic substances in *Daqu* (benzaldehyde, 4-methylphenol, benzeneethanol, ethyl phenylacetate, etc.) (He et al., 2019b). Thirdly, the study revealed ingredients that are good for health can be enriched in *Daqu*, for example, *Bacillus licheniformis* directly proliferated in *Daqu*,

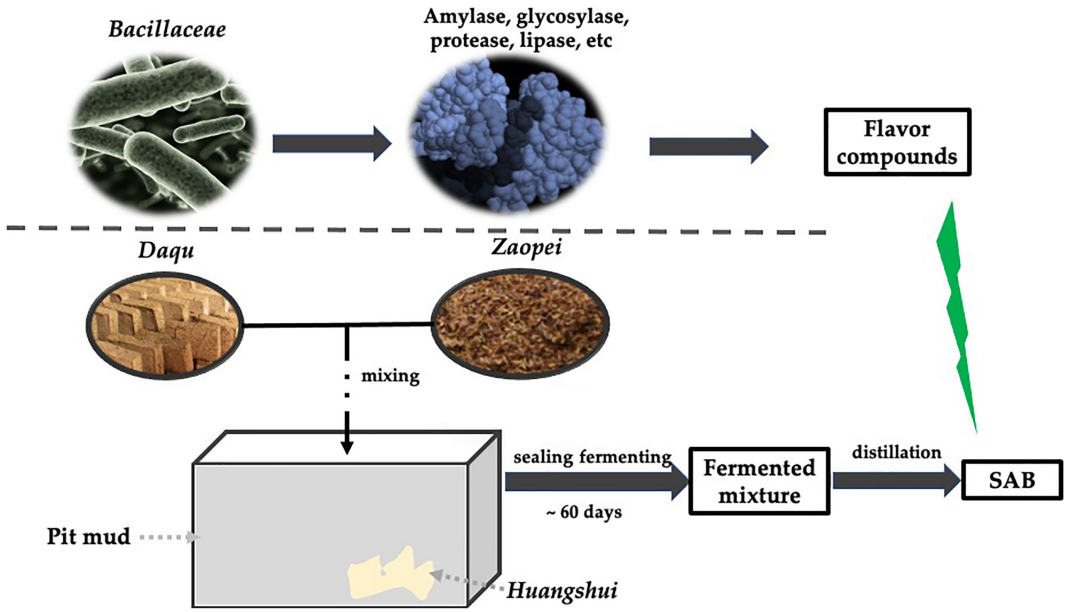


FIGURE 1 | *Bacillaceae* associated with the process of SAB brewing.

TABLE 1 | Difference distribution of *Bacillaceae* in SAB distilleries producing *Daqu*.

Sample	Places	Representative strains	References
Daqu of "Luzhou Laojiao"	Luzhou City, Sichuan Province	*Bacillus subtilis*, *Bacillus licheniformis*	Yao et al., 2005
Daqu of "Wuliangye"	Yibin City, Sichuan Province	*Bacillus subtilis*, *Bacillus cereus*	Zhao et al., 2009, Liang et al., 2017, Fan et al., 2021
Daqu of "Gujingong"	Haozhou City, Anhui Province	*Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens, Paenibacillus Castaneae, Bacillus pumilus, Bacillus subtilis SS Subtilis, Bacillus circosus, Bacillus cereus,* etc.	Liang et al., 2017
Daqu of "Yingjia Gongjiu"	Liuan city, Anhui Province	*Bacillus pumilus, Bacillus amyloliquefaciens, Bacillus licheniformis, Paenibacillus nicotianae*	Wang et al., 2021
pit mud of "Luzhou Laojiao"	Luzhou City, Sichuan Province	*Lysinibacillus sphaerieus, Brevibacillus brevis, Paenibacillus larvae subsup. pulvifaciens*	Liu et al., 2017c
pit mud of "Yanghe Daqu"	Suqian City, Jiangsu Province	*Clostridium Kluyverii, Ruminiclostridium (Clostridium tyrobutyricum, Clostridium butyricum)*	Wang et al., 2015a, Gou et al., 2020
pit mud of "Gujingong"	Haozhou City, Anhui Province	*Bacillus licheniformis, Bacillus coagulans, Bacillus amyloliquefaciens, Bacillus atrophus, Bacillus mohighi, Bacillus pumilus*	Wu et al., 2016
pit mud of "Zhijiang"	Zhijiang City, Hubei Province	*Bacillus, Sporolactobacillus*	You et al., 2009
pit mud of "Daohuaxiang"	Yichang City, Hubei Province	*Clostridium*	Wang et al., 2010
pit mud of "Jiannanchun"	Mianzhu City, Sichuan Province	*Bacillus, Clostridium* (*Clostridium sporogense, Clostridium aminophilum, Clostridium cochlearum, Clostridium innocuum*)	Liu et al., 2017c, Xu Z. et al., 2019
Zaopei of "Xufu"	Yibin City, Sichuan Province	*Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus drentensis, Bacillus stratosphericus, Bacillus anthracis, Bacillus safensis, Bacillus cereus, Bacillus vallismortis, Lysinibacillus fusiformis, Rummeliibacillus pycnu, Brevibacillus borstelensis*	Zhou et al., 2010
Huangshui of "Shuijingfang"	Chengdu city, Sichuan Province	Clostridia *(such as Clostridium_sensu_stricto, norank_f_Clostridiaceae),* Bacilli	Gao Z. et al., 2020

which could significantly increase the content of pyrazines, terpene, and zearin, etc. (Zhang R. et al., 2013; Wang et al., 2015b; Zhou et al., 2016), substances that are good for human health (i.e., anti-cancer, anti-virus, anti-inflammatory, anti-oxidation). Recently, two strains of high yield of fibrinolysin (function as anti-thrombus) were also identified from *Daqu* (*Bacillus velezensis, Bacillus licheniformis*) (Zhang et al., 2021b). Fourthly, promoting the distillation of flavor components from *Zaopei,* and reducing the harmful components of microbial metabolite, *Bacillus licheniformis* could produce lichenin to enhance the volatilization of esters and other substances, and markedly decrease the volatilization of phenol and phenylethanol (Wang et al., 2015b).

Bacillaceae in Pit Mud

The SAB fermentation process was carried out in a pit cellar, in which mud functioned as a fermentation carrier of SAB. Pit mud contains a variety of important microorganisms (Tao et al., 2014; Liang et al., 2015; Hu X. et al., 2016; Liu et al., 2017a), and the special pit aroma in SAB is mainly derived from pit mud microbes (Zhang et al., 2017). The bacteria in pit mud consist of *Bacillus, Clostridium, Pseudomonas, Sporolactobacillus, Lactobacillus,* and *actinomycetes*, etc. (Yue et al., 2007; Zou et al., 2018; Liu M. et al., 2020; Liang et al., 2021). PCR-DGGE analysis revealed that eight families of bacteria species existed in pit mud, e.g., *Clostridiaceae*, *Lactobacillaceae*, *Synergistaceae, Sphingomonadaceae, Ruminococcaceae, Clostridiales*_Incertae Sedis XI*, Lanchnospiraceae,* and *Plannococcaceae*. They belong to three main microorganisms of *Clostridiales, Lacotobacillaceae,* and *Bacillale,* separately (Zheng et al., 2013). Fifteen *Clostridium* species and one *Bacillus* were isolated from one of the best-known brands of SAB pit mud (Luzhou Lao Jiao liquor), all of them had the ability to produce organic acid (Guo et al., 2020). Recently, more bacteria of the *Bacillaceae* species were isolated from pit mud (e.g., *Aneurinibacillus migulanus*, *Bacillus pumilus*, *Lysinibacillus boronitolerans*, *Bacillus badius*, *Bacillus coagulans, Bacillus aerius, Bacillus subtilis, Bacillus licheniformis, Lysinibacillus sphaericus, Bacillus pulvifaciens, Bacillus pumilus*, *Virgibacillus pantothenticus, Sporolactobacillus, Brevibacillus* sp.*, Bacillus sphaericus, Bacillus niabensis, Bacillus bataviensis, Bacillus cereus*) (Zhang et al., 2010, 2019; Liu et al., 2013, 2017c, 2018c; Ye et al., 2013; Wang et al., 2018). The culturable bacteria's from pit mud were *Bacillus* species, and the predominant strains were *Bacillus licheniformis* and *Bacillus subtilis* (Zhang et al., 2010). Most of the *Bacillaceae* isolated from pit mud can produce acetoin, 2,3-butanediol, C4 compounds, pyrazines, volatile acid, etc., all the compounds listed above are important flavors, and are crucial to regulating the SAB flavor (Wu et al., 2019; **Table 2**).

Bacillaceae is the most abundant genus in pit mud, which plays a key role in SAB production. It is involved in the formation of major flavors in SAB, for example, ethyl caproate, ethyl butyrate, and caproic acid, which have been correlated positively with *Clostridium*, *Rummeliibacillus* in pit mud (Liu M. et al., 2020). Amylase, glycosylase and debranched enzyme could be generated by *Bacillus amylolitica*, and butyric acid, acetoin, volatile acid could be synthesized by *Clostridium* and *Bacillus* in pit mud (Liu et al., 2015; Wu et al., 2019). *Bacillaceae* can be employed as an indicator of pit mud quality: when the total number of *Bacillaceaes* in the new pit mud is at a high level, with the improvement of pit mud quality, the relative abundance of *Clostridium kluyver* and other *Clostridiums* increased significantly (Hu X. et al., 2016). The relative abundance of *Bacillus* decreased inversely in aged pit mud (Yu and Liu, 2016). By depressing the reproduction of harmful bacterial, one study revealed that *Clostridium butyricum* produced a variety of enzymes, which could decompose polysaccharides into oligosaccharides, and produce multiple secondary metabolites, such as antibacterial peptide, butyric acid, acetic acid, and vitamins, and that they could inhibit the propagation of harmful bacteria (Xu P. et al., 2019).

Bacillaceae in pit mud not only metabolizes caproic acid, butyric acid, acetic acid and other important aromatic components of SAB, but also could metabolize some secondary metabolites, which interact and regulate the related microorganisms in pit mud, thus affecting the aroma components of SAB.

Bacillaceae in Zaopei

Research has shown that there are various *Bacillaceae* in *Zaopei* (Yao et al., 2010; Wang et al., 2011; Hu et al., 2021a). 16S rDNA analysis found multiple microbe species in *Zaopei*, *Bacillus,* and *lactobacillus* (Liu F. et al., 2020; Hu et al., 2021a; Li and Qiu, 2021). The distribution species of microbes in *Zaopei* may be related to the specific environment in a mud pit, such as sealed hypoxia, low pH, high content of ethanol, so the growth of *Bacillaceae* is suited to this specific micro-ecological environment. As fermentation progresses, the relative abundance of *Bacillaceae* increases (Chen et al., 2010). Zhang et al. (2010) investigated bacteria flora from *Zaopei* and pit mud in Guizhou province, and 477 strains were isolated, *Bacillus Licheniformis* was identified as the dominant group of *Bacillus,* accounting for 32.96% of the total number of *Bacillus*, meanwhile, a small number of uncertain strains of *Brevi Bacillus* were isolated from *Zaopei*.

Bacillaceae are essential for *Zaopei*. They function by firstly secreting multiple hydrolases in the early fermentation phase, characteristics that ensure full utilization of *Daqu* and *Zaopei* (Li and Qiu, 2021). For example, organic matter such as starch, protein, and purine can be decomposed by *Clostridium* (Fan et al., 2007; Hahnke et al., 2014; Zhang et al., 2014; Yang and Chen, 2021). As they are involved in the formation of flavor matter in SAL, *Bacillus* and *Clostridium* are responsible for the production of ethyl hexanoate, caproic acid, butyric acid, lactic acid, benzaldehyde, alcohols, fatty acids, phenol, and other compounds (Hu et al., 2015; Liu et al., 2018a; Chai et al., 2019; **Table 2**). In summary, *Bacillaceae* of *Zaopei* was very similar to pit mud, because they were in the same container during the braving process of SAB.

Bacillaceae in Huangshui

Huangshui was the main byproduct in SAB production, and presents a dark brown viscous liquid form that seeps into the bottom of the pit cellar during the fermentation process

TABLE 2 | Main flavors produced by identified SAB *Bacillaceae* in various fermentation processes.

Bacillaceae	Substrate	Category	Test method	References
Bacillus subtilis	Soymilk/sorghum/ corn fermentation	**Hydrocarbons** (nonane, 5-methyl-1-heptene, 2,6,10-trimethyldodecane, 1,3-dimethylnaphthalene, 2,3-dimethylnaphthalene); **acids** (acetic acid, 2-methyl-propanoic acid, propanoic acid, butanoic acid, 3-mehtyl butanoic acid, 2-methyl butanoic acid, glyoxylic acid, tryptophan. lysine, leucine, isoleucine, aspartate, phenylalanine, valine, histidine, methionine, alanine, tyrosine, glutamate, glycine, taurine, γ-aminobutyrate, 6-phosphogluconic acid, lactate, succinate, pyruvate, fumarate, malonate, citrate, isobutyrate, isovalerate, 2-methylbutyrate, 2-hydroxyisobutyrate, 3-hydroxybutyrate, β-hydroxyphenylacetate, α-ketoisovalerate); **alcohols** (ethanol, isopropyl alcohol, butanol, pentanol, 2,3-butanediol, 1,3-butanediol, 2-butanol, β-phenylethanol, n-butanol, cedrol, phenylethyl alcohol, isooctanol, 2-(1-methoxyethoxy)ethanol, 1,2-propanediol, methanol, isopropanol); **aldoketones** (butanedione (diacetyl), 2,3-butanedione, 3-hydroxy-2-butanone, 5-hydroxy-4-octanone, benzeneacetaldehyde, acetoin, 2-heptanone, 2-non-anone); **esters** (butanoic acid butyl ester, 3-methyl butanoic acid butyl ester, 2-methyl-2-hydroxy-propanoic acid ethyl ester, vinyl acetate, diethyl phthalate, 1,2-phthalic acid ester, isopentyl nitrite, 2-isohexyl sulfurous essien ester, butyl-2-ethylhexyl 1,2-phthalate, DL-alanine ethyl ester, ethyl caproate, ethyl phenylacetate, 1-hydroxy-1-cyclopropanecarboxylic acid ester); **ethers** (3-tert-butyl-4-hydroxyanisole); **heterocycles** (2,5-dimethyl pyrazine, 5-methyl-2-furanmethanol, 5-methyl-2-furancarboxaldehyde, 2,3,5-trimethyl pyrazine, 2,3,5,6-tetramethylpyrazine, benzothiazole, 2,2′,5,5′-tetramethylbiphenyl, 1,1 ′- (1-butenyl) biphenyl, 3,4-diethyl-1,1 ′- biphenyl, pterin, trigonelline); **nitrogen-containing compounds** (2-formamide (2-aminoethyl) - *N*-methoxyaziridine, L-alanine acetamide, trimethylamine *N*-oxide, methylamine, histamine, choline); **phenolic compounds** (phenol, guaiacol, 2-methoxy-4-vinylphenol, 3,5-diisopropylphenol, genistein); **Miscellaneous** (α-glucose, β-glucose, glucose-1-phosphate, soybean lecithin)	Nuclear magnetic resonance (^{1}H NMR); headspace-solid phase microextraction-GC-MS (HS-SPME-GC-MS)	Yang et al., 2012, Lin et al., 2013a, Liu et al., 2018c, Gao Y. X. et al., 2020
Bacillus pumilus	Chicory roots fermentation	**Hydrocarbons** (hexadecane, 3,7-dimethylnonane, tetradecane, 2,2-dimethyl butane, 2,4-ditertbutyl-1,3-pentadiene, 1,4-cyclohexane); **Acids** (caproic acid, n-caprylic acid, palmitic acid, stearic acid, linoleic acid); **alcohols** (benzyl alcohol, phenethyl alcohol, phenethyl alcohol, isooctanol); **aldoketones** (phenylacetaldehyde, nonanal, decanal); **esters** (methyl palmitate, 2,6-bis (1,1-dimethyl ethyl) - 4-methylaminophenol formate, diethyl phthalate, dibutyl phthalate); **heterocycles** (trimethyl pyrazine, 2-pyrrolecarbaldehyde, γ-Hexalactone, 2-acetylpyrrole, 2-ethyl-3,5-dimethyl pyrazine, 5-methyl-1H-pyrrole-2-carbaldehyde, indole, eugenol, vanillin, 2,2′,5,5′-tetramethylbiphenyl, 3,4′-diethylbiphenyl, naphthofurans); **phenolic compounds** (o-methoxy-phenol, 4-vinylguaiacol); **nitrogen-containing compounds** (2-formamide (2-aminoethyl) - n-methoxyaziridine)	Simultaneous distillation extraction -gas chromatography-mass spectrometry (SDE/GC-MS); HS-SPME-GC-MS	Yang et al., 2012, Yang P. et al., 2019
Bacillus amyloliquefaciens	Soybean/wheat fermentation	**Hydrocarbons** (1-octene); **acids** (2-methylpropanoic acid, 3-methylbutanoic acid, 4-methylpentanoic acid, acetic acid, butanoic acid, 3-nitropropanoic acid, 3-methyl-butanoic acid, pentanoic acid, hexanoic acid, octanoic acid, nonanoic acid); **alcohols** (pent-1-en-3-ol, 3-methylbutan-1-ol, 2-phenylethanol, 1-octen-3-ol, 2,3-butanediol, 2-pentadecanol, 2-octanol, 2,3,4-trimethyl-3-pentanol, furfuryl alcohol, benzyl alcohol, phenylethyl alcohol, ethanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 1-hexanol); **aldoketones** [(*E*)-oct-2-enal, benzaldehyde, acetaldehyde, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, butane-2,3-dione, 4-ethenylphenol, hexanal, 2-phenylacetaldehyde, heptan-2-one, octan-2-one, nonan-2-one, 2-furancarboxaldehyde, benzaldehyde, benzacetaldehyde, 2-methyl-benzaldehyde, (*E*)-11-hexadecenal, acetone, 2-propanone, 2,3-butanedione, acetoin, 1-phenyl-ethanone, dihydro-5-pentyl-2(3H)-furanone, 5-hexyldihydro-2(3H)-furanone];		

(Continued)

TABLE 2 | (Continued)

Bacillaceae	Substrate	Category	Test method	References
		esters (ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, 3-methylbutyl acetate, methyl 4-methylpentanoate, ethyl hexanoate, ethyl acetate, butyl heptadecyl sulfuroate, 2-methyl-hexyl propanoate, 2-methyl-2-methylpropyl propanoate, 2-octyl benzoate, ethyl hexadecanoate, methyl 9,12-octadecadienoate, ethyl (9Z,12Z)-9,12-octadecadienoate); **heterocycles** (2-butylfuran, 2-pentylfuran, 2-ethylfuran, maltol, 2,5-dimethylpyrazine, trimethyl pyrazine, 2,3,5,7-tetramethyl pyrazine, 2-pentyl-furan, 2,3-dihydro-benzofuran); **phenolic compounds** (phenol, 2-methyl-phenol, 2-methoxy-phenol, 4-ethyl-2-methoxy-phenol, 2-methyl-4-vinylphenol, 2-methoxy-4-vinylphenol, 2,6-dimethoxy-phenol, 4-vinylphenol); **sulfur compounds** (methanesulfonic anhydride, 1-docosanethiol, *N*-ethyl-hydrazinecarbothioamide); miscellaneous (1,2-dimethoxy-benzene, 6-methyl-2-phenylindole)	HS-SPME-GC-MS	Hong et al., 2012, Seo et al., 2018
Bacillus atrophaeus	sorghum fermentation	**alcohols** (2,3-butanediol); **aldoketones** (acetoin, butane-2,3-dione, 2-heptanone); **phenolic compounds** (guaiacol)	GC-MS	Liu et al., 2018c
Bacillus atrophaeus	LB broth medium	**Hydrocarbons** (1-tetradecane, hexadecane, 1-octadecene, octadecane, 1-non-adecene, docosane, heptadecane, tetrapentacontane, eicosane); **acids** (hexadecanoic acid, phthalic acid, chloroacetic acid, *cis*-3-octyloxiraneoctanoic acid, hexanedioic acid, propanoic acid); **alcohols** (1-tetradecanol, 1-hexadecanol, cyclobutanol, erythritol, isophytol, L-alaninol); **aldoketones** (*O*-anisaldehyde, 3-buten-2-one); **esters** (methyl stearate); **heterocycles** (decamethyl-ccyclopentasiloxane, dodecamethyl-cyclohexasiloxane, hexadecamethyl-cyclooctasiloxane, octadecamethyl-cyclononasiloxane, 2-methylaminomethyl-1,3-dioxolane); **nitrogen-containing compounds** (dimethylamine, 2-octanamine, 2-hexanamine, propenamide, benzamide, *N,N*-dimethyl-methanesulfonamide, 2,3-dimethoxybenzamide, dimethyl methanesulfonamide); **phenolic compounds** (phenol); **Miscellaneous** (cyclopropyl carbinol, silane)	GC-MS	Zhang X. et al., 2013, Rajaofera et al., 2019
Bacillus fusiformis				-
Bacillus badius				-
Bacillus bataviensis	Lycium chinense Miller (Goji) fermentation	**Hydrocarbons** [(*Z*)-3-ethyl-2-methyl-1, 3-hexadiene, (*E, E*)-2, 6-Non-adienal, naphthalene]; **acids** (formic acid, acetic acid, hexanoic acid, octanoic acid, n-hexadecanoic acid); **alcohols** (1-hexanol, 1-octen-3-ol, benzyl alcohol, 3, 7-dimethyl-1, 6-octadien-3-ol, phenylethyl alcohol, (*E*) -2-nonen-1-ol, geraniol); **aldoketones** (hexanal, (*E*) -2-heptenal, benzaldehyde, (*E, E*) -2, 4-heptadienal, benzeneacetaldehyde, (*E*) -2-non-enal, 2, 4-dimethyl-benzaldehyde, 2, 6, 6-trimethyl-1, 3-cyclohexadiene-1-carboxaldehyde, acetoin, 6-methyl-5-hepten-2-one, 6-methyl-3,5-heptadiene-2-one, 2-n-hexylcyclopentanone, *(E)*-6, 10-dimethyl-5, 9-undecadien-2-one, *trans*-. beta. -Ionone, 5, 6, 7, 7a-tetrahydro-4, 4, 7a-trimethyl-2(4H)-benzofuranone); **ethers** (sulfurous acid-nonyl pentyl ester, Phthalic acid,isobutyl nonyl ester, hexadecanoic acid-methyl ester, hexadecanoic acid-ethyl ester); **heterocycles** (2-pentyl-furan); **nitrogen-containing compounds** (*N*, *N*-dibutyl-formamide); **phenolic compounds** (2-methoxy-phenol, 2, 3, 5-trimethyl-phenol, 2-methoxy-4-vinylphenol)	HS-SPME-GC-MS	Liu Y. et al., 2020
Bacillus brevis				
Bacillus circulans	Vinegar brewing mass fermentation	Six of high content amino acids were detected: arginine, alanine, glutamic acid, threonine, valine and leuconic acid; thirty-five volatile compounds were detected (data not shown), the high content compounds were as follows: **acids** (ethyl palmitate, iso-valerate, caprylic acid, hexanoic acid); **alcohols** (phenyl ethanol); **esters** (phenyl ethyl acetate, ethyl palmitate)	High performance liquid chromatography (HPLC)/HS-SPME-GC-MS	Wang et al., 2016

(Continued)

TABLE 2 | (Continued)

Bacillaceae	Substrate	Category	Test method	References
Bacillus cereus	Sorghums/corn fermentation	**Hydrocarbons** (3,7-dimethylnonane, 3-tetradecene, tetradecane, 6-methyl-1,3,5-cycloheptene, 1,3-dimethylnaphthalene, pentadecane, 1-pentadecyne); **acids** (isobutyric acid, acetic acid); **alcohols** (phenethyl alcohol, 2,3-butanediol, glycerol); **aldoketones** (acetoin furaldehyde); **esters** (DL-alanine ethyl ester, ethyl caproate, ethyl phenylacetate, 2-phenylethyl acetate, 1-hydroxy-1-cyclopropanecarboxylic acid ester); **heterocycles** (benzothiazole, 2-Methoxy-4-vinylphenol, 2′,5,5′-tetramethylbiphenyl, 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2,3,5,6-tetramethylpyrazine); **phenolic compounds** (guaiacol)	HS-SPME-GC-MS	Yang et al., 2012, Cheng G. et al., 2021
Bacillus coagulans				-
Bacillus endophyticus				-
Bacillus licheniformis	Sorghum/corn/ Lycium chinense Miller (Goji) fermentation	**Hydrocarbons** (n-hexadecane, 3,7-dimethylnonane, 3-tetradecane, 3,8-dimethylundecanone, nonadecane, (*Z*) -3-ethyl-2-methyl-1, 3-hexadiene, (*E, E*) -2, 6-non-adienal, naphthalene); **acids** (acetic acid, 2-methyl propanoic acid, propanoic acid, butanoic acid, 3-methyl butanoic acid, 2-methyl butanoic acid, 3-methyl-2-butenoic acid, 4-methyl-3-pentenoic acid, formic acid, hexanoic acid, octanoic acid, n-hexadecanoic acid); **alcohols** (ethanol, isopropyl alcohol, 2-methyl propanol, 3-methyl-butanol, butanol, pentanol, 1, 3-butanediol, isoborneol, borneol, α-terpineol, 2-butanol, β-phenylethanol, 2, 3-butanediol, cedrol, furfuryl alcohol, benzyl alcohol, 3, 7-dimethyl-1, 6-octadien-3-ol, phenylethyl alcohol, (*E*)-2-nonen-1-ol); **aldoketones** (2, 3-butanedione, 2-pentanone, 3-methy1-2-pentanone, acetoin, 4-hydroxy -2 -butanone, 2-heptanone, 5-hydroxy-4-octanone, benzeneace taldehyde, 2-pentanone, hexanal, (*E*)-2-heptenal, (*E, E*) -2, 4-heptadienal, benzaldehyde, benzeneacetaldehyde, (*E*)-2-non-enal, (*E, E*) -2, 4-decadienal, 6-methyl-5-hepten-2-one, 1-(1H-pyrrol-2-yl)-ethanone, 6-methyl-3, 5-heptadiene-2-one, (*E*)-6,10-dimethyl-5,9-undecadien-2-one, *trans*-.beta.-ionone, 5, 6, 7, 7a-tetrahydro-4, 4, 7a-trimethyl-2(4H)-benzofuranone); **esters** (2-methyl-2-hydroxy propanoic acid ethyl ester, butanoic acid butyl ester, 3-methyl butanoic acid butyl ester, vinyl acetate, dibutyl phthalate, 2-ethyl-isohexyl-sulfurous acid hexyl ester, ethyl caproate, 1-hydroxy-1-cyclopropanecarboxylic acid ester, benzoic acid 2-ethylhexyl ester, 6-ethyl-3-octyl butyl phthalate, *N*-methoxy-phenyl-oxime, phthalic acid-isobutyl nonyl ester, hexadecanoic acid-methyl ester, hexadecanoic acid-ethyl ester); **heterocycles** (2-methylpyridine, 2,3, 5-methyl pyrazine, 2, 5-dimethylpyrazine, 5-methyl-2-furanme thanol, 5-methyl-2-furancarboxaldehyde, 2, 3, 5, 6-tetramethyl pyrazine, benzothiazole, 2,2′,5,5′-tetramethylbiphenyl, 3,4-diethyl-1,1′-biphenyl, 2-pentyl-furan); **nitrogen-containing compounds** (L-alanine methylamide, L-alanine acetamide, 2-methyl-1-(2-nitrophenyl) -3 -(phenylmethoxy) benzene, *N*, *N*-dibutyl-formamide); **phenolic compound** (guaiacol, phenol, 2-methoxyphenol, 2, 3, 5-trimethyl-phenol, 2-methoxy-phenol)	HS-SPME-GC-MS	Yang et al., 2012, Lin et al., 2013b, Liu et al., 2018c, Liu Y. et al., 2020
Bacillus megaterium	Sorghum fermentation	**Alcohols (**isopentyl alcohol**); aldoketones** (acetoin); **phenolic compounds** (guaiacol)	GC-MS	Liu et al., 2018c
Bacillus niabensis				-
Bacillus pulvifaciens				-
Bacillus simplex				-
Bacillus sphaericus				-
Bacillus velezensis	Minced fish fermentation	**Hydrocarbons** (3,5,5-trimethyl-1-hexene); **acids** (3-methylbutanoic acid, 2-methylbutyric acid, caproic acid); **alcohols** (1-penten-3-ol, *cis*-2-penten-1-ol, 1-hexanol, 1-octen-3-ol, 2,7-octadienol, 1-nonen-3-ol, phenylethyl alcohol); **aldoketones** (isobutyraldehyde, isovaleraldehyde, 2-methylbutyraldehyde, (*E*) 2-methyl-2-butenal, caproaldehyde, 2-ethyl-2-butenal, *trans*-2-hexenal, 2-ethyl-2-hexenal, n-capryl(ic) aldehyde, benzaldehyde, (*E*)-4-oxohexyl-2-enoaldehyde, decanal, 4-ethylbenzaldehyde, 2-butanone, 3-pentanone, 5-methyl-2-hexanone, 3-hepten-2-one, 2,3-dimethyl-2-cyclopentene-1-one, (E, *E*)-3,5-octadiene-2-one, 2,3-dimethyl-2-cyclopentene-1-one); **esters** (hexyl formate,		

(Continued)

TABLE 2 | (Continued)

Bacillaceae	Substrate	Category	Test method	References
		γ-heptalactone); **heterocycles** (2-ethyl furan, *cis*-2-(2-pentenyl) furan, 2-methylpyridine, 2-ethylpyridine, 2-amino-4-methylpyridine); **nitrogen-containing compounds** (trimethylamine); **phenolic compounds** (4-ethylphenol)	HS-SPME-GC-MS	Yang H. et al., 2019
Bacillus velezensis	MOLP medium fermentation	**Hydrocarbons** (nonane, 8-methylheptadecane); **acids** (isovaleric acid); **alcohols** (1-butanol, isoamyl alcohol, 2-ethylhexanol, 2,3-butanediol, 1-phenylethanol); **aldoketones** (benzaldehyde, butane-2,3-dione, 2-heptanone, acetoin, 2-non-anone, 2-undecanone); **esters** (butyl formate); **heterocycles** (2,3-dimethylpyrazine, pyrazine, tetramethylpyrazine)	SPME-GC-MS	Calvo et al., 2020
Brevibacillus brevis	fermentation medium	**acids** (filicinic acid, diethyldithiophosphinic acid, 2-acetylamino-3-cyano-propionic acid); **alcohols** (3-octanol); **aldoketones** (6-dimethyl-6-nitro-2-hepten-4-one); **esters** (ethylparaben, dibutyl phthalate, 1,2-benzenedicarboxylic acid, butyl-2-methylpropyl ester, 1,2-benzenedicarboxylic acid-disooctyl ester, mono (2-ethylhexyl) phthalate, propylparaben, benzoic acid-3-amino-4-propoxy-2-(diethylamino)-ethyl ester); **heterocycles** (3-benzyl-hexahydropyrrole [1,2-a] pyrazine-1,4-dione, hexahydro pyrrole [1,2-a] pyrazine-1,4-dione, maltol, 3-isobutyl-hexahydropyrrole [1,2-a] pyrazine-1,4-dione, 3,6-diisobutyl-2,5-piperazine dione, 5-nitroso-2, 4, 6-triaminopyrimidine, dihydro-4,4-dimethyl-2,3-furandione); **nitrogen-containing compounds** (dihydroergotoxine); **phenolic compounds** (phenol, 3,5-dimethoxyphenol); **miscellaneous** [(4-acetylphenyl)phenylmethane, methoxyphenyl oxime]	GC-MS	Che et al., 2012
Brevibacillus brevis	Sorghum fermentation	**Alcohols** (2,3-butanediol, 3-pentanol, isopropanol); **aldoketones** (acetoin, butane-2,3-dione, 2-heptanone)	SPME-GC-MS	Liu et al., 2017c
Lysinibacillus sphaerieus	Sorghum fermentation	**Alcohols** (2,3-butanediol, 3-pentanol, ethanol, isobutanol, isoamyl alcohol); **aldoketones** (acetoin, butane-2,3-dione, 6-methyl-2-heptanone); **phenolic compounds** (guaiacol)	SPME-GC-MS	Liu et al., 2017c
Lysinibacillus boronitolerans				-
Paenibacillus Castaneae				-
Paenibacillus larvae subsup. Pulvifaciens	Sorghum fermentation	**Acids** (2-valeric acid); **alcohols** (isobutanol, isopropanol, 1-octen-3-ol); **aldoketones** (acetoin, butane-2,3-dione, 2-heptanone, 2-non-anone); **phenolic compounds** (guaiacol)	SPME-GC-MS	Liu et al., 2017c
Paenibacillus macerans				-
Paenibacillus nicotianae				-
Rummeliibacillus				-
Sporolactobacillus				-
Virgibacillus pantothenticu	Sorghum fermentation	**Alcohols (**isopentyl alcohol, 3-pentanol**); aldoketones** (acetoin, butane-2,3-dione, 2-heptanone, 2-non-anone); **phenolic compounds** (guaiacol)	GC-MS	Liu et al., 2018c
Clostridium butyricum	Cheeses fermentation	**Acids** (acetic acid, propionic acid, butyric acid, pentanoic acid); **alcohols** (1-butanol); **aldoketones** (2-butanone, 2,3-butanedione, acetoin); **esters** (ethyl acetate, ethyl butanoate)	SPME-GC-MS	Gómez-Torres et al., 2015
Clostridium kluyver				-

-, no published data. Bold indicates classification name of compound.

(Feng et al., 2017). Studies have shown that various organic matter can be produced by the microbes in *Huangshui* (e.g., alcohols, aldehydes, organic acid, esters, starch, reducing sugars, yeast autolysis, and other nutrients) (Zou et al., 2018; He F. et al., 2020). The microorganisms mainly consist of bacteria in *Huangshui*, among which the dominant genus was *Clostridium, Lactobacillus, and Serratia* (Li et al., 2015, 2020; Xie et al., 2020).

Bacillaceae are involved in *Huangshui* formation, which plays a key role in metabolizing nutrients, and could produce flavor components such as esters, acerbity, ketone, aldehyde (Li et al., 2015). Unfortunately, up to now, only a few studies have reported on the function of bacterial in *Huangshui*, especially for investigating *Bacillaceae*. At present, *Clostridium* spp. and *Bacillus* spp. had found the capacity of cellulose degradation in *Huangshui* (Desvaux et al., 2000; Sasaki et al., 2012). Six strains of *Bacillus* were isolated from *Huangshui*, which could produce cellulase, including *Bacillus cereus, Bacillus circulans, Bacillus megaterium, Bacillus endophyticus, Bacillus simplex,* and

Bacillus bataviensis, separately (Zeng et al., 2016). During the SAB production, cellulase producing bacteria could degrade the cellulose in mixed raw materials, and release the starch inside it, which is conducive to the action of the saccharifying enzyme, thus furthering the utilization rate of raw materials, improving the fermentation rate, and shortening the fermentation time (Hu D. et al., 2016). However, the structure and reaction mechanism of *Bacillaceae* cellulase are still unclear in *Huangshui*. Recently, a redundancy analysis of the microbe community structure and aroma components showed that SAB aroma components were positively correlated with Clostridia, but negatively correlated with Bacilli in *Huangshui*. Meanwhile, acidity was positively correlated with Bacilli and negatively correlated with Clostridia (Gao Z. et al., 2020).

CONCLUSION AND PERSPECTIVES

At present, little information is available on the specific metabolite of each *Bacillaceae* species. This information is crucial for applying *Bacillaceae* in SAB making. Other approaches to food fermentation by the bioaugmentation of special *Bacillaceae* may provide references for future research (**Table 2**). Some *Bacillus* species associated with SAB making showed the ability to produce various substances, such as hydrocarbons, organic acids, alcohols, aldoketones, esters, ethers, heterocycles, nitrogen-containing compounds, and phenolic compounds. All the metabolites listed above were identified by simulated fermentation (**Table 2**). Unfortunately, we still lack information about other *Bacillus* species, *Clostridium* species and metabolites, and further work is required.

In the process of making SAB, various microorganisms (associated with microbial proliferation and metabolism) interact, which contributes to the diversity of SAB flavor. The synergistic effect of diverse esters has been detected between pit mud microbes and *Daqu* microbes, for example, volatile acids and alcohols were provided by pit mud. Subsequently, esterifying reactions are achieved in SAB making by *Daqu* (Gao et al., 2021). As one of the main brewing microorganisms, *Bacillaceae* interact with other microorganisms, and this interaction effect has been studied by the method of single-strain bioaugmentation. However, there are at present few studies on the interaction among SAB brewing microorganisms. Previous research suggests that bioaugmentation of *Bacillus velezensis* and *Bacillus subtilis* in *Daqu,* which alter the microbial community and improve the flavor character of *Daqu* (He et al., 2019a). Bioaugmentation with *Hydrogenispora* could affect the abundance of *Clostridium* in pit mud (Chai et al., 2019). The study found that lactic acids were cut down by bioaugmentation with *Bacillus* during the SAB fermentation and the cause was *Bacillus* was negatively related to other bacterial species (Wang et al., 2017).

There are three main problems in the study of SAB *Bacillaceae* function: firstly, we lack studies on the interaction among SAB brewing microorganisms, to better understand the relationship between metabolic mechanism and the flavor production of brewing microorganisms, the interaction between *Bacillaceae* and other microbes should be further explored. Secondly, owing to the particularity of the mud pit environment, the anaerobic strains of *Bacillaceae* are difficult to isolate and culture from pit mud and *Huangshui*, so that functional properties of some *Bacillaceae* are difficult to determine. Thirdly, there are few studies on the isolation, culture, and flavor characteristics of a single strain of SAB *Bacillaceae*, especially the effect on solid-state fermentation. Fourthly, due to the different methods of metabolite detection on strains, as a result, different compounds were detected in different experiments with the same strain. Encouragingly, new technology can help solve the challenge, for instance, multi-omics approaches (metagenomes, metatranscriptomes, metaproteomes, and metabolomes) enable us to unravel the effects of *Bacillaceae* in the production of SAB. However, the high level of ethanol, acids, and humus in samples should be properly resolved. Isotope labeling and other biotechnology have been applied to study flavor formation pathways in *Bacillaceae* and will benefit from further exploration of the metabolic mechanism of flavor substances in SAB *Bacillaceae*. In the future, further study will promote the utilization of *Bacillaceae* in the brewing process, and improve the quality and stability of SAB.

AUTHOR CONTRIBUTIONS

WT, PH, and YY carried out the initial literature research and manuscript writing. ZQ, DH, and HL helped to provide expertise and insight relating to *Baijiu* microbiology. WT and XF revised the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by Talents Introduction Project from Sichuan University of Science and Engineering (2019RC31), Open Foundation from Key Laboratory of Biomaterials of CAS, and Postdoctoral Foundation from Wuliangye Yibin Co. Ltd., (294074).

REFERENCES

Beaumont, M. (2002). Flavouring composition prepared by fermentation with *Bacillus* spp. *Int. J. Food Microbiol.* 75, 189–196. doi: 10.1016/S0168-1605(01)00706-1

Calvo, H., Mendiara, I., Arias, E., Gracia, A. P., Blanco, D., and Venturini, M. E. (2020). Antifungal activity of the volatile organic compounds produced by *Bacillus velezensis* strains against postharvest fungal pathogens. *Postharvest Biol. Technol.* 166:111208. doi: 10.1016/j.postharvbio.2020.111208

Chai, L., Xu, P., Qian, W., Zhang, X., Ma, J., Lu, Z., et al. (2019). Profiling the Clostridia with butyrate-producing potential in the mud of Chinese liquor fermentation cellar. *Int. J. Food Microbiol.* 297, 41–50. doi: 10.1016/j.ijfoodmicro.2019.02.023

Che, J., Chen, Z., Shi, H., and Liu, B. (2012). Functional components in *Brevibacilus brevis* FJAT-0809-GLX determined by GC/MSD. *Fujian*

J. Agric. Sci. 27, 1106–1111. doi: 10.19303/j.issn.1008-0384.2012.10.016

Chen, M., Yang, D., Qian, Z., Zhen, D., Peng, D., and Fang, S. (2010). Analysis of microorganisms and physicochemical properties in *Zaopei* during the fermentation of Chinese zhijiang-flavor liquor. *Afr. J. Biotechnol.* 9, 3874–3882.

Cheng, G., Zheng, Z., Wei, C., and Huang, Z. (2021). Mixed fermentation effect of *Bacillus cereus* Frankland and *Saccharomyces cerevisiae*. *Liquor Mak. Sci. Technol.* 321, 53–59. doi: 10.13746/j.njkj.2020203

Cheng, T., Yin, X., Xia, Y., and Su, J. (2021). Study on the identification of ethyl caproate doped in Wuliangye strong aromatic liquor produced by solid-state fermentation process. *Packag. Food Mach.* 39, 46–51. doi: 10.3969/j.issn.1005-1295.2021.03.008

Deng, L., Mao, X., Liu, D., Ning, X., Shen, Y., Chen, B., et al. (2020). Comparative analysis of physicochemical properties and microbial composition in high-temperature Daqu with different colors. *Front. Microbiol.* 11:3010. doi: 10.3389/fmicb.2020.588117

Desvaux, M., Guedon, E., and Petitdemange, H. (2000). Cellulose catabolism by *Clostridium cellulolyticum* growing in batch culture on defined medium. *Appl. Environ. Microbiol.* 66, 2461–2470. doi: 10.1128/AEM.66.6.2461-2470.2000

Ding, X., Wu, C., Huang, J., and Zhou, R. (2015). Interphase microbial community characteristics in the fermentation cellar of Chinese Luzhou-flavor liquor determined by PLFA and DGGE profiles. *Food Res. Int.* 72, 16–24. doi: 10.1016/j.foodres.2015.03.018

Ding, X., Wu, C., Zhang, L., Zheng, J., and Zhou, R. (2014). Characterization of eubacterial and archaeal community diversity in the pit mud of Chinese Luzhou-flavor liquor by nested PCR–DGGE. *World J. Microbiol. Biotechnol.* 30, 605–612. doi: 10.1007/s11274-013-1472-4

Fan, W., Xu, Y., and Zhang, Y. (2007). Characterization of pyrazines in some Chinese liquors and their approximate concentrations. *J. Agric. Food Chem.* 55, 9956–9962. doi: 10.1021/jf071357q

Fan, W., Zhao, X., Du, G., Chen, J., Li, J., Zheng, J., et al. (2021). Metaproteomic analysis of enzymatic composition in Baobaoqu fermentation starter for Wuliangye baijiu. *Int. J. Food Sci. Technol.* 56, 4170–4181. doi: 10.1111/ijfs.15047

Feng, X., Deng, J., Xie, J., Wei, C., Luo, H., Huang, Z., et al. (2017). Brief analysis on current situation of comprehensive utilization of by-products yellow water from baijiu-making. *China Brew.* 36, 6–9. doi: 10.11882/j.issn.0254-5071.2017.02.002

Fu, X., Liu, X., Ao, Z., Han, G., Liu, X., Qiu, D., et al. (2011). Research progress in typical fermented grains of Chinese strong aromatic spirits and its influencing factors. *J. Beijing Technol. Bus. Univ. Nat. Sci. Ed.* 6, 54–57.

Gao, J., Liu, G., Li, A., Liang, C., Ren, C., and Xu, Y. (2021). Domination of pit mud microbes in the formation of diverse flavour compounds during Chinese strong aroma-type baijiu fermentation. *LWT* 137:110442. doi: 10.1016/j.lwt.2020.110442

Gao, Y. X., Xu, B., Fan, H. R., Zhang, M. R., Zhang, L. J., Lu, C., et al. (2020). ^{1}H NMR-based chemometric metabolomics characterization of soymilk fermented by *Bacillus subtilis* BSNK-5. *Food. Res. Int.* 138:109686. doi: 10.1016/j.foodres.2020.109686

Gao, Z., Wu, Z., and Zhang, W. (2020). Effect of pit mud on bacterial community and aroma components in yellow water and their changes during the fermentation of Chinese strong-flavor liquor. *Foods* 9:372. doi: 10.3390/foods9030372

Gómez-Torres, N., Garde, S., Peirotén, Á, and Ávila, M. (2015). Impact of *Clostridium* spp. on cheese characteristics: microbiology, color, formation of volatile compounds and off-flavors. *Food Control* 56, 186–194. doi: 10.1016/j.foodcont.2015.03.025

Gou, M., Wang, H., Yuan, H., Zhang, W., Tang, Y., and Kida, K. (2015). Characterization of the microbial community in three types of fermentation starters used for Chinese liquor production. *J. Inst. Brew.* 121, 620–627. doi: 10.1002/jib.272

Gou, W., Tian, Y., Kong, X., Wu, F., and Fang, F. (2020). Bacterial composition in pit mud of Yanghe liquor and identification of acid producing bacteria. *Microbiol. China* 47, 1651–1661.

Guan, T., Lin, Y., Chen, K., Ou, M., and Zhang, J. (2020). Physicochemical factors affecting microbiota dynamics during traditional solid-state fermentation of Chinese strong-flavor baijiu. *Front. Microbiol.* 11:2090. doi: 10.3389/fmicb.2020.02090

Guo, X., Fan, E., Ma, B., Li, Z., Zhang, Y., Zhang, Z., et al. (2020). Research progress in functional bacteria in solid-state fermented baijiu in China. *Food Ferment. Ind.* 46, 280–286. doi: 10.13995/j.cnki.11-1802/ts.022283

Hahnke, S., Striesow, J., Elvert, M., Mollar, X. P., and Klocke, M. (2014). *Clostridium bornimense* sp. nov., isolated from a mesophilic, two-phase, laboratory-scale biogas reactor. *Int. J. Syst. Evol. Microbiol.* 64, 2792–2797. doi: 10.1099/ijs.0.059691-0

He, F., Yang, H., Zeng, L., Hu, H., and Hu, C. (2020). Production and characterization of bacterial cellulose obtained by *Gluconacetobacter xylinus* utilizing the by-products from baijiu production. *Bioprocess Biosyst. Eng.* 43, 927–936. doi: 10.1007/s00449-020-02289-6

He, G., Huang, J., Wu, C., Jin, Y., and Zhou, R. (2020). Bioturbation effect of fortified Daqu on microbial community and flavor metabolite in Chinese strong-flavor liquor brewing microecosystem. *Food Res. Int.* 129:108851. doi: 10.1016/j.foodres.2019.108851

He, G., Huang, J., Zhou, R., Wu, C., and Jin, Y. (2019b). Effect of fortified Daqu on the microbial community and flavor in Chinese strong-flavor liquor brewing process. *Front. Microbiol.* 10:56. doi: 10.3389/fmicb.2019.00056

He, G., Dong, Y., Huang, J., Wang, X., Zhang, S., Wu, C., et al. (2019a). Alteration of microbial community for improving flavor character of Daqu by inoculation with *Bacillus velezensis* and *Bacillus subtilis*. *LWT* 111, 1–8. doi: 10.1016/j.lwt.2019.04.098

Hong, Y., Jung, H.-J., and Kim, H.-Y. (2012). Aroma characteristics of fermented Korean soybean paste (Doenjang) produced by *Bacillus amyloliquefaciens*. *Food Sci. Biotechnol.* 21, 1163–1172. doi: 10.1007/s10068-012-0152-8

Hu, D., Ju, X., Li, L., Hu, C., Yan, L., Wu, T., et al. (2016). Improved *in situ* saccharification of cellulose pretreated by dimethyl sulfoxide/ionic liquid using cellulase from a newly isolated *Paenibacillus* sp. LLZ1. *Bioresour. Technol.* 201, 8–14. doi: 10.1016/j.biortech.2015.11.039

Hu, X., Du, H., Ren, C., and Xu, Y. (2016). Illuminating anaerobic microbial community and cooccurrence patterns across a quality gradient in Chinese liquor fermentation pit muds. *Appl. Environ. Microbiol.* 82, 2506–2515. doi: 10.1128/AEM.03409-15

Hu, X., Du, H., and Xu, Y. (2015). Identification and quantification of the caproic acid-producing bacterium *Clostridium kluyveri* in the fermentation of pit mud used for Chinese strong-aroma type liquor production. *Int. J. Food Microbiol.* 214, 116–122. doi: 10.1016/j.ijfoodmicro.2015.07.032

Hu, X., Tian, R., Wang, K., Cao, Z., Yan, P., Li, F., et al. (2021b). The prokaryotic community, physicochemical properties and flavors dynamics and their correlations in fermented grains for Chinese strong-flavor baijiu production. *Food Res. Int.* 148:110626. doi: 10.1016/j.foodres.2021.110626

Hu, X., Tian, R., Li, B., Zhang, Y., Chi, L., He, P., et al. (2021a). Analysis of community structure and functional changes of active microorganisms in fermented grains for the Chinese strong-flavor baijiu production based on metatranscriptomics technology. *Food Sci.* 42, 1–12. doi: 10.7506/spkx1002-6630-20210324-293

Hu, X., Wang, K., Chen, M., Fan, J., Han, S., Hou, J., et al. (2020). Profiling the composition and metabolic activities of microbial community in fermented grain for the Chinese strong-flavor baijiu production by using the metatranscriptome, high-throughput 16S rRNA and ITS gene sequencings. *Food Res. Int.* 138:109765. doi: 10.1016/j.foodres.2020.109765

Li, K., Fan, Z., Wang, J., Lin, K., and Xiang, W. (2015). Microbial diversity in fermented yellow water of traditional intense flavor liquor. *J. Food Sci. Biotechnol.* 34, 1155–1161. doi: 10.3969/j.issn.1673-1689.2015.11.006

Li, X., Zhang, J., Yan, J., Xue, D., Chen, M., and Fang, S. (2014). Dynamic analysis of microbial communities in the fermenting process of Daohuaxiang liquor by use of wrapped starter. *Liquor Mak. Sci. Technol.* 235, 16–20. doi: 10.13746/j.njkj.2014.01.007

Li, Y., Yuan, S., Yong, X., and Liu, J. (2020). Research progress on small peptides in Chinese baijiu. *J. Funct. Foods* 72:104081. doi: 10.1016/j.jff.2020.104081

Li, Z., and Qiu, L. (2021). Changes of prokaryotic community succession process of fermented grains during strong-flavor baijiu fermentation. *J. Shandong Inst. Light Ind. Nat. Sci. Ed.* 35, 26–31. doi: 10.16442/j.cnki.qlgydxxb.2021.04.005

Liang, H., Li, W., Luo, Q., Liu, C., Wu, Z., and Zhang, W. (2015). Analysis of the bacterial community in aged and aging pit mud of Chinese Luzhou-flavour liquor by combined PCR-DGGE and quantitative PCR assay. *J. Sci. Food Agric.* 95, 2729–2735. doi: 10.1002/jsfa.7013

Liang, H., Xu, C., Tang, W., Ren, Y., and Zhu, I. (2021). Microbial community structure and diversity in Luzhou-flavor liquor cellar mud. *Mod. Agric. Sci. Technol.* 24, 203–206+220. doi: 10.3969/j.issn.1007-5739.2021.03.078

Liang, J., Li, A., Li, L., He, H., Tang, Y., Wu, W., et al. (2017). Separation,identification of microorganisms in GuJing liquor Daqu using biolog-ECO method. *Liquor Mak.* 44, 28–32.

Lin, Q., Dong, S., Fu, Q., Hu, C., Pan, Q., and Zhou, G. (2013a). Isolation of aroma-producing *Bacillus subtilis* and analysis of its fermentation metabolites. *Liquor Mak. Sci. Technol.* 11, 30–32. doi: 10.13746/j.njkj.2013.11.027

Lin, Q., Xiao, Z., Fu, Q., Fang, X., Mo, L., and Pan, Q. (2013b). Isolation of aroma-producing *Bacillus licheniformis* and analysis of its fermentation metabolites. *Liquor Mak. Sci. Technol.* 223, 49–52. doi: 10.13746/j.njkj.2013.12.020

Liu, F., Zhou, X., Chen, X., Chen, J., Du, G., and Fang, F. (2018b). Microbial community of fermented grains and its correlation with organic acids biosynthesis during Yanghe strong-aroma liquor manufacturing process. *Acta Microbiol. Sin.* 12, 2087–2098. doi: 10.13343/j.cnki.wsxb.20170616

Liu, Y., Wang, Y., Li, Y., Wang, W., Liu, M., Wu, S., et al. (2018c). The study of microorganisms in pit mud by Agilent GC and GC-MS. *Environ. Chem.* 37, 902–905.

Liu, F., Qiu, Y., Zhou, X., Chen, X., Li, Z., Chen, J., et al. (2018a). The correlation between organic acid producing bacteria and organic acids biosynthesis in fermented grains of Yanghe strong-aroma spirit. *Food Ferment. Ind.* 44, 22–29. doi: 10.13995/j.cnki.11-1802/ts.017898

Liu, F., Yang, K., Zhang, J., Qiao, Z., An, M., Zheng, J., et al. (2020). Research progress in microbial diversity of fermented grains of baijiu. *Liquor Mak. Sci. Technol.* 307, 89–97. doi: 10.13746/j.njkj.2020060

Liu, M., Tang, Y., Guo, X., Ke, Z., Penttinen, P., Tian, X., et al. (2020). Structural and functional changes in prokaryotic communities in artificial pit mud during Chinese baijiu production. *mSystems* 5:e00829-19. doi: 10.1128/mSystems.00829-19

Liu, M., Tang, Y., Zhao, K., Gu, Y., Ren, D., Yao, W., et al. (2017b). Recent advances in research on the community, isolation, and application of microbes in the pit mud used in manufacture of Chinese strong-flavor baijiu. *Microbiol. China* 44, 1222–1229. doi: 10.13344/j.microbiol.china.160559

Liu, Y., Wang, Y., Wang, W., Li, Y., Wu, S., Liu, M., et al. (2017c). Screening, identification and metabolites analyses of *Bacillus* in pit mud of Luzhou-flavor baijiu. *China Brew.* 36, 76–79. doi: 10.11882/j.issn.0254-5071.2017.07.017

Liu, M., Tang, Y., Guo, X., Zhao, K., Tian, X., Liu, Y., et al. (2017a). Deep sequencing reveals high bacterial diversity and phylogenetic novelty in pit mud from Luzhou Laojiao cellars for Chinese strong-flavor baijiu. *Food Res. Int.* 102, 68–76. doi: 10.1016/j.foodres.2017.09.075

Liu, M., Zhao, K., Tang, Y., Ren, D., Yao, W., Tian, X., et al. (2015). Analysis of *Clostridium* cluster I community diversity in pit mud used in manufacture of Chinese Luzhou-flavor liquor. *Food Sci. Biotechnol.* 24, 995–1000. doi: 10.1007/s10068-015-0127-7

Liu, S., Li, L., Li, K., Jia, B., Zhong, X., Che, Z., et al. (2013). Spatial heterogeneity of prokaryotic microbial communities in Luzhou-flavor liquor pit mud. *Food Sci.* 34, 221–226. doi: 10.7506/spkx1002-6630-201321045

Liu, Y., Cheng, H., Ye, X., Liu, H., and Fang, H. (2020). Changes of bioactive compounds and volatile compounds contents in goji juice fermented by different probiotics. *Acta Agric. Univ. Zhejiangensis* 32:499. doi: 10.3969/j.issn.1004-1524.2020.03.16

Luo, J., Zhu, S., Wang, L., He, J., Ouyang, L., and Zhou, J. (2020). Research progress on the composition of brewing microorganisms and flavor substances in strong-flavor baijiu. *China Brew.* 39, 1–6. doi: 10.11882/j.issn.0254-5071.2020.04.001

Ma, J., Lu, Z., Zhang, X., Wang, S., Shen, C., Shi, J., et al. (2016). Role of *Clostridia* community in fermented grains during Luzhou-flavor liquor production. *Microbiol. China* 43, 2322–2329. doi: 10.13344/j.microbiol.china.160110

Qian, W., Lu, Z., Chai, L., Zhang, X., Li, Q., Wang, S., et al. (2021). Cooperation within the microbial consortia of fermented grains and pit mud drives organic acid synthesis in strong-flavor baijiu production. *Food Res. Int.* 147:110449. doi: 10.1016/j.foodres.2021.110449

Rajaofera, M. J. N., Wang, Y., Dahar, G. Y., Jin, P., Fan, L., Xu, L., et al. (2019). Volatile organic compounds of *Bacillus atrophaeus* HAB-5 inhibit the growth of *Colletotrichum gloeosporioides*. *Pestic. Biochem. Physiol.* 156, 170–176. doi: 10.1016/j.pestbp.2019.02.019

Sasaki, D., Morita, M., Sasaki, K., Watanabe, A., and Ohmura, N. (2012). Acceleration of cellulose degradation and shift of product via methanogenic co-culture of a cellulolytic bacterium with a hydrogenotrophic methanogen. *J. Biosci. Bioeng.* 114, 435–439. doi: 10.1016/j.jbiosc.2012.05.002

Seo, H. S., Lee, S., Singh, D., Park, M. K., Kim, Y.-S., Shin, H. W., et al. (2018). Evaluating the headspace volatolome, primary metabolites, and aroma characteristics of koji fermented with *Bacillus amyloliquefaciens* and *Aspergillus oryzae*. *J. Microbiol. Biotechnol.* 28, 1260–1269. doi: 10.4014/jmb.1804.04017

Shi, X., Xu, Y., Cui, F., Zhong, Y., Xie, Y., and Xie, X. (2012).). Study on the application of *B. subtilis* in the production of liquor. *Liquor Mak. Sci. Technol.* 212, 49–53. doi: 10.13746/j.njkj.2012.02.016

Tao, Y., Li, J., Rui, J., Xu, Z., Zhou, Y., Hu, X., et al. (2014). Prokaryotic communities in pit mud from different-aged cellars used for the production of Chinese strong-flavored liquor. *Appl. Environ. Microbiol.* 80, 2254–2260. doi: 10.1128/AEM.04070-13

Wang, C., Chen, Q., Wang, Q., Li, C., Leng, Y., Li, S., et al. (2014). Long-term batch brewing accumulates adaptive microbes, which comprehensively produce more flavorful Chinese liquors. *Food Res. Int.* 62, 894–901. doi: 10.1016/j.foodres.2014.05.017

Wang, J., Wan, Z., Li, L., Li, X., and Chen, M. (2010). Study on the change of microflora in pit mud of Daohuaxiang. *Liquor Mak. Sci. Technol.* 11, 36–39. doi: 10.13746/j.njkj.2010.11.009

Wang, S., Yan, S., and Jin, X. (2021). Dynamic changes of bacteria and fungi in strong-flavor Daqu during fermentation. *Liquor Mak.* 48, 29–34.

Wang, T., Zhao, D., Tian, S., You, L., Wang, S., Feng, R., et al. (2011). Phylogenetic diversity of cultivable bacteria during the brewing process of the luzhou-flavor liquor in Yibin, Sichuan province, China. *Acta Microbiol. Sin.* 51, 1351–1357. doi: 10.13343/j.cnki.wsxb.2011.10.006

Wang, W., Wang, Y., Wu, S., Liu, M., Deng, J., Li, Y., et al. (2018). Isolation and identification of three anaerobic bacteria strains in Luzhou-flavor liquor and metabolites analysis. *Food Sci. Technol.* 43, 15–20. doi: 10.13684/j.cnki.spkj.2018.02.003

Wang, X., Du, H., and Xu, Y. (2017). Source tracking of prokaryotic communities in fermented grain of Chinese strong-flavor liquor. *Int. J. Food Microbiol.* 244, 27–35. doi: 10.1016/j.ijfoodmicro.2016.12.018

Wang, Y., Luo, H., Liu, Y., Wang, Y., and Ye, G. (2015b). Separation and identification of *Bacillus* spp. from Luzhou-flavor Daqu. *J. Sichuan Univ. Sci. Eng. Nat. Sci. Ed.* 28, 5–8. doi: 10.11863/j.suse.2015.02.02

Wang, Y., Li, Z., Gan, G., Jiang, L., Yang, Y., Sheng, X., et al. (2015a). Screening of acid production functional bacteria in pit mud and preliminary study on fermentation conditions. *Liquor Mak.* 42, 52–56.

Wang, Z., Lu, Z., Zhu, Q., Shi, J., and Xu, Z. (2016). Fermentation properties of *Bacilus circulans*, a functional microbe from Zhenjiang aromatic vinegar brewing mas. *China Condiments* 41, 24–28. doi: 10.3969/j.issn.1000-9973.2016.09.006

Wu, Q., Feng, X., Guo, W., Bao, X., and Ren, N. (2020). Long-term medium chain carboxylic acids production from liquor-making wastewater: parameters optimization and toxicity mitigation. *Chem. Eng. J.* 388:124218. doi: 10.1016/j.cej.2020.124218

Wu, W., Li, A., Tang, Y., and Li, L. (2016). The flavor compound in the fermentation of *Bacillus* from Gujing pit mud. *Food Sci. Technol.* 41, 27–30. doi: 10.13684/j.cnki.spkj.2016.03.006

Wu, W., Liu, F., Fang, S., and Pu, S. (2019). Flavor compounds produced by *Paenibacillus* from pit mud in northern Anhui. *Sci. Technol. Food Ind.* 40, 274–279. doi: 10.13386/j.issn1002-0306.2019.10.045

Xiang, H., Lin, Y., Guan, T., Zhang, J., Shang, H., Zhao, X., et al. (2020). Diversities of culturable yeast and *Bacillus* and their relationship with process parameters during the production of Sichuan Luzhou-flavor Daqu. *Food Sci.* 41, 196–201. doi: 10.7506/spkx1002-6630-20181217-179

Xie, J., Cheng, K., Zhao, D., Yang, G., Qiao, Z., Qiu, S., et al. (2020). *Bacillus aquiflavi* sp. nov., isolated from yellow water of strongly flavored Chinese baijiu. *Int. J. Syst. Evol. Microbiol.* 70, 3406–3412. doi: 10.1099/ijsem.0.004185

Xu, P., Chai, L., Qiu, T., Zhang, X., Lu, Z., Xiao, C., et al. (2019). *Clostridium fermenticellae* sp. nov., isolated from the mud in a fermentation cellar for the production of the Chinese liquor, baijiu. *Int. J. Syst. Evol. Microbiol.* 69, 859–865. doi: 10.1099/ijsem.0.003254

Xu, Y., Sun, B., Fan, G., Teng, C., Xiong, K., Zhu, Y., et al. (2017). The brewing process and microbial diversity of strong flavour Chinese spirits: a review. *J. Inst. Brew.* 123, 5–12. doi: 10.1002/jib.404

Xu, Z., Tang, Q., Xu, Z., Liu, M., and Qiu, X. (2019). Screening and application of special functional bacteria from aged pit mud in workshops of Jiannanchun Tianyilaohao. *Liquor Mak. Sci. Technol.* 295, 94–100. doi: 10.13746/j.njkj.2019202

Yan, Z., Zheng, X., Han, B., Han, J., Nout, M., and Chen, J. (2013b). Monitoring the ecology of *Bacillus* during Daqu incubation, a fermentation starter, using culture-dependent and culture-independent methods. *J. Microbiol. Biotechnol.* 23, 614–622. doi: 10.4014/jmb.1211.11065

Yan, Z., Zheng, X., Chen, J., Han, J., and Han, B. (2013a). Effect of different *Bacillus* strains on the profile of organic acids in a liquid culture of Daqu. *J. Inst. Brew.* 119, 78–83. doi: 10.1002/jib.58

Yang, C., Liao, Y., Liu, J., Hu, J., Hu, J., and Dou, S. (2012). Identification of *Bacillus* from Niulanshan erguotou fermented grain and analysis of flavor compounds in the fermentation. *Sci. Technol. Food Ind.* 33, 69–74. doi: 10.13386/j.issn1002-0306.2012.09.073

Yang, H., Ning, Y., Wang, C., Weng, P., Wu, Z., and Zhu, Y. (2019). Effects of inoculated fermentation on characters of anchovy fish sauce by *Bacillus velezensis* SW5. *J. Nucl. Agric. Sci.* 33, 2013–2022.

Yang, L., and Chen, L. (2021). Optimization of conditions for amylase and protease production of a thermotolerant *Bacillus licheniformis* strain. *Liquor Mak.* 48, 49–53.

Yang, P., Xu, S., Duan, Y., Li, Y., Wu, H., Wang, K., et al. (2019). Analysis of volatile components in fermentation extracts from chicory rootss fermented by *Bacillus pumilus*. *China Food Addit.* 30, 93–99. doi: 10.19804/j.issn1006-2513.2019.10.009

Yao, W., Chen, M., Zhen, D., and Guo, Y. (2010). Isolation of lactate-producing microbes from fermented grains of Luzhou-flavor liquor and their effect on simulative solid-state fermentation. *Liquor Mak.* 37, 37–41.

Yao, W., Tang, Y., Ren, D., Liao, J., Shen, C., Xu, D., et al. (2005). Study on the differences of microbes in the different layers of Guojiao Daqu. *Liquor Mak.* 32, 35–37.

Ye, G., Luo, H., Yang, X., Li, D., Wang, Y., and Ni, B. (2013). Community structure of prokaryotes in pit mud of lu-flavor liquor from luzhou prefecture based on culture-independent approach. *J. Food Sci.* 34, 194–199. doi: 10.7506/spkx1002-6630-201317038

Yin, X., Zhang, S., Ao, Z., Shui, L., Li, D., Liu, Y., et al. (2014). Effects of seasons on the fermentation of Nong-flavor liquor. *Liquor Mak. Sci. Technol.* 235, 51–54+58. doi: 10.13746/j.njkj.2014.01.017

You, J., Chen, M., Fang, S., Liu, Q., and Li, J. (2009). Isolation and preliminary identification of dominsnt microbe in Daqu and mud pit of Zhijiang Daqu liquor. *Liquor Mak.* 36, 31–34.

Yu, C., and Liu, C. (2016). Analysis of bacterial diversity in different areas of strong aromatic Chinese spirit in pit mud. *Food Res. Dev.* 37, 148–151. doi: 10.3969/j.issn.1005-6521.2016.24.035

Yue, Y., Zhang, W., Liu, X., Hu, C., and Zhang, S. (2007). Isolation and identification of facultative anaerobes in the pit mud of Chinese Luzhou-flavor liquor. *Microbiol. China* 34, 251–255. doi: 10.13344/j.microbiol.china.2007.02.013

Zeng, L., Tan, X., Yuan, C., Zhang, Q., Yang, Y., Zhao, T., et al. (2016). Isolation and identification of cellulose-degrading strains from yellow water of baijiu (Chinese liquor) and its cellulase activity. *China Brew.* 35, 59–63. doi: 10.11882/j.issn.0254-5071.2016.11.012

Zhai, L., Yu, X., Feng, H., Zhang, C., Zhou, L., Qiu, S., et al. (2020). Study on bacterial community structure in the brewing habitats of strong flavour Chinese baijiu from Yibin region. *Food Ferment. Ind.* 46, 18–24. doi: 10.13995/j.cnki.11-1802/ts.022600

Zhang, C., Li, J., Liu, Z., Sun, L., Dong, X., and Li, R. (2019). Study of a caproic acid-producing functional strain from the pit mud of Nongxiang baijiu. *Liquor Mak. Sci. Technol.* 295, 50–53. doi: 10.13746/j.njkj.2019171

Zhang, L., Luo, H., Huang, D., Zhang, Q., Zheng, S., and Tong, W. (2021a). Screening of a *Bacillus* strain with high acetoin-producing ability and optimization of its fermentation conditions. *Mod. Food Sci. Technol.* 37, 71–81. doi: 10.13982/j.mfst.1673-9078.2021.5.0919

Zhang, L., Ran, M., Wang, S., Liu, G., Ma, Z., Ren, J., et al. (2021b). Isolation and identification of strains with high yield of fibrinolysin from Daqu of Luzhou Laojiao. *Liquor Mak. Sci. Technol.* 319, 17–24. doi: 10.13746/j.njkj.2021033

Zhang, R., Wu, Q., and Xu, Y. (2013). Aroma characteristics of Moutai-flavour liquor produced with *Bacillus licheniformis* by solid-state fermentation. *Lett. Appl. Microbiol.* 57, 11–18. doi: 10.1111/lam.12087

Zhang, R., Wu, Q., and Xu, Y. (2014). Lichenysin, a cyclooctapeptide occurring in Chinese liquor Jiannanchun reduced the headspace concentration of phenolic off-flavors via hydrogen-bond interactions. *J. Agric. Food Chem.* 62, 8302–8307. doi: 10.1021/jf502053g

Zhang, X., Li, B., Wang, Y., Guo, Q., Lu, X., Li, S., et al. (2013). Lipopeptides, a novel protein, and volatile compounds contribute to the antifungal activity of the biocontrol agent *Bacillus atrophaeus* CAB-1. *Appl. Microbiol. Biotechnol.* 97, 9525–9534. doi: 10.1007/s00253-013-5198-x

Zhang, X., Wu, Z., Zhang, S., Hu, C., and Zhang, W. (2010). Phylogenetic analysis of cultured bacteria in Luzhou-flavor liquor pits in Guizhou. *Liquor Mak. Sci. Technol.* 198, 23–27. doi: 10.13746/j.njkj.2010.12.001

Zhang, Y., Zhu, X., Li, X., Tao, Y., Jia, J., and He, X. (2017). The process-related dynamics of microbial community during a simulated fermentation of Chinese strong-flavored liquor. *BMC Microbiol.* 17:196. doi: 10.1186/s12866-017-1106-3

Zhao, D., Niu, G., Peng, Z., Chen, L., and Yang, R. (2009). Change in microflora and the evolution of physical and chemical factors in Wuliangye wrapped starter. *Liquor Mak. Sci. Technol.* 12, 38–40. doi: 10.13746/j.njkj.2009.12.003

Zhao, H., Chang, Y., Wang, W., Ling, H., Ping, W., Zhao, Z., et al. (2012). Isolation and identification of facultative anaerobic strains with high yield of hexanoic acid from Luzhou-flavor liquor pit mud. *Food Sci.* 33, 177–182.

Zheng, J., Liang, R., Zhang, L., Wu, C., Zhou, R., and Liao, X. (2013). Characterization of microbial communities in strong aromatic liquor fermentation pit muds of different ages assessed by combined DGGE and PLFA analyses. *Food Res. Int.* 54, 660–666. doi: 10.1016/j.foodres.2013.07.058

Zheng, X., and Han, B. (2016). Baijiu, Chinese liquor: history, classification and manufacture. *J. Ethn. Foods* 3, 19–25. doi: 10.1016/j.jef.2016.03.001

Zheng, X., Tabrizi, M. R., Nout, M. R., and Han, B. (2011). Daqu—a traditional Chinese liquor fermentation starter. *J. Inst. Brew.* 117, 82–90. doi: 10.1002/j.2050-0416.2011.tb00447.x

Zhi, Y., Wu, Q., Du, H., and Xu, Y. (2016). Biocontrol of geosmin-producing *Streptomyces* spp. by two *Bacillus* strains from Chinese liquor. *Int. J. Food Microbiol.* 231, 1–9. doi: 10.1016/j.ijfoodmicro.2016.04.021

Zhou, P., Luo, H., Huang, D., Deng, B., Wang, Q., Huang, Z., et al. (2016). Separation and identification of thermoduric bacteria strains in medium/high temperature Daqu and the analysis of the flavor metabolites. *Sci. Technol. Food Ind.* 15, 215–220. doi: 10.13386/j.issn1002-0306.2016.24.033

Zhou, R., Chen, Y., and Tang, D. (2010). Research on microbial diversity & distribution in Yibin multiple-grains Luzhou-flavor liquor distillery. *Liquor Mak. Sci. Technol.* 5, 60–64. doi: 10.13746/j.njkj.2010.05.001

Zou, W., Zhao, C., and Luo, H. (2018). Diversity and function of microbial community in Chinese strong-flavor baijiu ecosystem: a review. *Front. Microbiol.* 9:671. doi: 10.3389/fmicb.2018.00671

Conflict of Interest: WT and ZQ were employed by the company Wuliangye Yibin Co. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 February 2022
doi: 10.3389/fmicb.2022.820539

Dimensional Analysis Model Predicting the Number of Food Microorganisms

Cuiqin Li[1,2,3], Laping He[1,2], Yuedan Hu[1,2], Hanyu Liu[1,2], Xiao Wang[1,2]*, Li Chen[1,2] and Xuefeng Zeng[1,2]*

[1] Key Laboratory of Agricultural and Animal Products Storage and Processing of Guizhou Province, Guizhou University, Guiyang, China, [2] College of Liquor and Food Engineering, Guizhou University, Guiyang, China, [3] School of Chemistry and Chemical Engineering, Guizhou University, Guiyang, China

Predicting the number of microorganisms has excellent application in the food industry. It helps in predicting and managing the storage time and food safety. This study aimed to establish a new, simple, and effective model for predicting the number of microorganisms. The dimensional analysis model (DAM) was established based on dimensionless analysis and the Pi theorem. It was then applied to predict the number of *Pseudomonas* in Niuganba (NGB), a traditional Chinese fermented dry-cured beef, which was prepared and stored at 278 K, 283 K, and 288 K. Finally, the internal and external validation of the DAM was performed using six parameters including R^2, R^2_{adj}, root mean square error (RMSE), standard error of prediction (%SEP), A_f, and B_f. High R^2 and R^2_{adj} and low RMSE and %SEP values indicated that the DAM had high accuracy in predicting the number of microorganisms and the storage time of NGB samples. Both A_f and B_f values were close to 1. The correlation between the observed and predicted numbers of *Pseudomonas* was high. The study showed that the DAM was a simple, unified and effective model to predict the number of microorganisms and storage time.

Keywords: dimensional analysis, Pi theorem, predicting microorganism model, storage time, validation

OPEN ACCESS

Edited by:
Laurent Dufossé, Université de la Réunion, France

Reviewed by:
Muhammet Irfan Aksu, Eskişehir Osmangazi University, Turkey
Veronica Ortiz Alvarenga, Federal University of Minas Gerais, Brazil

***Correspondence:**
Laping He
helaping@163.com; lphe@gzu.edu.cn
Xiao Wang
38114047@qq.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *23 November 2021*
Accepted: *04 January 2022*
Published: *08 February 2022*

Citation:
Li C, He L, Hu Y, Liu H, Wang X, Chen L and Zeng X (2022) Dimensional Analysis Model Predicting the Number of Food Microorganisms. Front. Microbiol. 13:820539. doi: 10.3389/fmicb.2022.820539

INTRODUCTION

At present, predictive microbiological models are desirable tools in the food industry. They combine microbial growth, mathematical model, and statistics (Mcmeekin et al., 2002; Tarlak et al., 2018). These models can assess the dynamic changes in the growth of certain microorganisms in food using microbial predictive methods, providing a sound basis for the rapid assessment and prediction of storage time (shelf life) and food safety. The predictive microbiological models are a valuable tool for managing and guaranteeing food safety and promoting progress in the food industry (McMeekin et al., 2006; Tamplin, 2018).

Predictive microbiological models are classified into primary, secondary, and tertiary models for predicting microbial growth (Whiting and Buchanan, 1993; Isabelle and André, 2006). Primary models do not include factors but focus on the time behavior. Secondary models deal with the response of parameters that appear in primary modeling approaches such as temperature, pH, and so forth (Giannuzzi et al., 1998; Molina and Giannuzzi, 1999; Xiong et al., 1999; Davey, 2010; Zimmermann et al., 2013). The tertiary level combines the first two types of models with user-friendly applications (Dyer, 1996;

McKellar and Lu, 2003; Longhi et al., 2014). The growth of microorganisms requires nutrients, but none of the existing models consider the impact of nutrients on the growth of microorganisms. The nutrition of microorganisms in food is the food itself. Therefore, food mass should be included to build a microbial prediction model. Temperature is the key factor affecting microbial growth. In statistical mechanics, the absolute temperature is proportional to the average kinetic energy of the molecules or atoms of a system (Xu et al., 2012; Bormashenko, 2020). Food microorganisms and food are also composed of molecules or atoms. Therefore, the influence of absolute temperature on microorganisms can be regarded as the influence of the average kinetic energy E_K of the molecules or atoms of the food-microbial system, and this E_K is proportional to the absolute temperature θ (Xu et al., 2012; Bormashenko, 2020), that is, $E_K = c\theta$,where c is scale coefficient, $KJ \cdot K^{-1}$.

From a professional point of view, the main factors affecting the growth of microorganisms are food mass, temperature (reflection of the average translational kinetic energy of internal particles in the system), pH, water activity (a_w) (Sautour et al., 2003), and time. Considering the growth of food microorganisms in the environment, the surface area of the food affects the heat and mass transfer between the food and the environment (Mohos, 2017). The large surface area per unit mass of food helps in heat transfer and contributes to microbial growth within the growth temperature range. In the meantime, the surface area affects the mass transfer and the transfer of nutrient molecules or particles. When the temperature of a substance is greater than absolute 0 K, its molecules or atoms have average kinetic energy (Xu et al., 2012; Bormashenko, 2020). Therefore, mass transfer affects the transfer of molecules or atoms, and then influences temperature diffusion. The temperature obviously affects the growth or metabolism of microorganisms. In addition, mass transfer will affect the absorption of substances by microorganisms, thus influencing the metabolism and growth of microorganisms. Therefore, mass transfer affects temperature diffusion and microbial growth or metabolism. Thus, the surface area of food is also used as a factor affecting the growth of microorganisms. Therefore, the main factors influencing microbial growth include food mass, temperature θ, time T, surface area S_T at time T, pH_T (pH at time T), and a_{wT} (water activity at time T). But how are so many variables put into a model? Considering that the dimensional analysis combined with the π theorem is an effective method to solve multivariate problems, the present study attempted to use dimensional analysis and the π theorem to construct a microbial prediction model.

The dimensional analysis method (Kuneš, 2012; Singh, 2017) is a well-developed methodology in physics, chemical engineering, food engineering, and so on. It is used for diluting complex physical phenomena to the most simplified form (Buckingham, 1914; Chandarana et al., 2010). It reduces the number of variables in the problem by combining dimensional variables to form nondimensional parameters. Moreover, dimensionless groups allow the application of empirical correlation to a wide range of conditions. A classic example of this aspect is discovering the Reynolds number and the relationship between friction coefficient, Reynolds number, and relative roughness in the fields of chemical engineering and food engineering using dimensional analysis. Meanwhile, dimensional analysis is also applied to characterize some indicators in biological systems (Mcmahon, 1973; Pilbeam and Gould, 1974; Ruzicka, 2008). For example, the square of characteristic body dimension L must support a weight that increases with L^3; the surface density is M/L^2 (kg/m^2); and elastic criteria impose limits on biological proportions and, consequently, on metabolic rates. However, it is true that biological laws are not derivable from physical laws in any simple sense (Mcmahon, 1973). Therefore, dimensional analysis is seldom used in biology. The dimensional analysis has several advantages, and hence it is necessary to consider its application in predicting microbial growth in food. It can be applied more widely in biology with the development of cognition. Buchanan (2010) commented, "Dimension clearly matters more than we might naively think, and perhaps biology awaits a similar explosion." However, the predictive model based on the combination of temperature and dimensional analysis on microbial growth has not been reported. Under some conditions, the Pi theorem can also be written in the form of a power function (Nagy, 2019), which is undoubtedly helpful in its application. Therefore, investigating and modeling the microbial growth of foods are important.

Niuganba (NGB) is a traditional Chinese fermented beef (Tian et al., 2021). It has a unique flavor, delicious taste, and nongreasy texture. NGB has a shelf life, and *Pseudomonas* is usually the spoilage bacteria in meat products.

The objective of this study was to introduce dimensionless numbers by dimensional analysis and then to establish a predictive microbiological model based on the Pi theorem. Then, the model was used to predict *Pseudomonas* in NGB during storage. This new unified predictive microbiological model anticipated the microbial growth of foods, as well as the storage time. It might support the development of food microbiology and predict the storage time.

MATERIALS AND METHODS

Materials

Fresh rump, salt, garlic, ginger, fresh red pepper, sesame, tea polyphenols, glucose, peppercorns, monosodium glutamate (MSG), and white liquor were purchased from local supermarkets. *Lactobacillus pentosus* MT-4 (China Center for Type Culture Collection M2016001) was preserved in the laboratory. *Staphylococcus xylosus* American Type Culture Collection 2997 was collected from the Guangdong Industrial Microbiology Collection, China. *Wickerhamomyces anomalus* yeast was purchased from Yichang Angel Yeast Co., Ltd., China. The De Man, Rogosa, and Sharpe (MRS) liquid medium was prepared as described in a previous study (de-Man et al., 1960). The MRS agar medium was prepared by adding agar (20 g/L) to the MRS liquid medium. A yeast extract peptone dextrose (YPED) medium was prepared using peptone (20 g), glucose (10.0 g), yeast extract (10.0 g), and distilled water (1,000 mL).

A mannitol salt (MS) medium was prepared using peptone (10.0 g), beef extract (1.0 g), D-mannitol (10.0 g), sodium chloride (75.0 g), phenol red (0.025 g), and distilled water (1,000 mL). The YPED agar medium was prepared by adding agar (20 g/L) to the YPED medium, while the MS agar medium was prepared by adding agar (20 g/L) to the MS medium. A *Pseudomonas* agar medium (Gospavic et al., 2008; ISO, 2010) was prepared using peptone (16.0 g), hydrolyzed casein (10.0 g), anhydrous potassium sulfate (10.0 g), magnesium chloride (1.4 g), glycerol (10.0 mL), *Pseudomonas* medium selection agent (five vials), cephaloridine-fucidin-cetrimide (Oxoid, United Kingdom), agar (20 g), and distilled water (1,000 mL). The pH of the medium was adjusted to 7.0 ± 0.2. All the chemicals used in this study were of analytical grade and commercially available.

Preparation of Niuganba

Preparation of Starters

Lactobacillus pentosus MT-4 was anaerobically cultured in the MRS medium at 37°C for 24 h. *W. anomalus* yeast was grown in the YPED medium at 30°C for 24 h, and *S. xylosus* was grown in the MS medium at 37°C for 24 h. After culturing, the number of living cells was measured by spreading them on the corresponding agar medium under the same conditions as the corresponding liquid culture. The cells were harvested at 5,000 × *g* at 4°C for 10 min, washed with sterilized physiological saline three times, and resuspended in sterilized physiological saline. Then, the cell concentration of *L. pentosus* MT-4, *W. anomalus* yeast, and *S. xylosus* was adjusted to 6.0×10^7, 7.0×10^7, and 7.0×10^7 CFU/mL, respectively.

Selection and Treatment of Raw Rump

Rump with dark red, long fibers, less fat fascia, elastic luster, natural odor, slightly dry appearance, and nonsticky hand with a "marble pattern" was selected as the raw material based on the National Food Hygiene Standard GB 2707-2005 (Ministry of Health, 2005). The raw rump was sliced along muscle lines, fascia and fat were removed, and the blood was washed out with water. The rump strips were divided into rump chunks of $3 \times 2 \times 2$ cm^3, which were neat and uniform in thickness and stored at 4°C for no more than 3 h.

Inoculation, Fermentation, Baking, and Packaging of Niuganba

The washed and cut rump chunks were soaked in white liquor for about 20 min and inoculated with 0.8% MT-4 (6.0×10^7 CFU/mL), 0.8% *W. anomalus* yeast (7.0×10^7 CFU/mL), and 1% *S. xylosus* (7.0×10^7 CFU/mL). Then, the following ingredients (g/100 g rump) were added to the meat: glucose (1.5), salt (1.5), *Perilla* seed (2), tea polyphenols (0.01), ginger (6), red pepper (0.6), garlic (6) orange peel (1) and pepper (1). Repeated marination was carried out until the surface of the meat was wet and soft. The mixture was then fermented in closed containers at 20°C for 36 h. Subsequently, the fermented NGB was taken out and placed in an oven at 70°C for drying until the moisture content of NGB was approximately 30%. Subsequently, it was cooled to room temperature and packed under vacuum.

Storage and Sample Analysis

Vacuum-packaged NGB samples were stored in the incubator at 278, 283, and 288 K for different storage times. The samples were randomly taken out at frequent intervals appropriate for each storage time to determine the number of *Pseudomonas* ($\log_{10}$ CFU/g), S_T, and pH_T.

Colony Count of Pseudomonas

In the aseptic operation room, each NGB sample (25 g) was homogenized in sterile saline (225 mL). The solution made using sterile saline was spread onto the *Pseudomonas* agar plate and incubated at 30°C for 72 h to determine the number (CFU/g) of *Pseudomonas* (Gospavic et al., 2008; ISO, 2010).

Determination of Surface Area and pH

For determining the surface area (*S*), a paper towel was wrapped around NGB samples, and then the area of the paper towel was measured in m^2 or cm^2. The pH was determined with a pH meter.

Principle and Hypothetical Model of Dimensional Analysis

Principle of Dimensional Analysis

As mentioned in the introduction, the main factors affecting N_T/N_0 (where N_T is the number of microorganisms per unit mass of food at a certain time T, and N_0 is the initial number of microorganisms per unit mass of food) include food mass M, temperature θ (reflection of the translational kinetic energy of the internal molecules or atoms of the food microorganism system), time T, surface area S_T at time T, pH_T (pH at time T), and a_{wT} (water activity at time T).

When the microorganisms are in lag time (λ). N_T is obviously equal to N_0. As food is deteriorated by spoilage microorganisms at a later stage, this study focused on the changes in food microorganisms after the lag time. Therefore, $N_T/N_0 = f$ (M, S_T, θ, T-λ, pH_T, a_{wT}) ($T > \lambda$). N_T/N_0 is obviously dimensionless. The pH_T, and a_{wT} are also dimensionless. M, S_T, θ, and T-λ mean food mass (kg), surface area (m^2), temperature (K), and time (S), respectively. The growth of microorganisms is related to energy. Absolute temperature is proportional to the average kinetic energy of molecules or atoms of a system. As mentioned in the introduction, the influence of food temperature on microorganisms can be regarded as the influence of the average kinetic energy [$c\theta$(KJ, c is scale coefficient, KJ·K^{-1}.)] of molecules or atoms in the food–microorganism system. Then, the parameters M, S_T, $c\theta$, and T-λ can be combined into the formula $\frac{c\theta(T-\lambda)^2}{MS_T}$, and hence its dimension is expressed as follows:

$$\frac{[\mathrm{KJ} \bullet \mathrm{K}^{-1} \times K \times \mathrm{S}^2]}{[\mathrm{Kg} \times \mathrm{m}^2]} = \frac{[\mathrm{KJ}]}{[\mathrm{Kg} \times \mathrm{m}^2 \times \mathrm{S}^{-2}]} = \frac{[\mathrm{KJ}]}{[\mathrm{KJ}]} = [\mathrm{KJ}]^0$$

It is a dimensionless number. Let He = $\frac{c\theta(T-\lambda)^2}{MS_T}$. Relative to the storage time T of microorganisms, λ is usually small. Subsequently, only the case where the storage time T is much

greater than λ is considered, so T-λ is approximately equal to t, that is He = $\frac{c\theta(T-\lambda)^2}{MS_T} \approx \frac{c\theta T^2}{MS_T}$. According to the Pi theorem, $N_T/N_0 = \varphi$ (He, pH_T, and a_{wT}). In natural convection under some conditions, the Nusselt number Nu was correlated by a power function with Reynolds number Re, Prandtl number Pr, and Grashof number Gr; $Nu = CRe^m Pr^n Gr^k$ (where C, m, n, and k are undetermined constants) (McAdams, 1954; Nagy, 2019). Hence, whether the dimensionless number (N_T/N_0) is correlated by a power function with He, pH_T, and a_{wT} needs exploration. Suppose they are related through the following hypothetical Equation 1:

When the storage time T is much greater than λ,

$$N_T/N_0 = f(He,\ pH_T,\ a_{wT}) = j_1[\frac{c\theta T^2}{MS_T}]^{n_1} pH_T^{n_2} a_{wT}^{n_3} (T \gg \lambda) \quad (1)$$

where c and j1 are constants. Under normal circumstances, the food quality M can be considered basically unchanged before the food is spoiled. Hence M is also a constant. Therefore, Equation 1 becomes Equation 2:

$$N_T = j_1 N_0 [c/M]^{n_1} [\frac{\theta T^2}{S_T}]^{n_1} pH_T^{n_2} a_{wT}^{n_3} \quad (2)$$

Because N_0 is also a constant, let $j_1 N_0 [c/M]^{n1} = j_0$. Then, Equation 2 becomes:

$$N_T = j_0 (\theta T^2/S_T)^{n_1} pH_T^{n_2} a_{wT}^{n_3} \quad (3)$$

where T is the time (S), N_T is the number of microorganisms (CFU/g) at time T; θ is the temperature (K); N_0 is the initial number of microorganisms (CFU/g); S_T is the surface area (m^2); j_0, n_1, n_2, and n_3, are unknown constants; and j_0 closely correlated with food nutrition and initial microbial concentration.

Hypothetical Model for Predicting Microbes in Niuganba

Considering the sealed packaging of NGB, it was believed that a_w changed little, and its effects on the number of *Pseudomonas* remained unchanged within shelf life. Therefore, for simplifying the calculation, the effects of a_w on microbial growth were considered as constant j_0 in the microbial growth prediction in the subsequent experiment. Thus, Equation 3 became:

$$N_T = j_2 j_0 (\theta T^2/S_T)^{n_1} pH_T^{n_2} \quad (4)$$

Let $_{j2j0 = j}$, then, Equation 4 became:

$$N_T = j\ (\theta T^2/S_T)^{n_1} pH_T^{n_2} \quad (5)$$

Let $y = N_T$, $x_1 = \theta T^2/S_T$, and $x_2 = pH_T$. After taking the logarithm of both sides of Equations 5, 6 were obtained:

$$Logy = Logj + n_1 Logx_1 + n_2 Logx_2 \quad (6)$$

Both Equations 5, 6 were referred to the dimensional analysis model (DAM).

Application of Hypothetical Model Dimensional Analysis Model for Predicting Microbial Growth in Niuganba

The vacuum-packaged NGB was stored at a certain temperature for some days. Then, some samples were randomly taken out (sealed after sampling) to determine the number of *Pseudomonas*, S_T, and pH for predicting microbial growth and storage time.

Validation of the Model

In this study, two validation procedures were performed. First, for internal validation, the model was validated against the same data used to build the model (Dong et al., 2007). It ensured that the model accurately described the data from which it was generated and represented any biological trends in the data. For external evaluation (Giffel and Zwietering, 1999), new data from the storage of NGB samples selected randomly within the range of experimental design were used. The accuracy of the models describing microbial growth was evaluated using the following seven criteria: coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}), median relative error (RE) (Equation 12) of model predictions, root mean square error (RMSE) (Hu et al., 2018; Antunes-Rohling et al., 2019; Park et al., 2020), %SEP, accuracy factor (A_f), and bias factor (B_f) (Baranyi et al., 1993; Ross, 1996; Tarlak et al., 2018), expressed as Equations 7–13, respectively.

$$R^2 = 1 - \frac{\sum_{i=1}^{n} (N_{oberseved} - N_{predicted})^2}{\sum_{i=1}^{n} (N_{oberseved} - N_{\overline{oberseved}})^2} \quad (7)$$

$$R^2_{adj} = 1 - \frac{(1 - R^2)(n - 1)}{(n - N - 1)} \quad (8)$$

$$RE = \frac{N_{predicted} - N_{observed}}{N_{observed}} \quad (9)$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n} (N_{observed} - N_{predicted})^2}{n}} \quad (10)$$

$$\%SEP = \frac{100}{\overline{N_{observed}}} \sqrt{\frac{\sum_{i=1}^{n} (N_{observed} - N_{predicted})^2}{n}} \quad (11)$$

$$A_f = 10^{(\sum_{i=1}^{n} \left|\log(N_{predicted}/N_{observed})\right|/n)} \quad (12)$$

$$B_f = 10^{(\sum_{i=1}^{n} log(N_{predicted}/N_{obseerved})/n)} \quad (13)$$

$N_{predicted}$ and $N_{observed}$ refer to the predicted number of microorganisms (log_{10} CFU/g) and the observed number of microorganisms (log_{10} CFU/g), respectively, and $N_{\overline{observed}}$ represents the mean of the observed number of microorganisms (log_{10} CFU/g). Also, n represents the number of observations, and N is the number of variable parameters in the predictive model. The goodness of fit (R^2) and RMSE were used as a quantitative means of measuring the performance of the model. The success of the model in predicting the

dependent variables from the independent variables increased when the values of R^2 were closer to 1. The RMSE values approached zero, indicating that the data closely fitted the model. R^2_{adj} was based on the squared Pearson correlation coefficient considering the number of experimental points and parameters. Good fits were obtained when R^2_{adj} values were almost one.

The B_f estimates a mean variation between the predicted and observed values. A_f, which is analogous to RMSE, estimates the mean difference between the predicted and observed values, disregarding whether the difference is positive or negative. A value of 1 for A_f and B_f indicates an exact agreement between predicted and observed values.

Data Analysis

Each experiment was repeated independently three times, and the data were presented as mean ± standard deviation. Statistical software SPSS 19.0 and Origin 2018 were used for correlation analysis, regression analysis, calculation of the goodness-of-the-fit parameters, and plotting.

RESULTS

Application of Dimensional Analysis Model in Niuganba

In the first week, *Pseudomonas* in NGB was not detected when it was stored at 5 or 10°C, and the content of *Pseudomonas* detected was very low when it was stored at 15°C (only 2.67 CFU/g). The plate method could not accurately detect such a low content of *Pseudomonas*, that is, for the low content of *Pseudomonas* during the initial first week of storage of NGB, the plate method could not detect it or the detected data were very low, and its accuracy was difficult to meet the requirements. Additionally, as shown in **Figure 1**, the number of *Pseudomonas* in NGB stored at 5°C for 20 days, 10°C for 10 days, and 15°C for 10 days was 16.6 CFU/g, 10.2 CFU/g, and 10.3 CFU/g, respectively. This indicated that the content of *Pseudomonas* in the first week was very low and difficult to detect accurately. The content of *Pseudomonas* was so low that it would not certainly result in the corruption of NGB in the first week. Therefore, the data of *Pseudomonas* in the first week were unnecessary to include in the model in this study. In addition, during the experiment, the growth characteristics of

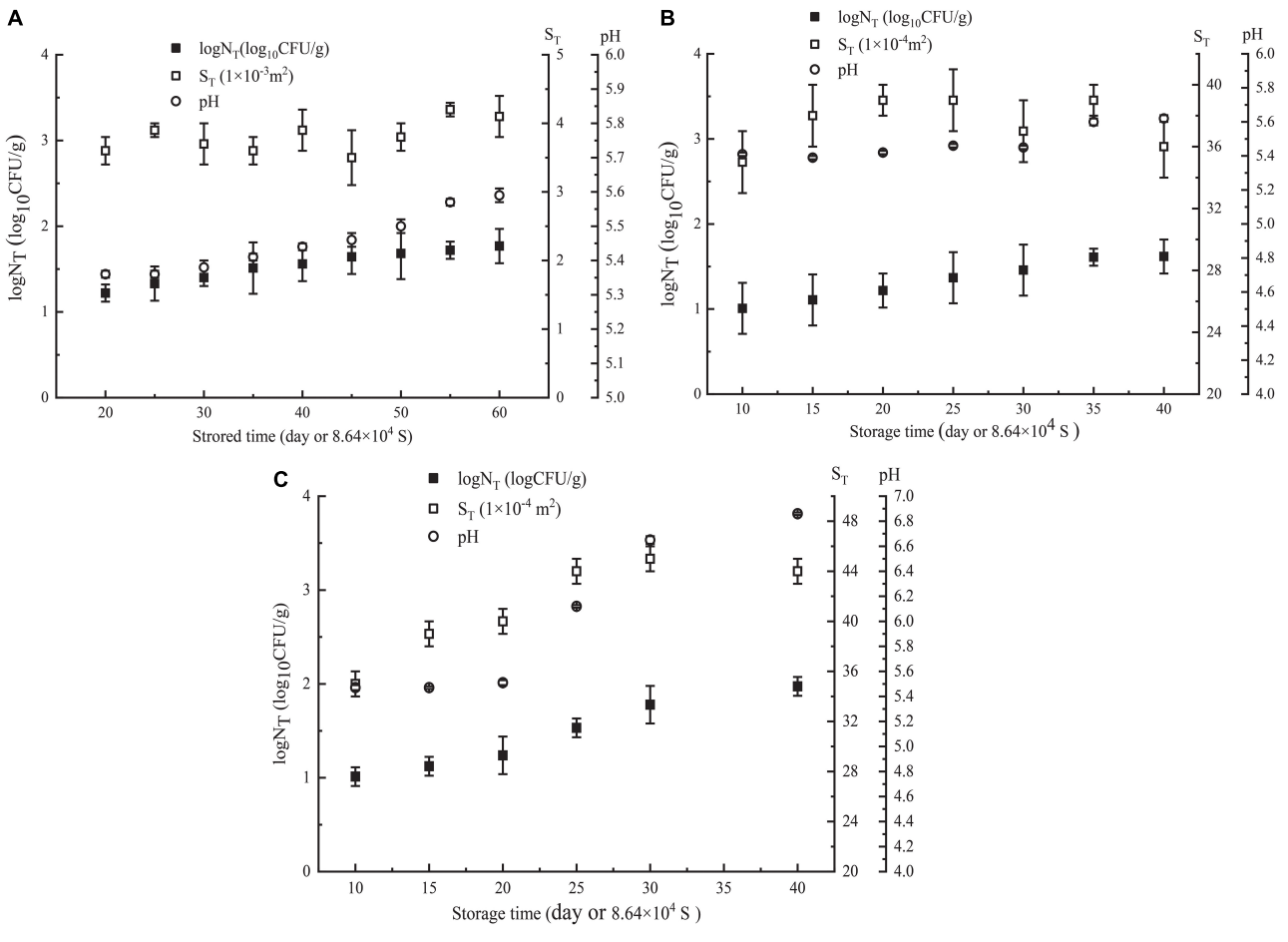


FIGURE 1 | Parameters of NGB (logN_T, S_T, storagetime, and pH values) obtained at different θ. **(A)** θ = 278 K, **(B)** θ = 283 K, and **(C)** θ = 288 K. N_T means observed *Pseudomonas* number (CFU/g). Error bars represent the standard deviation of three independent samples.

microorganisms exhibited significant differences in the early and late stages of storage. This was because food spoilage occurred mainly in the late stage of storage, and the early stage was generally short. The microbial growth data were taken just after the early stage.

Establishment of Dimensional Analysis Model on the Growth of Pseudomonas in Niuganba

The number of *Pseudomonas* increased with increasing storage time; the effect of storage time on pH showed a similar trend as the number of *Pseudomonas* (**Figure 1**). This was in agreement with the relationship between the number of microorganisms, pH value, and storage time of this type of food. Moreover, **Figure 2** shows that LogN_T and He number ($\theta T^2/S_T$) had a linear relationship with pH_T. The data in **Figure 1** were used in Equation 5. Using SPSS software, Equation 14 for predicting the number of *Pseudomonas* in NGB was obtained.

$$\text{Log}y = -10.445 + 0.495\,\text{Log}x_1 + 4.243\,\text{Log}x_2$$
$$(R^2 = 0.992 \text{ and } R^2_{\text{adj}} = 0.991) \quad (14)$$

Equation 14 was equivalently changed to Equations 15, 16.

$$N_T = 10^{-10.445}(\theta T^2/S_T)^{0.495} pH_T^{4.243} (T \geq 10 \text{ days}, T \gg \lambda) \quad (15)$$

$$\text{Therefore, } T^{0.99} = \frac{10^{10.445} N_T}{pH^{4.243}} \left(\frac{S_T}{\theta}\right)^{0.495} (278K \leq \theta \leq 288K) \quad (16)$$

where N_T and T are the number of *Pseudomonas* and storage time (second) of NGB, respectively.

The model achieved R^2 values higher than 0.992 and very good R^2_{adj} = 0.991. It indicated that the DAM based on the dimensional analysis and the Pi theorem could be used to predict the number of *Pseudomonas* N_T and storage time T of NGB with high accuracy and precision. Equations 15, 16 were validated as follows.

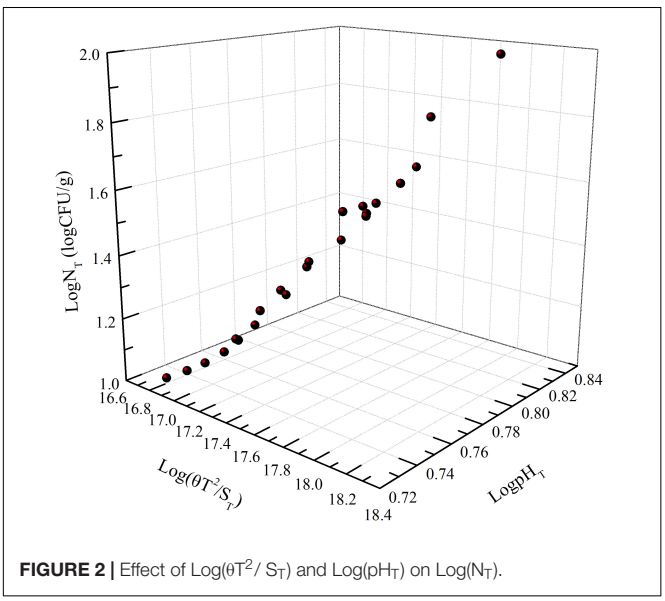


FIGURE 2 | Effect of Log(θT^2/ S_T) and Log(pH_T) on Log(N_T).

Validation of the Dimensional Model

Validation is a vital step for assessing the ability of a new model to interpolate. In this study, R^2, R^2_{adj}, RE, RMSE, standard error of prediction (%SEP), A_f, and B_f were used to evaluate and validate the DAM. R^2 and R^2_{adj} were between 0 and a value close to 1, indicating that they were less than 1; and the closer they got to 1, the better, and the smaller the values of | RE|, RMSE, and %SEP, the better.

R^2 and R^2_{adj} for the Dimensional Model

The goodness of fit of the DAM used was evaluated by considering R^2 and R^2_{adj} values using Equations 6, 7, respectively. **Figures 3**, **4** show that the range of R^2 and R^2_{adj} of all predicted and observed values was 0.966 − 0.992, irrespective of external (data from **Figure 5**) or internal verification (data from **Figure 1**), and observed and predicted values of the microbial growth or storage time. These results indicated that the predicted value was very close or equal to the observed value, suggesting that a DAM could predict the microbial growth (or storage time, shelf life) based on the principles of dimensional analysis and Pi theorem.

MRE, Root Mean Square Error, Standard Error of Prediction, A_f, and B_f for Dimensional Analysis Model

Validation parameters for DAM describing the growth of microorganisms or storage time for NGB, MRE, RMSE, %SEP, A_f, and B_f, were easily calculated based on the predicted number of *Pseudomonas* and the observed number of *Pseudomonas* in **Figures 3**, **4**, as shown in **Table 1**. The MRE and RMSE values for validation ranged from 0.60 to 5.31% and from 0.0302 to 2.8846, respectively. The SEP for validation ranged from 2.0838 to 9.4016%. These results indicated that the predictive model yielded the lowest MRE, RMSE, and SEP. Therefore, the predictive model was selected as the best model to fit the number of *Pseudomonas* and the storage time of NGB.

Model validation was also performed by considering the bias (B_f) and accuracy (A_f) using Equation 9, 10, respectively. Ross et al. (2000) reported that the predictive models should ideally have an A_f = 1.00, indicating a perfect model fit, where the predicted and actual response values were equal. The acceptable A_f values were in the range of 1.10–1.15. The B_f value of 1 indicated no structural deviation of the predictive model. Ross (1996) concluded that the range of B_f from 0.9 to 1.05 could be considered perfect for the models, while 0.7–0.9 or 1.06–1.15 was considered to be acceptable, and < 0.7 or > 1.15 was considered to be unacceptable.

The A_f values ranged from 1.0127 to 1.0941, most of which were less than 1.0; the maximum A_f value was less than 1.10. The B_f values for both equations ranged from 0.9985 to 1.0617, all of which were less than 1.06, indicating that the observed data were very close to the equivalence line of fail-safe and fail-dangerous regions. These results revealed that the models could be safely used because the error rates were relatively low.

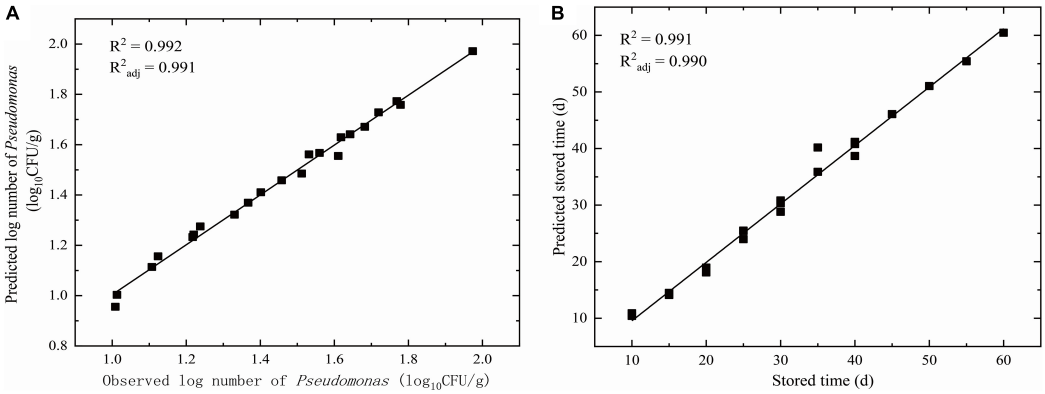


FIGURE 3 | Linear fit for observed and predicted numbers based on DAM for NGB from internal data. **(A)** Observed and predicted numbers of *Pseudomonas*; **(B)** Observed and predicted storage time.

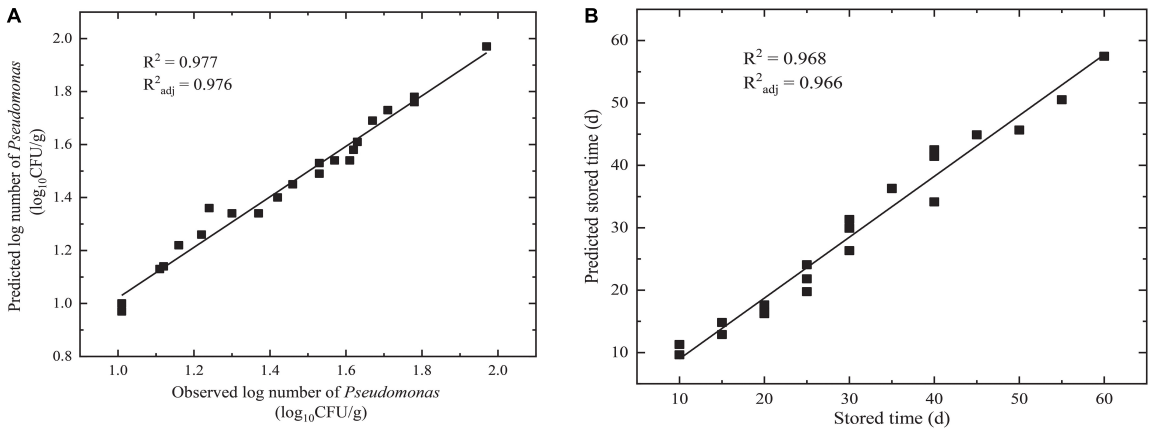


FIGURE 4 | Linear fit for observed and predicted numbers based on DAM for NGB from external data. **(A)** Observed and predicted numbers of *Pseudomonas*; **(B)** observed and predicted storage times.

TABLE 1 | Validation parameters for DAM describing the growth of microorganisms or storage time for NGB.

Sample	Equation	Data	MRE /%	RMSE	%SEP	A_f	B_f
NGB	Equation 15 (predicted logN_T)	Internal	0.60	0.0302	2.0838	1.0127	1.0001
		External	1.60	0.0524	3.6231	1.0228	0.9985
	Equation 16 (predicted storage time)	Internal	2.83	1.4540	4.7388	1.0423	0.9990
		External	5.31	2.8846	9.4016	1.0941	1.0617

DISCUSSION

The prediction of the number of microorganisms is of great value for food quality and safety. The microbial growth model based on dimensional analysis and Pi theorem was presented in this study, considering that dimensional analysis and the Pi theorem are widely applied in physics and chemistry, providing a method for diluting complex phenomena to the most simplified form (Buckingham, 1914; Chandarana et al., 2010): $N_T/N_0 = f(M, S_T, \theta, T, pH_T$, and $a_{wT})$. Also, a hypothetical DAM was established for predicting the number of microorganisms: $N_T = j_1 N_0 [c/M]^{n_1} [\frac{\theta T^2}{S_T}]^{n_1} pH_T^{n_2} a_{wT}^{n_3}$ $or N_T = j_0 (\theta T^2/S_T)^{n_1} pH_T^{n_2} a_{wT}^{n_3}$. The DAM reflected the effect of food mass M and environmental variables, such as temperature θ, time T, initial microbe quantity per unit mass N_0, surface area S_T, and pH_T on N_T. The DAM introduced a dimensionless number He ($c\theta T^2/MS_T$), which was an interesting feature of this model. The DAM could be further simplified as $N_T = 10^{-10.445} (\theta T^2/S_T)^{0.495} pH_T^{4.243}$, when it was used to predict the number of *Pseudomonas* in sealed NGB. The results showed that DAM had a high R^2_{adj}. The internal and external verifications confirmed that DAM could be used to predict well the number of *Pseudomonas* and the storage time of NGB. They also validated the hypothesis that the dimensionless number

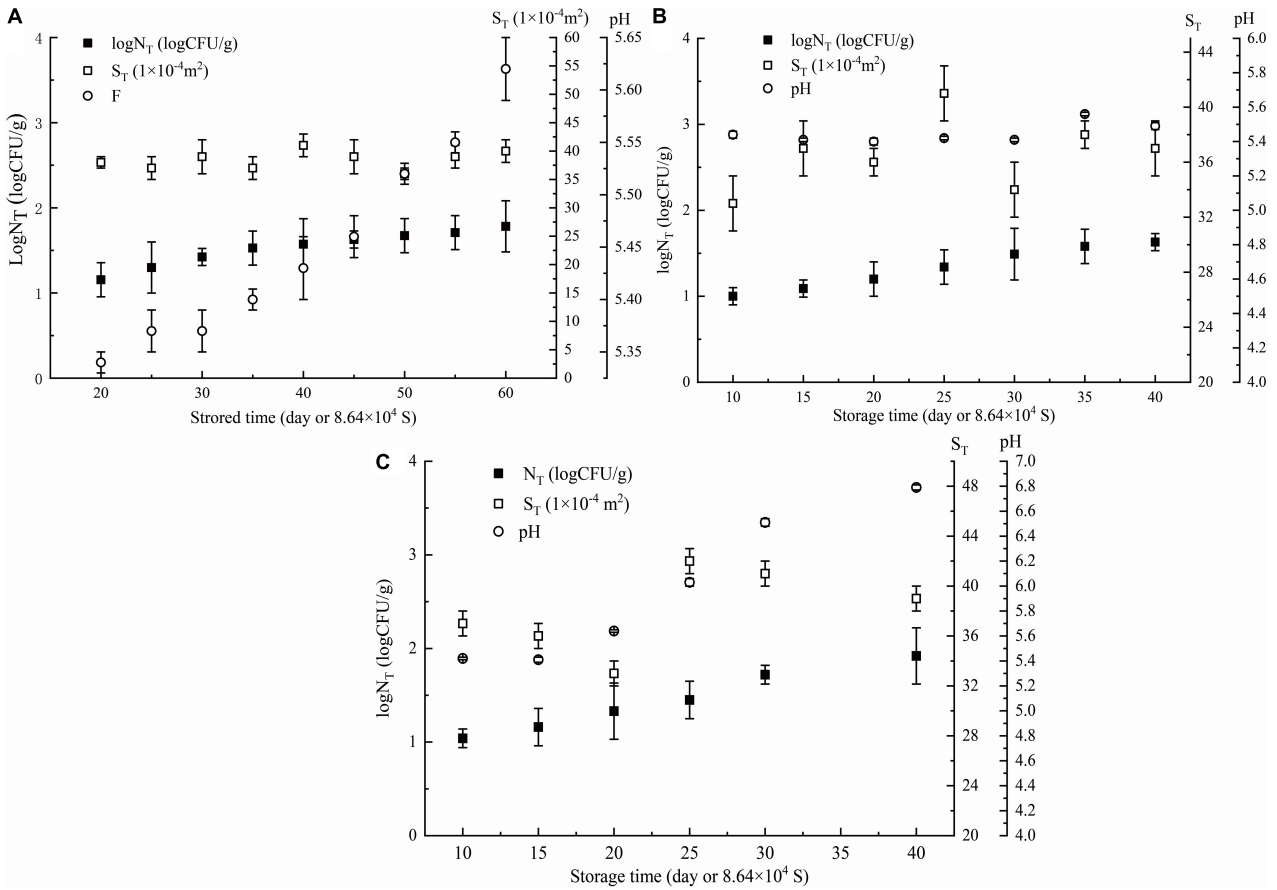


FIGURE 5 | External data: parameters of NGB (logN_T, S_T, storage time, and pH) obtained at different θ, **(A)** θ = 278 K, **(B)** θ = 283 K, **(C)** θ = 288 K. N_T means observed *Pseudomonas* number (CFU/g). Error bars represent the standard deviation of three independent samples.

N_T/N_0 was correlated by a power function with the He, pH_T, and a_{wT}.

Of course, biologically useful energy also affects microorganism growth. This energy must be stored in food nutrients, and food nutrients are rich relative to a small number of spoilage microorganisms. It can be considered that their content remains basically unchanged before food spoilage. Therefore, their impact on microorganisms can be regarded as a constant and can be combined with the constant term of DAM. In this way, the main contradictions can be grasped and the efficiency of solving problems can be greatly improved. Otherwise, if every aspect is considered, the problem cannot be started and solved.

One advantage of the DAM was that it did not require the initial number of microorganisms. It was emphasized that the value of N_0 was not needed. However, it did not mean that N_0 had no impact on the number of spoilage microorganisms during storage. Its impact on spoilage microorganisms must be the greatest because N_T was the result of the growth of N_0, that is, N_T equaled $j_1 N_0[c/M]^{n_1}[\frac{\theta T^2}{S_T}]^{n_1} pH_T^{n_2} a_{wT}^{n_3}$. Just because N_0 was a constant, it could be incorporated into the constant term of the equation, that is $N_T = j_0(\theta T^2/S_T)^{n_1} pH_T^{n_2} a_{wT}^{n_3}$. Therefore, this undoubtedly simplified the method of predicting microorganisms. However, microbial prediction models generally required the N_0 value (Park et al., 2020; Tarlak et al., 2020; Yu et al., 2020), which was obtained by direct measurement or curve fitting. Fitting was required at this time because the initial number of spoilage microorganisms in food was usually very low and difficult to detect. However, the fitting usually had errors. Therefore, the model proposed in this study, which did not require the N_0 value (of course, this model did not object to the known N_0), undoubtedly improved the prediction accuracy. Another advantage of this model was that the specific growth rate that was required to be calculated in the general prediction model was not required here (Park et al., 2020; Tarlak et al., 2020; Yu et al., 2020), thereby simplifying the calculation process. Moreover, general models require multiple equations to be combined for prediction. However, one equation of the proposed model could be used to predict the number of microorganisms and the storage time.

Although the number of *Pseudomonas* in NGB was predicted using the DAM, it is conceivable that the DAM can be extended to more general cases: replace NGB with any other food,

and replace the *Pseudomonas* with any other microorganism. The growth of any microorganism in any food can be determined using a similar method. The growth inhibition prediction model of any microorganism in any food can also be obtained. Most importantly, the DAM can have a general expression:$N_T = j_0(\theta T^2/S_T)^{n_1} pH_T^{n_2} a_{wT}^{n_3}$. By adjusting the DAM parameters, the prediction model of changes in specific microorganisms in specific food and under specific storage conditions can be established. This unified the prediction model of food microorganisms from the perspectives of biology, physics, and food science. These advantages can undoubtedly help promote the application of the model and reveal the nature of the biophysical mathematical principles behind the growth-inhibitory properties of food microorganisms.

CONCLUSION

A DAM based on dimensionless analysis and Pi theorem was introduced in this study. The internal and external verifications suggested the perfect prediction of the number of *Pseudomonas* in sealed NGB and the storage time of NGB, thus proving the rationality and feasibility of this model. An important characteristic of the model was that this model introduced the He number ($\frac{c\theta T^2}{MS_T}$). This model could be used to predict both the number of microorganisms and the storage time. Most importantly, based on the same principle, it was also inferred that this model could be used to predict the growth inhibition of any microorganism, and it might represent a universal model based on biology, physics, and food science. These advantages simplified the prediction process. This study laid a strong foundation for applying this model in predicting the number of microorganisms in food products.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

CL performed the data analysis and wrote the original manuscript. LH proposed the dimensional analysis model, supervised the experiment, wrote, reviewed and edited the manuscript. YH prepared the NGB. HL performed the experiment of storage. XW performed the data analysis and reviewed the manuscript. LC and XZ reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was financially supported by the Key Agricultural Project of Guizhou Province [QKHCG-(2021)YB031, QKHZC-(2019)2382, QKHZC-(2021)YB184, QKHZC-(2021)YB278, QKHZC-(2021)YB142, and QKHZC-(2016)2580], the National Key R&D Program of China (Grant no. 2018YFD0401200), the National Natural Science Foundation of China (31870002, 31160002, and 31801509), the Qiankehe talents project [(2018)5781, (2017)5788, and (2017)5788-11], the Guizhou Science and Technology Program [QKHPTRC (2020) 5004, (2019)1078], and the High-level innovative talents training project of Guizhou province [QKHPTRC(2016)5662].

REFERENCES

Antunes-Rohling, A., Artaiz, Á, Calero, S., Halaihel, N., Guillén, S., Raso, J., et al. (2019). Modelling microbial growth in modified-atmosphere-packed hake (*Merluccius merluccius*) fillets stored at different temperatures. *Food Res. Int.* 122, 506–516. doi: 10.1016/j.foodres.2019.05.018

Baranyi, J., McClure, P. J., Sutherland, J. P., and Roberts, T. A. (1993). Modelling bacterial growth responses. *J. Ind. Microbiol. Biotechnol.* 12, 190–194. doi: 10.1007/BF01584189

Bormashenko, E. J. E. (2020). What is the temperature? Modern outlook on the concept of temperature. *Entropy* 22:1366. doi: 10.3390/e22121366

Buchanan, M. (2010). Dimensional analysis. *Nat. Phys.* 6, 555–555. doi: 10.1038/nphys1744

Buckingham, E. (1914). On physically similar systems; illustrations of the use of dimensional equations. *Phys. Rev.* 4, 345–376. doi: 10.1103/PhysRev.4.345

Chandarana, D. I., Iii, A. G., and Wheaton, F. W. (2010). Particle/fluid heat transfer under UHT conditions at low particle/fluid relative velocities. *J. Food Proc. Eng.* 13, 191–206. doi: 10.1111/j.1745-4530.1990.tb00067.x

Davey, K. R. (2010). A predictive model for combined temperature and water activity on microbial growth during the growth phase. *J. Appl. Microbiol.* 67, 483–488. doi: 10.1111/j.1365-2672.1989.tb02519.x

de-Man, J. C., Rogosa, M., and Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* 23, 130–135. doi: 10.1111/j.1365-2672.1960.tb00188.x

Dong, Q., Tu, K., Guo, L., Li, H., and Zhao, Y. (2007). Response surface model for prediction of growth parameters from spores of *Clostridium sporogenes* under different experimental conditions. *Food Microbiol.* 24, 624–632. doi: 10.1016/j.fm.2006.12.003

Dyer, J. M. (1996). The sorting sequence of the peroxisomal integral membrane protein PMP47 is contained within a short hydrophilic loop. *J. Cell Biol.* 133, 269–280. doi: 10.1083/jcb.133.2.269

Giannuzzi, L., Pinotti, A., and Zaritzky, N. (1998). Mathematical modelling of microbial growth in packaged refrigerated beef stored at different temperatures. *Int. J. Food Microbiol.* 39, 101–110. doi: 10.1016/s0168-1605(97)00127-x

Giffel, M. C. T., and Zwietering, M. H. (1999). Validation of predictive models describing the growth of Listeria monocytogenes. *Int. J. Food Microbiol.* 46, 135–149. doi: 10.1016/S0168-1605(98)00189-5

Gospavic, R., Kreyenschmidt, J., Bruckner, S., Popov, V., and Haque, N. J. (2008). Mathematical modelling for predicting the growth of *Pseudomonas* spp. in poultry under variable temperature conditions. *Int. J. Food Microbiol.* 127, 290–297. doi: 10.1016/j.ijfoodmicro.2008.07.022

Hu, J., Lin, L., Chen, M., and Yan, W. (2018). Modeling for predicting the time to detection of staphylococcal enterotoxin a in cooked chicken product. *Front. microbiol.* 9:1536. doi: 10.3389/fmicb.2018.01536

Isabelle, L., and André, L. (2006). Quantitative prediction of microbial behaviour during food processing using an integrated modelling approach: a review. *Int. J. Refrig.* 29, 968–984. doi: 10.1016/j.ijrefrig.2006.04.008

ISO (2010). *Meat And Meat Products — Enumeration of Presump-Tive Pseudomonas* Spp. ISO 13720. Geneva: ISO.

Kuneš, J. (2012). *Dimensionless Physical Quantities in Science and Engineering*. Amsterdam: Elsevier, 427–441.

Longhi, D. A., Tremarin, A., Carciofi, B. A. M., Laurindo, J. B., and Aragão, G. M. F. D. (2014). Modeling the growth of Byssochlamys fulva on solidified apple juice at different temperatures. *Braz. Arch. Biol. Technol.* 57, 971–978. doi: 10.1590/S1516-8913201402619

McAdams, W. H. (1954). *Heat Transmission*, 3rd Edn. New York, NY: McGraw-Hill Book Co Inc.

McKellar, R. C., and Lu, X. (2003). *Modeling Microbial Responses in Food*, 1st Edn. Boca Raton, FL: CRC.

Mcmahon, T. A. (1973). Size and shape in biology. *Science* 179, 1201–1204.

McMeekin, T. A., Baranyi, J., Bowman, J., Dalgaard, P., Kirk, M., Ross, T., et al. (2006). Information systems in food safety management. *Int. J. Food Microbiol.* 112, 181–194. doi: 10.1016/j.ijfoodmicro.2006.04.048

Mcmeekin, T. A., Olley, J., Ratkowsky, D. A., and Ross, T. (2002). Predictive microbiology: towards the interface and beyond. *Int. J. Food Microbiol.* 73, 395–407. doi: 10.1016/S0168-1605(01)00663-8

Ministry of Health (2005). *Hygienic Standards For Fresh (Frozen) of Livestock, National Standards of The People's Republic of China, (GB 2707-2005)*. Beijing: China Standards Press

Mohos, F. (2017). "Principles of food engineering," in *Confectionery and Chocolate Engineering* (Hoboken, NJ: Wiley), 1–22. doi: 10.1002/9781118939741.ch1

Molina, M., and Giannuzzi, L. (1999). Combined effect of temperature and propionic acid concentration on the growth of *Aspergillus parasiticus*. *Food Res. Int.* 32, 677–682. doi: 10.1016/S0963-9969(99)00146-5

Nagy, E. (2019). "Chapter 19 – membrane distillation," in *Basic Equations of Mass Transport Through A Membrane Layer*, 2nd Edn, ed. E. Nagy (Amsterdam: Elsevier), 483–496.

Park, J. H., Kang, M. S., Park, K. M., Lee, H. Y., Ok, G. S., Koo, M. S., et al. (2020). A dynamic predictive model for the growth of *Salmonella* spp. and Staphylococcus aureus in fresh egg yolk and scenario-based risk estimation. *Food Control* 118:107421. doi: 10.1016/j.foodcont.2020.107421

Pilbeam, D., and Gould, S. J. (1974). Size and scaling in human evolution. *Science* 186, 892–901. doi: 10.1126/science.186.4167.892

Ross, T. (1996). Indices for performance evaluation of predictive models in food microbiology. *J. Appl. Bacteriol.* 81, 501–508. doi: 10.1111/j.1365-2672.1996.tb03539.x

Ross, T., Dalgaard, P., and Tienungoon, S. (2000). Predictive modelling of the growth and survival of Listeria in fishery products. *Int. J. Food Microbiol.* 62, 231–245. doi: 10.1016/s0168-1605(00)00340-8

Ruzicka, M. C. (2008). On dimensionless numbers. *Chem. Eng. Res. Des.* 86, 835–868. doi: 10.1016/j.cherd.2008.03.007

Sautour, M., Mary, P., Chihib, N. E., and Hornez, J. P. (2003). The effects of temperature, water activity and pH on the growth of *Aeromonas hydrophila* and on its subsequent survival in microcosm water. *J. Appl. Microbiol.* 95, 807–813. doi: 10.1046/j.1365-2672.2003.02048.x

Singh, R. (2017). Modelling the cast component weight in hot chamber die casting using combined Taguchi and Buckingham's π approach. *J. Inst. Eng.* 99, 1–8. doi: 10.1007/s40032-017-0345-2

Tamplin, M. L. (2018). Integrating predictive models and sensors to manage food stability in supply chains. *Food microbiol.* 75, 90–94. doi: 10.1016/j.fm.2017.12.001

Tarlak, F., Johannessen, G., Bascón Villegas, I., Bolívar Carrillo, A., Posada-Izquierdo, G. D., and Pérez-Rodríguez, F. (2020). Modelling of the behaviour of *Salmonella enterica* serovar reading on commercial fresh-cut iceberg lettuce stored at different temperatures. *Foods* 9:946. doi: 10.3390/foods9070946

Tarlak, F., Ozdemir, M., and Melikoglu, M. (2018). Mathematical modelling of temperature effect on growth kinetics of *Pseudomonas* spp. on sliced mushroom (*Agaricus bisporus*). *Int. J. Food Microbiol.* 266, 274–281. doi: 10.1016/j.ijfoodmicro.2017.12.017

Tian, X., Liu, H., Wang, X., Li, C., He, L., and Zeng, X. (2021). Using combined optimization and vacuum freeze drying technology to prepare directed vat set starter for "Niuganba", a fermented beef. *J. Food Process. Preserv.* 45, doi: 10.1111/jfpp.15694

Whiting, R. C., and Buchanan, R. L. (1993). Letter to the editor: a classification of models in predictive microbiology - a reply to K. *R. Davey. Food Microbiol.* 10, 175–177. doi: 10.1006/fmic.1993.1017

Xiong, R., Xie, G., Edmondson, A. S., Linton, R. H., and Sheard, M. A. (1999). Comparison of the Baranyi model with the modified Gompertz equation for modelling thermal inactivation of Listeria monocytogenes Scott A. *Food Microbiol.* 16, 269–279. doi: 10.1006/fmic.1998.0243

Xu, R., Liu, B., He, X., and Li, D. (2012). Kinetic energy-based temperature computation in non-equilibrium molecular dynamics simulation. *J. Comput. Theor. Nanosci.* 9, 428–433. doi: 10.1166/jctn.2012.2042

Yu, H. H., Song, Y. J., Kim, Y. J., Lee, H. Y., Choi, Y.-S., Lee, N.-K., et al. (2020). Predictive model of growth kinetics for Staphylococcus aureus in raw beef under various packaging systems. *Meat Sci.* 165:108108. doi: 10.1016/j.meatsci.2020.108108

Zimmermann, M., Massaguer, P. R. D., and Aragão, G. M. F. (2013). Byssochlamys nivea growth in papaya juice as influenced by water activity and ascospore age. *Focus. Mod. Food Ind.* 2, 17–25.

Conflict of Interest: The authors declarethat the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

REVIEW
published: 07 March 2022
doi: 10.3389/fmicb.2022.845166

Two Faces of Fermented Foods—The Benefits and Threats of Its Consumption

Krzysztof Skowron[1], Anna Budzyńska[1], Katarzyna Grudlewska-Buda[1], Natalia Wiktorczyk-Kapischke[1], Małgorzata Andrzejewska[2], Ewa Wałecka-Zacharska[3] and Eugenia Gospodarek-Komkowska[1]*

[1] Department of Microbiology, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Bydgoszcz, Poland, [2] Department of Hygiene, Epidemiology, Ergonomy and Postgraduate Education, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Bydgoszcz, Poland, [3] Department of Food Hygiene and Consumer Health, Wrocław University of Environmental and Life Sciences, Wrocław, Poland

In underdeveloped and developing countries, due to poverty, fermentation is one of the most widely used preservation methods. It not only allows extending the shelf life of food, but also brings other benefits, including inhibiting the growth of pathogenic microorganisms, improving the organoleptic properties and product digestibility, and can be a valuable source of functional microorganisms. Today, there is a great interest in functional strains, which, in addition to typical probiotic strains, can participate in the treatment of numerous diseases, disorders of the digestive system, but also mental diseases, or stimulate our immune system. Hence, fermented foods and beverages are not only a part of the traditional diet, e.g., in Africa but also play a role in the nutrition of people around the world. The fermentation process for some products occurs spontaneously, without the use of well-defined starter cultures, under poorly controlled or uncontrolled conditions. Therefore, while this affordable technology has many advantages, it can also pose a potential health risk. The use of poor-quality ingredients, inadequate hygiene conditions in the manufacturing processes, the lack of standards for safety and hygiene controls lead to the failure food safety systems implementation, especially in low- and middle-income countries or for small-scale products (at household level, in villages and scale cottage industries). This can result in the presence of pathogenic microorganisms or their toxins in the food contributing to cases of illness or even outbreaks. Also, improper processing and storage, as by well as the conditions of sale affect the food safety. Foodborne diseases through the consumption of traditional fermented foods are not reported frequently, but this may be related, among other things, to a low percentage of people entering healthcare care or weaknesses in foodborne disease surveillance systems. In many parts of the world, especially in Africa and Asia, pathogens such as enterotoxigenic and enterohemorrhagic *Escherichia coli, Shigella* spp., *Salmonella* spp., enterotoxigenic *Staphylococcus aureus, Listeria monocytogenes,* and *Bacillus cereus* have been detected in fermented foods. Therefore, this review, in addition to the positive aspects, presents the potential risk associated with the consumption of this type of products.

Keywords: contaminated fermented products, fermented foods, food safety, foodborne, outbreaks

OPEN ACCESS

Edited by:
Laurent Dufossé,
Université de la Réunion, France

Reviewed by:
Folarin Anthony Oguntoyinbo,
Appalachian State University, United States
Anna Ligocka,
UTP University of Science and Technology, Poland
Giau Van Vo,
Gachon University, South Korea

****Correspondence:***
Krzysztof Skowron
skowron238@wp.pl

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *29 December 2021*
Accepted: *15 February 2022*
Published: *07 March 2022*

Citation:
Skowron K, Budzyńska A, Grudlewska-Buda K, Wiktorczyk-Kapischke N, Andrzejewska M, Wałecka-Zacharska E and Gospodarek-Komkowska E (2022) Two Faces of Fermented Foods—The Benefits and Threats of Its Consumption. Front. Microbiol. 13:845166. doi: 10.3389/fmicb.2022.845166

INTRODUCTION

Fermentation is one of the oldest processes that allows the preservation of food stability with the participation of microorganisms. The term itself comes from the Latin word fervere, which means "to cook." The action of microorganisms is based on the breakdown of complex compounds (carbohydrates and other macromolecules) into simple ones, which is accompanied by the formation of various types of beneficial catabolites, such as B vitamins, minerals, or Omega-3 fatty acids (Sivamaruthi et al., 2018). In most fermented products, lactic acid bacteria (LAB) play a major role in production. Also, several dozen types of bacteria, yeast, and filamentous fungi participate in the food fermentation (Laranjo et al., 2017; Rezac et al., 2018; Dimidi et al., 2019; van Reckem et al., 2019; Vilela et al., 2020; Zang et al., 2020; García-Díez and Saraiva, 2021; Xu et al., 2021).

A variety of single or mixed raw materials of plant origin (including cereals), meat and fish, and dairy products can be fermented. Such food can be eaten as a main course, drink, or snack.

Fermented foods can include processed foods on a small scale (household, craft industry) and large scale (industrially processed foods). A relevant role in traditionally fermented food play available plant or animal raw materials but also the customs, culture, and religion of indigenous peoples. Techniques of the fermentation process in some geographic areas are passed only orally, from generation to generation, and therefore are known to communities living close to each other (Anyogu et al., 2021).

Since ancient times, fermented foods have been produced by a process of natural (wild, spontaneous) fermentation, carried out by indigenous microorganisms naturally present in the raw material or processing environment (Campbell-Platt, 1987). The dominance of fermenting microorganisms, their metabolites and the changing pH of the raw material inhibit the growth of pathogenic microorganisms. Natural fermentation occurs also when a component containing a large number of microorganisms that initiate the fermentation process is added to the raw material. In both cases, the microorganisms involved in fermentation and the microclimate impact a product quality. The backsloping method, involving the use of a previously fermented product to inoculate a new batch, has also been used. This approach increases the chances of the desired microorganisms domination and competition with microorganisms that responsible for the product spoilage or disease. These traditional fermentation methods are still used today, primarily in home-based, local food production, or small-scale production. However, in the twentieth century, the development of microbiology, including food microbiology, has led to starter cultures introduction, which initiate the fermentation process and at the same time ensure greater product standardization. Such method results in products with constant organoleptic properties. Fermentation with well-defined cultures has found application, especially in the case of products obtained on an industrial scale. The process conducted under controlled conditions, it allows increasing the pace of the process and its throughput. The predominance of native microbiota allows limiting the growth of undesirable strains or species of microorganisms, as well as to reducing the toxic compounds they produce, ensuring the food safety. In developed countries, fermentation with the use of starter cultures also aims to achieve new health goals (Hesseltine and Wang, 1980; Holzapfel, 1997; Tamang et al., 2016, 2020; Voidarou et al., 2020; Mannaa et al., 2021). Research aimed at improving the starter cultures properties, carried out using the innovative CRISPR/Cas9 technology (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) which allows modification of the genome of any microorganism, may play an essential role here (Wu et al., 2020; Vilela, 2021). On the other hand, due to the current trend toward organic and biodynamic production, and the "flat" taste of products made with the participation of bacterial and fungal starter cultures, the strategy of traditional, spontaneous fermentation, and artisanal returns. This, however, increases the risk of the dangerous microorganisms presence in food (Capozzi et al., 2017).

BENEFITS

The health-promoting effect of fermented products is due to the presence of functional microorganisms in them. Microorganisms can occur naturally in various products (e.g., genera *Lactobacillus, Lacticaseibacillus, Levilactobacillus*) or, having GRAS (Generally Recognized As Safe) status, can be added to them (e.g., bacteria of the genus *Bifidobacterium*). The beneficial effect of microorganisms present in fermented products can be multidirectional (**Figure 1**). Since the consumption of functional foods can play a positive role in gut dysfunction, research is being conducted to determine their use in intestinal diseases. A study by Zhang et al. (2016), has shown that microorganisms present in fermented foods can transiently affect the gut microbiome. This allows for its modification and modulation of intestinal function, improving the health or reducing the risk of diseases associated with dysbiosis. Food can be a vehicle for probiotics, prebiotics, or synbiotics (Milani et al., 2019). The beneficial effects of probiotic strains include normalization of the gastrointestinal microbiota, antagonistic effects against pathogens, protection against pathogens' colonization, short-chain fatty acid production, or metabolism of bile acid salts. Such properties make probiotics useful in intestinal diseases treatment (including *Clostridioides difficile* etiology), in the treatment and prevention of obesity, lactose intolerance, diabetes, osteoporosis, and cardiovascular diseases. An example of the positive effects of fermented foods on the intestinal al microbiota is alleviation of symptoms of irritable bowel syndrome resulting from the consumption of fermented probiotic milk containing *Bifidobacterium lactis* CNCM I-2494 (Marteau et al., 2013). Studies have also confirmed an improvement in gastrointestinal passage and a decrease in common complaints in the human population, such as bloating and flatulence. This may be related to changes in the expression of bacterial genes that encode enzymes involved, among others, in carbohydrate metabolism (Agrawal et al., 2009; McNulty et al., 2011). The importance of probiotics in enhancing non-specific and specific immunity (modulation of the host immune response) is also highlighted. Probiotic bacteria stimulate

the mucosa-associated lymphoid tissue (MALT) immune system, formed, among others, by gut-associated lymphoid tissue (GALT) immune elements. Due to the production of chemokines, cytokines, growth factors, or immunoglobulins, MALT acts as a microbial fighter. Furthermore, probiotics influence the balance of the gut microbiome composition, reducing the risk of disease gut (Tokarz-Deptuła and Deptuła, 2017; Azad et al., 2018; Li et al., 2019; Uusitupa et al., 2020; Zhang et al., 2021). As microbes are able to produce neurochemicals, as well as respond to them, they can play a crucial role in the treatment of depressive and anxiety disorders (Romijn et al., 2017). Furthermore, the consumption of fermented products has a positive impact on the oral microbiota. The functional bacteria in the food reduce tooth decay, gum disease, and oral inflammation by lowering pH and producing antioxidants that inhibit plaque growth. They are also used in the treatment of halitosis, as they metabolize volatile sulfur compounds the source of unpleasant mouth odor (Gungor et al., 2015; Voidarou et al., 2020).

The microorganisms present in fermented products with high titers can interact with microorganisms that inhabit the digestive tract and colonize it temporarily or permanently (Davoren et al., 2019; Nemska et al., 2019; Roselli et al., 2021). Currently, due to the high degree of the variability of studies (heterogeneity of study design and methods used), and interindividual variability in the composition of the gastrointestinal microbiota, there is insufficient evidence for permanent colonization of the human gut by food microorganisms. However, the transient colonization shown in some cases indicates the need for permanent introduction of fermented products into the diet to maintain the positive effects of strains on the human body (Roselli et al., 2021).

The fermentation process is generally carried out to obtain a nutrient-enhanced product. However, in some cases, the overriding purpose is to prevent food spoilage. The metabolites produced by microorganisms (lactic acid, acetic acid, hydrogen peroxide, ethanol, compounds with antagonistic properties to other microorganisms) inhibit the growth of pathogenic microorganisms or those responsible for food spoilage. Fermented food is of great importance in low- and middle-income countries (subregions of Africa and Southeast Asia), which typically lack access to refrigeration facilities. In countries where the interplay of the dry season and the growing season results in a lack of availability of fresh food, preservation is a necessary solution to protect the population from starvation. The advantage of fermentation is also the ability to eliminate various types of toxic components present in raw materials, such as polyphenols (e.g., pachyrrhizine, rotenone, catechin derivatives), phytates, and tannins (Montagnac et al., 2009; Lautié et al., 2013). An example is the reduction of up to 95% of lectins and other toxic components in *tempe* produced from soybeans (Uzogara et al., 1990; Evans et al., 2013). It is possible to eat fermented products that would not be suitable for consumption without proper preparation (e.g., *cassava*, due to its cyanogen content) (Agbor-Egbe and Mbome, 2006). The increased digestibility of vegetable protein, by its partial breakdown, decreases the risk of food allergies and gastrointestinal disorders. An additional advantage of fermented products is their reduced mass, compared to the initial raw material, resulting from the processes (e.g., grating, soaking, squeezing) it undergoes before fermentation. This facilitates the transport of products which, especially in developing countries, is at a low level. In addition, the heat treatment time relative to the cooking time of the raw substrate is reduced (Uzogara et al., 1990; Nkhata et al., 2018).

Fermentation as a food processing technique can influence the level of mycotoxins in food. Mycotoxins pose a serious threat to human health due to their demonstrated carcinogenic, mutagenic, nephrogenic, hepato-, cytotoxic, neurotoxic, and teratogenic effects, and induction of immunosuppression. Toxins detected in foods produced by genera such as *Aspergillus, Penicillium,* and *Fusarium* include, among others, ochratoxin A, aflatoxins, zearalenone, and trichothecenes (Raiola et al., 2015; Ostry et al., 2017; Pei et al., 2021). Many strains of LAB producing antifungal metabolites (lactic acid, phenyllactic acid, hydroxyphenyllactic acid, indole, bioactive peptides) can reduce both fungal growth and mycotoxin synthesis. In addition to the inhibitory effect of bacterial organic compounds, antifungal activity may also be related to competition for the occupied niche and nutrients needed for growth. Modification of the external environment is also important here, as well as the binding of mycotoxins by components of the cell wall (polysaccharides, peptidoglycans) of bacteria. The species for which such properties have been demonstrated include strains of the genus *Lactobacillus* (e.g., *L. rossiae, L. fermentum L. sanfranciscensis*), as well as other bacterial genera such as *Bifidobacterium, Lactococcus, Pediococcus* (Valerio et al., 2009; Adedokun et al., 2016; Luz et al., 2017; Aarti et al., 2018; Guimarães et al., 2018; Khattab et al., 2018; Sivamaruthi et al., 2018; Sadiq et al., 2019).

Consumption of fermented foods also alleviate the severity of symptoms of COVID-19. This is due to the lactobacilli that are potent activators of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a major regulator of the cellular oxidative stress response (Bousquet et al., 2021). However, more this correlation merits further studies.

PROBLEMS

External and internal factors affect the growth capacity of pathogenic microorganisms in fermented foods (**Figure 1**). The risk of obtaining a contaminated fermented product increases when low-quality ingredients are used for its production, initially containing a sufficiently high number of bacteria, fungi, or toxins produced by them. An example is the pork used to make *nem chua*, a traditional raw sausage eaten in Vietnam after a short spontaneous fermentation process. Research by Le et al. (2012) showed repeatedly exceeded the level of microbiological contamination in meat intended for *nem chua* production. The presence of *Escherichia coli* and *Staphylococcus aureus* detected in the raw material did not meet the requirements for hygiene and safety. In countries with high poverty, raw materials of better quality are used mainly for export, as they are the primary source of income. On the contrary, secondary crops are used in

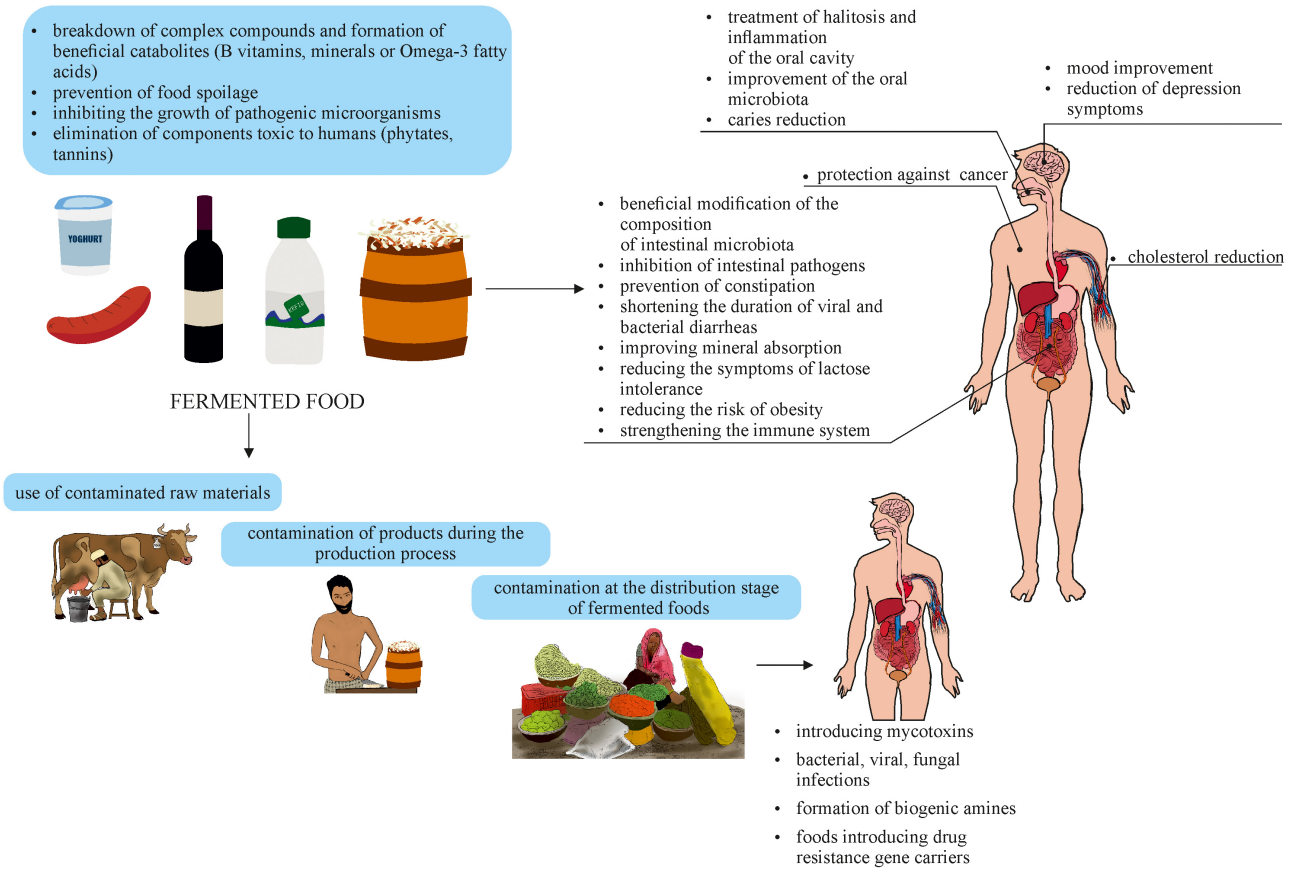


FIGURE 1 | Effects of consuming fermented foods.

household or small-scale food production, resulting in products of inappropriate microbiological standards.

The water used in the dilution stage or in the fermentation itself should also be free from microbiological contamination. Unfortunately, limited access to water in some regions, especially in rural areas and the use of potentially contaminated water from streams or rivers for production, increases the risk *E. coli* and *Salmonella* spp. presence in food (Anyogu et al., 2021).

In developing countries, the lack of Good Manufacturing Practices (GMPs), has a major impact on the safety of traditional, home-made, or cottage-made food (Oguntoyinbo, 2014). Their sale under unsanitary conditions without the use of protective coverings, such as gloves, is also a public health risk. Moreover, in developing countries, due to poverty and low consumer awareness, fermented foods sold locally are usually packaged in non-sterile utensils, used jute bags, or paper (e.g., newspaper), as well as gourds, or leaves. The inability to buy adequate packaging to limit microbial spoilage, even with a properly executed production process, poses a significant additional risk of food contamination (Oguntoyinbo, 2014).

Despite the positive impact of fermented products on human health, there is a risk that their consumption can introduce into the body carriers of antibiotic and chemotherapeutic resistance genes, leading to the selection of multidrug resistant strains responsible for infections that are difficult to treat. This selection also occurs as a result of the overuse of antibiotics in agriculture and livestock farming. Bacterial resistance mechanisms can be generated by the presence of drugs or their residues in food products at concentrations below the minimum inhibitory concentration (MIC). Due to horizontal gene transfer (transformation, conjugation, or transduction), there is a risk of spreading drug resistance among gastrointestinal microorganisms or foodborne pathogens (**Figure 2**; Tóth et al., 2020, 2021; Miranda et al., 2021). Therefore, in the case of fermented food production, in addition to the prudent use of antibiotics by breeders and farmers, it is important to use strains that do not contain drug resistance genes in their genetic material or to monitor the content of these genes in starter cultures and manufactured products.

The negative aspects of fermented foods also include biogenic amines (BAs) (**Figure 3**), which are formed during fermentation with both microorganisms naturally present in the raw material and with the participation of starter cultures. BAs are a product of amino acid decarboxylation, amination of aldehydes or ketones, or their transamination, and therefore their formation is influenced not only by the type of microorganism that performs these processes, but also by the composition of the raw material (e.g., free amino acid content), the fermentation time, and the

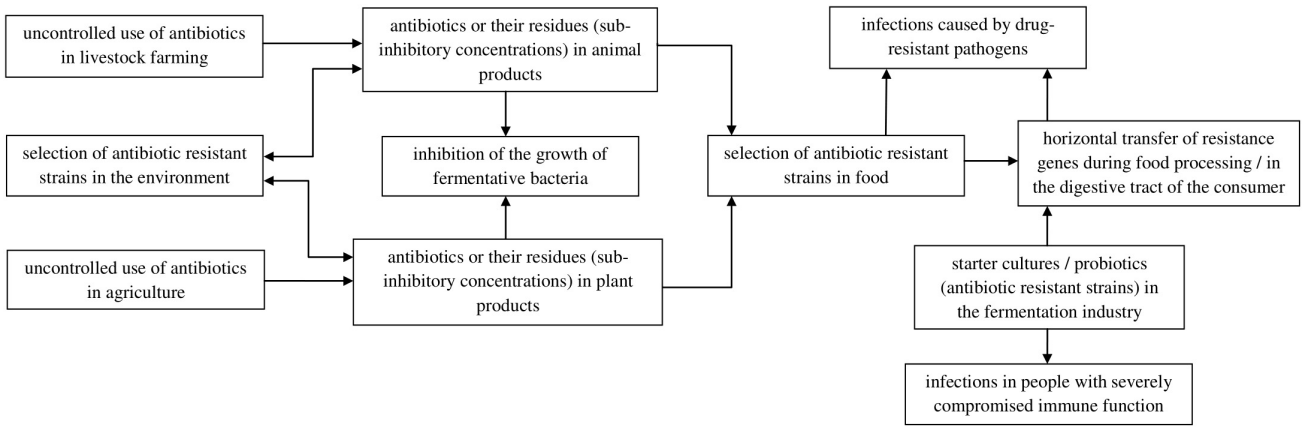


FIGURE 2 | Factors responsible for strain resistance to antibiotics in food and its effect.

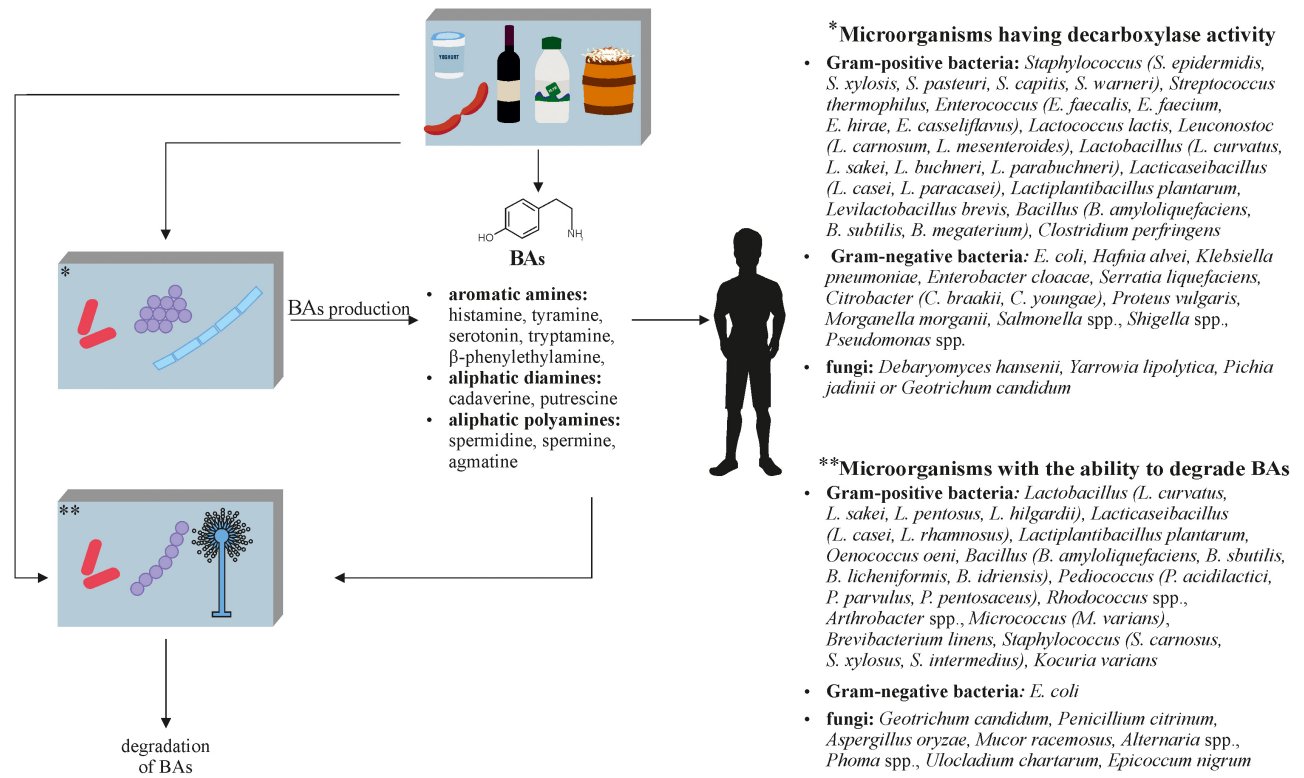


FIGURE 3 | The role of microorganisms in the metabolism of biogenic amines (BAs) in fermented foods (Smith, 1980; Latorre-Moratalla et al., 2010; Lorenzo et al., 2010; Spano et al., 2010; Cueva et al., 2012; Alvarez and Moreno-Arribas, 2014; De Mey et al., 2014; Zaman et al., 2014; Ladero et al., 2015; Ordóñez et al., 2016; Sun et al., 2016; Pugin et al., 2017; Ekici and Omer, 2018; Li et al., 2018; Barbieri et al., 2019; Mah et al., 2019; Park et al., 2019; Ruiz-Capillas and Herrero, 2019).

conditions under which food processing takes place. Due to the higher accumulation of BAs in products that do not meet microbiological standards, it is believed that the amounts of these compounds may reflect the degree of spoilage (Doeun et al., 2017; Xiang et al., 2019). In order to reduce the BA content, it is necessary to use microorganisms or processes leading to the degradation of these potentially toxic compounds in food production. However, the main activities are to control the quality of the raw materials that undergo fermentation and its conditions, but they are not always carried out.

Adverse health effects associated with the consumption of fermented foods can include isolated illnesses, outbreaks, and even deaths. Among other things, due to the mycotoxins present in some products, long-term consequences are also possible in the form of diseases of the gastrointestinal tract, kidney stones, or cancer (Singh et al., 1986; Phukan et al., 2006;

Gajamer and Tiwari, 2014; Keisam et al., 2019). The actual number of infections and outbreaks resulting from the consumption of fermented foods is probably underestimated. In developed countries, difficulties in detecting outbreaks that may originate from a variety of food products are associated with incomplete epidemiological data (mild cases of infection are not reported or documented) and the frequent lack of information exchange between diagnostic laboratories. This leads to delays in investigations and the inability to confirm hypotheses about a potential source of infection.

The composition of some fermented products may also cause some minor health disadvantages. An example of a product of this type can be kombucha made from tea and sugar. Its consumption can lead to excess sugar and calorie intake, which may also lead to bloating and gas.

DAIRY PRODUCTS

Many food products, such as yogurts, kefirs, and cheese, are obtained from milk, usually cow's milk, as a result of the fermentation process. Due to the diversity of the microbiome, various fermentation routes can be carried out, which leads to the production of thousands of final products (such as cheeses), differing in consistency, taste, and aroma (Voidarou et al., 2020).

Raw and fermented camel milk, which has high nutritional value, is consumed by the populations of Asian and African countries. It is considered a product with therapeutic properties because of the immunoglobulins, lysozyme, and lactoferrin content, which have antimicrobial activity. In some regions, it is used to treat diseases such as chronic hepatitis (Saltanat et al., 2009; Sharma and Singh, 2014). *Suusac*, consumed by the people of the arid and semi-arid areas of Kenya, is obtained from the spontaneous fermentation of fresh unpasteurized milk in cleaned smoke-treated gourds (Lore et al., 2005). A study by Maitha et al. (2019) on milk samples collected from different areas of northeastern Kenya found that *suusac* consumption also poses health risks to consumers. Researchers found *E. coli* in all samples analyzed, while *Shigella* spp. and *Klebsiella* spp. were present in 88.1 and 77.4% of the samples, respectively. Furthermore, bacteria of the species *S. aureus*, which are often responsible for animal udder infections, including camels, were detected in more than half of the samples. Another study reported on the detection of *Mycobacterium* strains other than *tuberculosis* (MOTT) (*Mycobacterium avium, M. intracellulare, M. kansasii, M. malmoense*) in *suusac* (8.2% of positive samples) (Mwangi et al., 2017). The authors of the above study also highlighted the problem related to the widespread distribution of *Brucella* spp. rods in dairy products in African countries. They showed that the risk of these microorganisms increases from 14 to 26% in *suusac* production. The ability of some *Brucella* species to survive at 4°C and at pH below 4.0 indicates the need for vaccination of livestock against brucellosis (Zúñiga Estrada et al., 2005; Dadar et al., 2019). Pasteurization of milk before *suusac* production or heat treatment before consumption of the finished product is also crucial to consumer health. Also, Iran by Yam et al. (2014) conducted a research aimed at determining the microbiological quality of fermented camel milk, *chal*out in (2014). *Chal* is obtained, like *suusac*, by spontaneous fermentation, carried out in leather bags or bottles. In samples collected in the province of Golestan, *Staphylococcus* spp. and rods from the Enterobacterales order were identified, while no bacteria of the genus *Salmonella* and *Shigella* were detected.

In addition to *mastitis*, poor hygiene of the milking personnel, the milking environment (milking is usually conducted in the open areas without prior washing and disinfection of the teats), and the fermentation tanks also contribute to milk contamination. The quality of the water used for washing is important, as it should be drinking water quality. Greater risk of infection poses a cheap product, kept in poor sales conditions. Fermented milk can be a particularly significant source of infection, as it is often consumed without heat treatment.

Among the microorganisms present in fermented milk are strains of *E. coli*. A study by Yakubu et al. (2018) showed the presence of the most pathogenic serotype *E. coli* O157: H7 in samples of this product from 100 points of sale in the area of Nigeria. The main source of *E. coli* strains, including those with a high pathogenic potential, is the gastrointestinal tract of cattle (animals do not show clinical signs of infection). These microorganisms, excreted in the feces, can contaminate milk, especially if premilking hygiene is not practiced (Leedom, 2006).

Many other studies have confirmed the contamination of products obtained by cow's milk fermentation. An example is *roub*, a drink obtained by inoculating milk with starter culture from the previous day's fermentation. It is drunk when diluted with water or added to make a soup to be eaten with pudding. In studies conducted on traditionally prepared *roub* samples collected in three regions of Sudan, a high level of contamination and the presence of *S. aureus* and coliform bacteria were demonstrated (Abdalla and Hussain, 2010). This confirms previously reported results (Abdalla and El Zubeir, 2006). Dehkordi et al. (2014) assessing the presence of shiga toxin-producing *E. coli* strains in traditionally produced dairy products (yogurt, *doogh,* and *kashk*) sold in supermarkets or retailers in Iran. The characteristics of the strains isolated from more than 8% of the products showed diversity in terms of serogroups, among which the serogroup reported the most frequently was O157 (26%) and O26 (12%). The relatively high frequency of strains responsible for various forms of infection, including bloody and non-bloody diarrhea or hemolytic uremic syndrome (HUS), may indicate failure to maintain the appropriate temperature or time parameters at the dairy production stage. It may also result from the use of contaminated water by the local population, who do not have access to water that meets microbiological requirements.

Nahidul-Islam et al. (2018) analyzed traditional hand-made fermented dairy products (*dahi, chanar-misti, paneer,* and *borhani*), produced in India but also popular in other Middle East to South East Asian countries. Their research with the use of pyrosequencing showed the presence in food, in addition to the dominant LAB (*Lactobacillus* spp. and *Streptococcus* spp.), differently numerically (depending on the product) bacteria, including *Acinetobacter* spp. and *Pseudomonas* spp., rods from Enterobacterales, as well as fungi of the genus *Aspergillus*.

The probable sources of these microorganisms are skin, bovine intestine, and *mastitis*.

Ras and *karish* cheese showed significant contamination with *E. coli* strains (8–21.7% and 74.5%, respectively) (Ombarak et al., 2016; Hegab et al., 2020). These cheeses are consumed in Egypt, a country where fresh milk and products thereof are important components of the daily diet. The cheeses are usually obtained in artisanal rural areas from raw cow's milk or a mixture of cow's and buffalo milk. Fermentation and ripening, which take 3–8 months, usually involve only microorganisms that are native to the milk microbiota. *E. coli* strains isolated from cheese possessed different pathogenic potential. Some strains, were responsible for *gastroenteritis*. The most common source of these microorganisms in raw milk and dairy products derived from it is feces that contaminates the milk at the milking stage. Hegab et al. (2020) also noted *S. aureus* strains in 26% of *ras* cheese samples, 15% of which were enterotoxigenic (demonstrated presence of *seb* and *sed* genes). Abdel-Hameid Ahmed et al. (2019) found an even higher percentage (50%) of enterotoxin producing *S. aureus* strains isolated from this type of cheese. The above results may indicate poor hygiene of people involved in the production of cheese.

In developing countries, pathogens that have been largely eliminated in other parts of the world may also be present in fermented dairy products. Examples include the previously mentioned *Brucella* spp., or MOTT, found in milk. Based on a study by Michel et al. (2015), *Mycobacterium bovis*, an etiological agent of bovine tuberculosis, can survive in souring cow's milk. This species is isolated in Africa from unpasteurized milk, but data on the incidence of the disease in humans are insufficient to conclude how high the risk of consuming contaminated products with *M. bovis* is. Inappropriate veterinary control of livestock in some countries is certainly a significant cause of approximately 70,000 cases of zoonotic tuberculosis reported annually in Africa (Olea-Popelka et al., 2017; Owusu-Kwarteng et al., 2020).

Despite the presence of many viruses in food, infections of this etiology caused by the consumption of contaminated fermented products are recorded less frequently than bacterial infections. This is due to the fact that viruses present in food are primarily bacteriophages and yeast-infecting viruses (Pringsulaka et al., 2011; Kleppen et al., 2012). The low number of these microorganisms in food products and the greater difficulty of detecting them may also be a reason. Foodborne viruses include *hepatitis* A and E virus, noroviruses, and rotaviruses (Maske et al., 2021). In 2016, two cases of tick-borne encephalitis were reported in Germany after consumption of unpasteurized goat cheese (Brockmann et al., 2018). Infections caused by tick-borne *encephalitis* virus transmitted by this type of cheese, although rarely, have also been found in other countries of Central and Eastern Europe (Croatia, Czech Republic, Austria) (Holzmann et al., 2009; Kríz et al., 2009; Markovinović et al., 2016). The work of Rehfeld et al. (2017) suggests that in Brazil, where cow's milk contamination with the Vaccinia virus (VACV) is noted, consumption of artisanal cheese from unpasteurized milk may also lead to illnesses of this etiology. The route of transmission of SARS-CoV-2 through food consumption has not been confirmed, but is considered unlikely (Center for Disease Control and Infection, 2020).

PRODUCTS OF PLANT ORIGIN

Various substrates of plant origin, such as cereals, oil seeds, nuts, roots, tubers, and plant juice, are fermented. Some of them are an important and inexpensive source of protein, which provides energy for the body. Additionally, the breakdown of proteins into amino acids during the fermentation process increases the digestibility of the product.

Sorghum is the second most commonly grown cereal in sub-Saharan Africa, thanks to its tolerance to drought, its ability to grow under harsh conditions, and its nutritional value (high starch content, among other things). Due to its benefits, it is an ingredient in the main meals consumed by the inhabitants of Africa. In addition, it is an important component of the diet of people with gluten intolerance. However, a fermentation process is required to convert the plant into an edible form (Odunmbaku et al., 2017; Adebo, 2020). The most common fermentation process in sorghum is lactic fermentation, in which primarily LAB participate, although certain fermented products involves also fungi. One of the traditionally produced sorghum-based products (or millet) is *obushera*. Due to its widespread use (in weaning, as a thirst-quenching drink and as a source of energy), its production is a source of income for households, but it is also being commercialized. *However, obushera* sold on the market does not always meet microbiological requirements. In the study by Byakika et al. (2019), despite the absence of *Salmonella* spp. rods in the samples tested, most did not meet the standards for coliforms and *Staphylococcus* spp. Microbiological contamination could result from the use of poor quality raw materials in production, but also by the lack of pasteurization processes.

Adedeji et al. (2017) and Ademola et al. (2018) conducted studies on *iru* and *ogiri*, traditional food condiments used in Nigeria and some parts of West Africa. They are popular due to the aromas and flavors, resulting from the primary and secondary metabolites of microorganisms produced during fermentation, and their high protein content, which is of a particular importance in the poor regions of the country. The first of these condiments is obtained from carob seeds [African locust bean (*Parkia biglobosa*) seeds], while the raw materials for *ogiri* may be melon seeds or castor bean seeds. The production of condiments is preceded by spontaneous fermentation of seeds, usually carried out at the household level. Previously conducted analysis of these products, using classical methods of bacterial identification, based on their biochemical characteristics, showed the presence of potentially pathogenic bacteria, such as *Bacillus cereus, Bacillus subtilis, S. aureus, E. coli, Proteus* spp., *Pseudomonas* spp. (Falegan, 2011; Ajayi, 2014). Adedeji et al. (2017) and Ademola et al. (2018) extended the research to include genotyping methods, confirming the participation of various microorganisms in condiments, representing both actual fermenters and undesirable species introduced at different stages of production. Ademola et al. (2018), analyzing the dynamics

of the bacterial population during the production of *iru* and *ogiri*, showed that species belonging to the genera *Bacillus (B. encimensis* and *B. safensis*), *Enterococcus (E. dispar),* and *Lysinibacillus* are present at almost every stage of spice processing. Therefore, they can be potential starters in the fermentation process. Other microorganisms, including potential pathogens, detected only at certain stages of processing are evidence of poor hygiene practices leading to contamination of foods that play an important role in the diets of rural populations in poor countries.

Douchi is a Chinese condiment obtained from black beans. In its production, strains of *Aspergillus* spp., *Mucor* spp. or *B. subtilis* are used, carrying out the first fermentation process, while the second process is an anaerobic spontaneous fermentation, with the participation of bacteria and fungi (Zhang and Liu, 2000). *Douchi* was the cause of an outbreak of food poisoning that occurred in Kunming, China (Zhou et al., 2014). The symptoms of food poisoning that occurred in 139 people who consumed a dish containing *douchi* were caused by cereulide or Nhe enterotoxin produced by strains of *B. cereus*. However, this was not the first case of *douchi*-related food poisoning reported in China. A two other previously reported outbreaks, *B. cereus* strains were also the etiological agent (Shen et al., 2005; Liu et al., 2006).

Species such as *B. cereus, Clostridium botulinum*, *Proteus mirabilis*, and *E. coli* have been detected in fermented soy products. Keisam et al. (2019) carried out analysis of such products sold in the northeastern region of India was using the next generation sequencing technique (MiSeq) combined with qPCR and immunoassays. The first two species mentioned were found in all samples taken for testing in the amount of $> 10^7$ cells/g. In addition, diarrheal or emetic toxins (hemolysin BL (HBL) and non-hemolytic enterotoxin (NHE) were detected in all isolated strains of *B. cereus,* while cereulide (an emetic toxin) was detected in less than half. In turn, the strains of *P. mirabilis* produced hemolysins, urease, and also showed multidrug resistance. The common presence of these intestinal rods in fermented foods could explain the high percentage of cases of *urolithiasis* in India.

The contamination of fermented soybean products with *B. cereus* strains is also a significant problem in Korea, where these products are common in the daily diet. *Doenjang, kochujang, meju*, or *cho-kochujang* are obtained by natural fermentation, either at home or with factory-made starter cultures. Its pro-health properties, such as its anticancer effect, has led to an increase in interest and frequency of its consumption not only among Koreans, but also among people around the world (Jung et al., 2006; Lee N. et al., 2017). However, numerous studies have confirmed the presence of strains of *B. cereus* in such products (Kim et al., 2015; Yim et al., 2015; Park et al., 2016; Lee N. et al., 2017). Although the detection rate of this bacilli species, as well as the level of product contamination, were varied (not always exceeding the acceptable standards of the Korean Food and Drug Administration), the NHE and/or HBL toxins detected in the strains indicate the need to monitor the level of *B. cereus* contamination in soybean products.

In South Korea, two outbreaks of *gastroenteritis* caused by enterotoxigenic *E. coli* (ETEC) O6 strains were reported in 2013–2014 (Shin et al., 2016). The first included 167 children attending a middle school in the Jeollanam-do province, and the other involved 1,022 cases in 10 schools in the Incheon Provence. *Kimchi* was suspected to be a carrier of pathogenic strains. This traditional Korean dish is prepared by fermenting various types of vegetables, the most popular of which is cabbage. The product is consumed about 1 week to several months after its preparation and contains beneficial bacteria that carry out the fermentation process, mainly from the genera *Leuconostoc, Lactobacillus,* and *Weissella*. Epidemiological and laboratory investigations reported ETEC O6 strain in *kimchi* prepared from cabbage in the school canteen and young radish prepared by a food company. A potential carrier of the bacteria could be poor quality water used for food preparation. Both, too short fermentation process and established environmental conditions may allow pathogens to grow of intestinal pathogen. Furthermore, excessive pH does not inhibit the secretion of the heat-labile (LT) toxin responsible for diarrhea by ETEC strains, as shown in a study by Gonzales et al. (2013). *Kimchi* prepared from cabbage or radish for the canteens of 7 schools in Incheon, Korea, was also likely the cause of a previous large outbreak of 1,642 cases of *enteritis* (Cho et al., 2014). Retrospective cohort studies carried out allowed the isolation of ETEC O169 strains indistinguishable in pulsed field gel electrophoresis (PFGE) from 230 students and kimchi produced by one food company. However, the presence of these pathogens was not found in raw vegetables or other food products. The reported outbreaks are evidence that not only homemade fermented foods, but also foods produced by food companies, may pose a risk of infection for the consumer. Hence, there is a need for continuous monitoring of food safety and the need to define precise criteria for the production process in food establishments. This is especially important in the case of a wide distribution of products due to the risk of causing a large outbreak in a relatively short period of time.

The concentration of mycotoxins depends on their initial content in the raw material. Therefore, in low-income countries, due to the frequent use of low-quality cereals, mycotoxins are found in products obtained from fermented crops. The presence of fungi producing toxic metabolites is influenced by the timely failure to clean crops from the soil, as the well as long-term and improper (at high humidity) storage of grains, especially crops without hulls. Also, crops damage during harvesting or storage increases the risk of mycotoxins in raw material. Favorable environmental conditions, such as temperature or water activity, are important factors that contribute to mycotoxins production (Milani and Maleki, 2014; Viaro et al., 2017). The risk of consuming fermented products prepared from raw material contaminated with mycotoxins is high, even with previous heat treatment, due to the stability of these toxins at processing and cooking temperatures.

People's awareness, mainly in developing countries, of the food contamination risk with fungi and mycotoxins is low (Siegrist and Cvetkovich, 2001; Ezekiel et al., 2013; Matumba et al., 2016; Adekoya et al., 2017). Therefore, few activities, such as sorting moldy seeds or proper drying, are undertaken to reduce the

contamination of raw materials used in food production, as well as the contamination that can occur during its preparation or storage. Ignorance or neglect by sellers, in the turn, results in mixing products that meet microbiological requirements with moldy food. Mycotoxins are often found in various types of products typically consumed in African countries, produced based on spontaneous fermentation or small-scale rural processing. In a study by Adekoya et al. (2017), more than 80% of the samples analyzed of foods produced in Nigeria and obtained by fermentation of raw materials such as melon seed (*Citrullus colocynthis*), oil bean seed (*Pentaclethra macrophylla*), maize or sorghum contained single toxins or combinations thereof. Some of them (fumonisin, aflatoxin, ochratoxin A, and zearalenone) exceeded the limit specified by the European Commission.

In China, fermented pastes, made from soybeans, broad beans, flour, and chili, are of great interest. Contamination with fungi and the formation of mycotoxins often occur during the cultivation stage if the species of fungi are compatible with the crop. However, studies have shown that mycotoxin production in this type of food can also occur during a long fermentation process (Shukla et al., 2014). Aflatoxins (present in soybean pods and seeds) and ochratoxins (present in wheat and soybeans) are frequently detected in fermented pastes (Zhao et al., 2020). The presence of aflatoxins was also found in soybean sauces obtained with the participation of *Aspergillus orizae* (Vu and Nguyen, 2016).

In Korea in 2013, *kimchi* produced by a food company was the source of acute *gastroenteritis outbreaks* caused by the GI.4 human norovirus (HNoV) genotype (Park et al., 2015). The analysis showed that the *kimchi* was probably contaminated with groundwater used by the company during the production stage. Spreading the product before the completion of the fermentation process may not have lowered the pH sufficiently, with the result that HNoV, which is resistant to pH $>$ 5, was able to survive in *kimchi*. Research by Lee H. M. et al. (2017) also showed that despite the reduction of the HNoV titer in experimentally contaminated cabbage *kimchi*, the fermentation conditions (acidity, salinity, organic acid content) may be an insufficient factor to eliminate this virus.

MEAT PRODUCTS

Due to the high likelihood of pathogens in raw meat, the risk of infection after consumption of fermented meats not heat treated prior to consumption is high. The addition of nitrite to meat helps reduce the growth of microorganisms such as *C. botulinum, S. aureus, L. monocytogenes,* and *Salmonella* spp. (Hospital et al., 2016; Majou and Christieans, 2018). However, since these compounds are an important risk factor for colorectal cancer, there is a trend toward eliminating them from the meat industry and introducing alternative preservation methods. This, however, may lead to a lower microbiological safety of the product (Christieans et al., 2018; Gonzalez-Fandos et al., 2021).

One pathogen most frequently reported responsible for foodborne disease outbreaks associated with meat products is *Salmonella* spp. (Patarata et al., 2020). In Italy, an outbreak involving 79 cases occurred in 2009–2010, caused by the Goldcoast *Salmonella enterica* serotype (Scavia et al., 2013). It is believed that its source could have been salami, dry, fermented sausages. Nonetheless, a delayed investigation, carried out after the peak of the outbreak, did not allow to confirm this suspicion. Stool samples collected from the patients for diagnostic testing were too small. It was also difficult to take samples of suspect food products and examine the trace-back activity of food, especially in the case of salami, produced from the meat of various species of animals. Furthermore, various typing methods were used in different laboratories. Despite low pH and water activity, as well as high salinity, *Salmonella* may remain in salami due to the too short fermentation period. The salami production process reduces significantly *Salmonella* spp. levels but the scale of reduction with high primary meat contamination may be insufficient. Cases of increased infections (60 cases of diarrhea, abdominal cramps, or fever) of *Salmonella* Goldcoast etiology were reported at a similar time in Hungary, where contaminated pork was the likely source (Horváth et al., 2013). In turn, raw pork and fermented raw pork sausage, Zwiebelmettwurst, were the likely vehicle for the transmission of *Salmonella enterica* serovar Bovismorbificans during the German outbreak (Gilsdorf et al., 2005). At that time, 525 cases of *gastroenteritis* were reported, with one death.

Dry fermented sausages are highly diversified, influenced, among others, by the degree of grinding meat and fat, its acidity, or the presence of mold on the surface (Meloni, 2015). Although listeriosis outbreaks associated with the consumption of these types of meat are rarely reported (Meloni, 2019), *L. monocytogenes* is relatively frequently detected in final products, demonstrating the ability of these bacteria to overcome barriers at the production stage. The source of *L. monocytogenes* in fermented sausages can be raw meat, the slaughterhouse environment, or people in contact with the raw material unprocessed or after processing. Resistance to disinfectants and the ability to form biofilms on various types of surfaces increase the risk of contamination of ready-to-eat (RTE) products (Martin et al., 2011; Meloni et al., 2014; Meloni, 2015).

Díez and Patarata (2013) studied the pathogen's survival during the production of *chouriço,* a dry fermented sausage. This product, which is produced both on a farm scale (using natural fermentation) and on an industrial scale (fermentation carried out under controlled conditions, using starter cultures), differs not only in the spices added, but also in the smoking, drying, and maturation times, which last between 1 and 4 weeks. An important role in meat preservation plays the amount of salt, which reduction for health reasons in recent years simultaneously decreases the microbiological safety of the product. The provocation tests carried out by Díez and Patarata (2013) showed that although the levels of *L. monocytogenes, S. aureus,* and *Salmonella* spp. were reduced in the early stages of drying, all pathogens were undetectable in *chouriço* after a longer period (30 days) of this process. In addition to salt concentration, a sufficiently high glucose content, the addition of antimicrobial compounds, or a low pH affect the survival of microorganisms (Piras et al., 2019). The conditions under which fermentation occurs are also important. The fermentation temperature and

postproduction processing, including storage temperature and time, as well as product freezing and thawing processes, are crucial in reducing the content of toxigenic strains of *E. coli* (Heir et al., 2013; McLeod et al., 2016; Shane et al., 2018). Therefore, to ensure the microbiological safety of fermented meat, while maintaining its taste, it is necessary to optimize the ingredients added to it and the process parameters.

Lee H. S. et al. (2017) have suggested that feeding animals with feed contaminated with mycotoxins can contaminate meat products. The research was carried out in Vietnam, where the tropical climate (high temperature and humidity) is particularly conducive to fungal growth in a variety of agricultural products, including pig feed products. Evaluation of exposure of pigs to aflatoxins has shown their presence in the urine of animals, suggesting a risk of toxins' presence in pork meat. However, the authors did not conduct any research in this regard.

Fermented meat products can also be a source of *hepatitis* E virus (HEV) infection, which is transmitted through meat from infected pigs or wild boar. In fact, a study by Wolff et al. (2020b) showed that despite the inhibitory effect of an acidic environment on many microorganisms, HEV shows a minimal decrease in infectivity at pH 2. Other studies by Wolff et al. (2020a) indicate that high salt concentrations that are usually applied for fermented raw sausages are also not sufficient to limit HEV survival in food products. Furthermore, an assessment of the prevalence of HEV genotype 3 in RTE products containing raw meat and originating from the Swiss retail market showed the presence of genetic material of these viruses in approximately 6% of samples (Moor et al., 2018). The RNA of HEV genotype 3 was also confirmed in a study of raw sausage samples collected from retail stores in the Netherlands (14.6% of samples) (Boxman et al., 2020). The greatest risk of infection with HEV etiology occurs when consumed raw pork products contain the liver of a contaminated animal (Pavio et al., 2014). This exemplified the hepatitis E outbreak reported in France associated with the consumption of figatelli, raw pig liver sausages (Renou et al., 2014). Since there is a high risk of pork meat contamination with the virus, it would be advisable to consider the HEV test, and the obligation to report cases of hepatitis E, which is currently not mandatory in many countries. In some European countries, raw sausages do not contain a pork liver (Vignolo et al., 2010). Another option is to inform people about the risk of consuming such foods without heat treatment.

FISH PRODUCTS

Fermented fish products, due to their protein content, are an important part of the diet in some countries such as Thailand, the Philippines, Cambodia and Indonesia. Salt and sometimes sun-drying are used for preservation, while microorganisms play a primarily role in developing the fish's characteristic flavor and aroma. In Norway, *rakfisk*, a traditionally produced fermented fish product, is very popular. Its raw material is freshwater salmonid fish, which are stored in brine at 3–8°C for 3–12 months, during which the fermentation process takes place. The finished product does not require heat treatment before consumption and therefore microorganisms that exhibit tolerance to increased salt concentrations can pose a risk to the consumer (Skåra et al., 2015; Bjerke et al., 2019). An example of such microorganisms is *L. monocytogenes*, which adapts to environments with high salinity and low temperatures in which it can multiply. A study by Axelsson et al. (2020) showed that temperature and salt may not sufficiently reduce the growth of *L. monocytogenes* in *rakfisk*. Therefore, additional strategies in the production of fermented fish are necessary, such as the use of the P100 bacteriophage, which reduces the pathogen's number and has GRAS status (generally recognized as safe) (Allende et al., 2016).

Raw oysters are one of the foods that transmit noroviruses and are responsible for several foodborne outbreaks of *gastroenteritis* (Le Guyader et al., 2006). The virus accumulates in the shellfish bodies during water filtration and is able to survive for a long time in oyster tissues. In South Korea and other Asian countries, raw oysters can be fermented before consumption. The process is carried out at room temperature for about 2 weeks in the presence of 5–10% salt. The HNoV outbreak, which affected 8 students at a high school in Gyeonggi Province (Cho et al., 2016), indicates that although the viral load decreases significantly during oyster fermentation, this reduction may not be sufficient to eliminate the risk of *gastroenteritis*, as confirmed by research by Seo et al. (2014).

Hepatitis A outbreaks that occurred in South Korea in 2019 have contributed to the research on the causal source of this disease. Jeong et al. (2021) showed that hepatitis A virus (HAV) strains were present in yogaejot, a traditional fermented food from that country that contains raw clams. Phylogenetic analysis confirmed that they are closely related to the strains prevalent in East Asia. The results obtained indicate the need for proper hygiene practices in the production stage. Furthermore, since an important source of HAV infection is fecally contaminated coastal waters where bivalve mollusks are harvested, it is necessary to improve regulations protecting against this activity.

ALCOHOLIC BEVERAGES

Alcoholic beverages containing more than 0.5% v/v of alcohol are made from raw materials such as cereals, vegetables and fruits, palm juice or honey (Voidarou et al., 2020).

Bacteria can develop tolerance to the acid and alcohol contained in various fermented products, as exemplified by the results obtained by Gómez-Aldapa et al. (2012). These researchers evaluated the behavior of *E. coli* O157: H7 during the fermentation from nectar of maguey agave plants used to make *pulque*, a traditional Mexican alcoholic beverage. The presence of *E. coli* O157: H7 strains in the *pulque*, although not proven, is highly probable. Although some companies industrialized the production of this beverage, the most commonly consumed is *pulque* made by artisans, also involved in cattle and sheep farming. These animals constitute an important reservoir of pathogenic *E. coli* strains and, often grazed on agave plantations, can contaminate the plant (Moxley, 2004; Varela-Hernández et al., 2007). Also importantly, *E. coli* O157: H7 strains can

develop an adaptive response to acidic environmental conditions and survive the fermentation process (Bachrouri et al., 2006). Moreover, Gómez-Aldapa et al. (2012) showed that these bacteria can exhibit tolerance to alcohol at low pH, and thus can be present in finished *pulque* posing a high potential risk to the consumers' health.

Pombe is an alcoholic beverage obtained from a variety of raw materials such as corn, millet, bananas, and pineapples (Kubo, 2016). In 2015, there was an outbreak in a village in Mazambique associated with the consumption of *pombe*, prepared from maize flour (Falconer et al., 2017; Gudo et al., 2018). Of the more than 230 people who developed symptoms of food poisoning and respiratory problems, 75 people died. The analysis revealed the presence of potentially lethal levels of bongkrekic acid, a potent toxin produced by *Burkholderia gladioli* pv. cocovenenans strains, in the collected beverage samples. This highly unsaturated tricarboxylic fatty acid can block the mitochondrial adenine nucleotide translocator (ANT) and prevent respiratory chain phosphorylation. The severity of symptoms caused by bongkrekic acid depends primarily on the amount of toxin-containing product consumed (Peng et al., 2021). Although *B. cocovenenans* shows sensitivity to high temperatures, the toxin itself is thermostable contributing to high mortality rate. Therefore, cooking foods containing bacteria does not protect against the effects of bongkrekic acid. Furthermore, Falconer et al. (2017) concluded from their research that the presence of *Rhizopus oryzae* in the raw material could enhance the toxin synthesis. Mass illnesses associated with the consumption of fermented foods containing bongkrekic acid had been reported many years earlier, in Indonesia (following the consumption of coconut-based tempeh) (van Veen, 1967) and in China (following the consumption of homemade fermented corn flour products) (Meng et al., 1988). However, many more similar outbreaks could not have been detected due to the smaller scale or the lack of research. Also in China, homemade sour soup prepared from fermented corn flour caused the death of all nine people consuming it (Yuan et al., 2020). Inadequate storage and processing conditions of raw materials, especially those rich in oleic acid, which create a good environment for the production of toxins by these bacteria may enable growth of *B. cocovenenans*.

A study by Scussel et al. (2013) demonstrated the presence of various types of toxins in wines randomly collected from a retail market in the Netherlands. Ochratoxin A and penicillinic acid produced by *Aspergillus* spp. and *Penicillium* spp. as well as alternariol and alternariol methyl produced by the genus *Alternaria* were detected in the samples tested by liquid chromatography based on tandem mass spectrometry. Primarily the region in which the grapes are grown and the associated climatic conditions affect the presence of mycotoxins in wine. The type of wine is also important because, unlike white wine, fermentation in red wines begins with the peel, which may contain mycotoxins. Therefore, the level of toxins detected by the researchers varied and depended on the type of sample tested and the country from which the wine originated. Hence, even low contamination of wine, with regular or frequent consumption, characteristic for some countries (e.g., France, Italy), due to the carcinogenic properties of mycotoxins, can pose a significant threat to human health. Also cork can be a source of toxins in wine. Treatment of corks with fungicides can reduce the risk of toxigenic fungi, but contamination can also occur after processing. A consequence of this is the transfer of mycotoxins from the cork to the wine (Centeno and Calvo, 2002).

Drinking *tari*, the fermented sap of the date palm, may contribute to the transmission of Nipah virus. Research in Bangladesh (Islam et al., 2016) found that this product could have been responsible for three outbreaks that occurred between 2011 and 2014 in the country. As sap collection usually consists of hanging clay vessels on the palm, into which sap drips, it can easily become contaminated with excrements and bat secretions, which are the source of the virus. Despite the sensitivity of the Nipah virus to alcohol solutions, its content (5–8%) in the sap after fermentation is too low to eliminate this microorganism from the beverage. It is important to remember that the lack of strategies to prevent sap contamination during harvest by bat droppings and secretions can also lead to the development of other diseases caused by viruses for which these animals are an important reservoir. The use of protective bamboo covers as a barrier for animals can be an effective solution.

CONCLUSION

The benefits of consuming fermented foods may be particularly important to people in developing countries where there is no access to probiotic. Such foods can be a source of good microbes, help reduce diarrhea, and stimulate the immune system to fight other microbes. However, despite many advantages that result from the fermentation processes with the participation of various microorganisms, especially functional ones, the lack of good production practices creates the risk of microbiological contamination of food products. This phenomenon is particularly visible in developing countries, where food processing is highly dispersed and individual, or there is no system and institutions supervising the processes of food production, including fermented ones. In this case, the risk of consuming fermented foods should be considered, especially if they are contaminated with pathogens, including viruses. The purchase and consumption of such foods by tourists poses a real risk of spreading infection around the world. Therefore, it is advisable to inform food handlers of the risks associated with the consumption of food contaminated with fungi, bacteria, the toxins they produce, and viruses. This will make individual producers want to pay more attention to the sanitary safety of the food they produce. Concerns about the quality of the raw material should also be recommended. A contaminated raw material will not produce a safe product, especially a fermented product that is made without heat treatment. Hence, it is necessary to ensure appropriate harvesting dates, pay attention to the weather during harvesting, reject batches of raw material, which visual quality deviates from the expected (serious mechanical damage, mold, discoloration, etc.), and avoid

obtaining milk from animals manifesting symptoms of disease. Equally important is the concern for personal hygiene of those who work in harvesting/collecting, processing, packaging, and distributing food. For this purpose, it is necessary to increase access to clean water sources or to enable the use of portable sources. It is also crucial to ensure optimal storage conditions, especially the cleanliness of storage rooms, and to optimize its temperature. These relatively simple treatments will significantly reduce the spread of foodborne pathogens. Also, in developed countries, it is possible to improve certain procedures by strictly adhering to the HACCP system, good manufacturing and hygiene practices, and the appropriate design of food processing plants. It is also worth trying to introduce various types of innovative solutions, mainly in food packaging, but not only. Reducing the contamination of food products with mycotoxins is possible by using adsorption materials in animal husbandry or toxin-degrading microbial catalysts. Following these rules will allow producing microbiologically safe food and enjoying the benefits associated with the consumption of fermented products.

AUTHOR CONTRIBUTIONS

KS, AB, and KG-B: conceptualization and supervision. AB, EW-Z, and NW-K: writing—original draft preparation. KS, KG-B, MA, EW-Z, and EG-K: writing—review and editing. KS and AB: visualization. EG-K: funding acquisition. All authors have read and agreed to the published version of the manuscript.

FUNDING

This article was financed with a contest fund in the "Excellence Initiative Research University" program at Nicolaus Copernicus University in Toruń.

REFERENCES

Aarti, C., Khusro, A., Varghese, R., Arasu, M. V., Agastian, P., Al-Dhabi, N. A., et al. (2018). *In vitro* investigation on probiotic, anti-Candida, and antibiofilm properties of *Lactobacillus pentosus* strain LAP1. *Arch. Oral Biol.* 89, 99–106. doi: 10.1016/j.archoralbio.2018.02.014

Abdalla, M. O., and Hussain, S. K. (2010). Enumeration and identification of microflora in Roub, a Sudanese traditional fermented dairy product. *Br. J. Dairy Sci.* 1, 30–33.

Abdalla, W. M., and El Zubeir, I. E. M. (2006). Microbial hazards associated with fermented milk (*Roub* and *Mish*) processing in Sudan. *Int. J. Dairy Sci.* 1, 21–26.

Abdel-Hameid Ahmed, A., Saad Maharik, N. M., Valero, A., and Kamal, S. M. (2019). Incidence of enterotoxigenic *Staphylococcus aureus* in milk and Egyptian artisanal dairy products. *Food Control* 104, 20–27.

Adebo, O. A. (2020). African sorghum-based fermented foods: past, current and future prospects. *Nutrients* 12:1111. doi: 10.3390/nu12041111

Adedeji, B. S., Ezeokoli, O. T., Ezekiel, C. N., Obadina, A. O., Somorin, Y. M., Sulyok, M., et al. (2017). Bacterial species and mycotoxin contamination associated with locust bean, melon and their fermented products in south-western Nigeria. *Int. J. Food Microb.* 258, 73–80. doi: 10.1016/j.ijfoodmicro.2017.07.014

Adedokun, E. O., Rather, I. A., Bajpai, V. K., and Park, Y. H. (2016). Biocontrol efficacy of *Lactobacillus fermentum* YML014 against food spoilage moulds using the tomato puree model. *Front. Life Sci.* 9, 64–68. doi: 10.1080/21553769.2015.1084951

Adekoya, I., Njobeh, P., Obadina, A., Chilaka, C., Okoth, S., De Boevre, M., et al. (2017). Awareness and prevalence of mycotoxin contamination in selected Nigerian fermented foods. *Toxins* 9:363. doi: 10.3390/toxins9110363

Ademola, O. M., Adeyemi, T. E., Ezeokoli, O. T., Ayeni, K. I., Obadina, A. O., Somorin, Y. M., et al. (2018). Phylogenetic analyses of bacteria associated with the processing of iru and ogiri condiments. *Lett. Appl. Microbiol.* 67, 354–362. doi: 10.1111/lam.13040

Agbor-Egbe, T., and Mbome, I. (2006). The effects of processing techniques in reducing cyanogen levels during the production of some Cameroonian cassava foods. *J. Food Compost. Anal.* 19, 354–363. doi: 10.1016/j.jfca.2005.02.004

Agrawal, A., Houghton, L. A., Morris, J., Reilly, B., Guyonnet, D., Goupil Feuillerat, N., et al. (2009). Clinical trial: the effects of a fermented milk product containing *Bifidobacterium lactis* DN-173 010 on abdominal distension and gastrointestinal transit in irritable bowel syndrome with constipation. *Aliment. Pharmacol. Ther.* 29, 104–114. doi: 10.1111/j.1365-2036.2008.03853.x

Ajayi, O. A. (2014). Bacteriology and qualitative study of African locust bean (*Parkia biglobosa*). *Open J. Soc. Sci.* 2, 73–78. doi: 10.4236/jss.2014.211010

Allende, A., Bolton, D., Chemaly, M., Davies, R., Escamez, P. S. F., Girones, R., et al. (2016). Evaluation of the safety and efficacy of Listex (TM) P100 for reduction of pathogens on different ready-to-eat (RTE) food products. *EFSA J.* 14:e04565. doi: 10.2903/j.efsa.2016.4565

Alvarez, M. A., and Moreno-Arribas, M. V. (2014). The problem of biogenic amines in fermented foods and the use of potential biogenic amine-degrading microorganisms as a solution. *Trends Food Sci. Technol.* 39, 146–155. doi: 10.1016/j.tifs.2014.07.007

Anyogu, A., Olukorede, A., Anumudu, C., Onyeaka, H., Areo, E., Adewale, O., et al. (2021). Microorganisms and food safety risks associated with indigenous fermented foods from Africa. *Food Control* 129:108227. doi: 10.1016/j.foodcont.2021.108227

Axelsson, L., Bjerke, G. A., McLeod, A., Berget, I., and Holck, A. L. (2020). Growth behavior of *Listeria monocytogenes* in a traditional Norwegian fermented fish product (*Rakfisk*), and its inhibition through bacteriophage addition. *Foods* 9:119. doi: 10.3390/foods9020119

Azad, M., Sarker, M., Li, T., and Yin, J. (2018). Probiotic species in the modulation of gut microbiota: an overview. *Biomed Res. Int.* 2018:9478630. doi: 10.1155/2018/9478630

Bachrouri, M., Quinto, E. J., and Mora, M. T. (2006). Kinetic parameters of *Escherichia coli* 0157:H7 survival during fermentation of milk and home-made yogurt. *Int. Dairy J.* 16, 474–481.

Barbieri, F., Montanari, C., Gardini, F., and Tabanelli, G. (2019). Biogenic amine production by lactic acid bacteria: a review. *Foods* 8:17. doi: 10.3390/foods8010017

Bjerke, G. A., Rudi, K., Avershina, E., Moen, B., Blom, H., and Axelsson, L. (2019). Exploring the brine microbiota of a traditional Norwegian fermented fish product (*Rakfisk*) from six different producers during two consecutive seasonal productions. *Foods* 8:72. doi: 10.3390/foods8020072

Bousquet, J., Anto, J. M., Czarlewski, W., Haahtela, T., Fonseca, S. C., Iaccarino, G., et al. (2021). Cabbage and fermented vegetables: from death rate heterogeneity in countries to candidates for mitigation strategies of severe COVID-19. *Allergy* 76, 735–750. doi: 10.1111/all.14549

Boxman, I. L. A., Jansen, C. C. C., Zwartkruis-Nahuis, A. J. T., Hägele, G., Sosef, N. P., and Dirks, R. A. M. (2020). Detection and quantification of hepatitis E virus RNA in ready to eat raw pork sausages in The Netherlands. *Int. J. Food Microbiol.* 333:108791. doi: 10.1016/j.ijfoodmicro.2020.108791

Brockmann, S. O., Oehme, R., Buckenmaier, T., Beer, M., Jeffery-Smith, A., Spannenkrebs, M., et al. (2018). A cluster of two human cases of tick-borne encephalitis (TBE) transmitted by unpasteurised goat milk and cheese in Germany, May 2016. *Euro Surveill.* 23:17-00336. doi: 10.2807/1560-7917.ES.2018.23.15.17-00336

Byakika, S., Mukisa, I. M., Byaruhanga, Y. B., Male, D., and Muyanja, C. (2019). Influence of food safety knowledge, attitudes and practices of processors on microbiological quality of commercially produced traditional fermented cereal

beverages, a case of Obushera in Kampala. *Food Control* 100, 212–219. doi: 10.1016/j.foodcont.2019.01.024

Campbell-Platt, G. (1987). *Fermented Foods of the World: A Dictionary and Guide*. London: Butterworths.

Capozzi, V., Fragasso, M., Romaniello, R., Berbegal, C., Russo, P., and Spano, G. (2017). Spontaneous food fermentations and potential risks for human health. *Fermentation* 3:49. doi: 10.3390/fermentation3040049

Centeno, S., and Calvo, M. (2002). Mycotoxins produced by fungi isolated from wine cork stoppers. *Pak. J. Nutr.* 1, 267–269. doi: 10.3923/pjn.2002.267.269

Center for Disease Control and Infection (2020). *Food and Coronavirus Disease 2019 (COVID-19)*. Available Online at: https://www.cdc.gov/coronavirus/2019-ncov/daily-life-coping/food-and-COVID-19.html [accessed February 4, 2022].

Cho, H. G., Lee, S. G., Lee, M. Y., Hur, E. S., Lee, J. S., Park, P. H., et al. (2016). An outbreak of norovirus infection associated with fermented oyster consumption in South Korea, 2013. *Epidemiol. Infect.* 144, 2759–2764. doi: 10.1017/S0950268816000170

Cho, S. H., Kim, J., Oh, K. H., Hu, J. K., Seo, J., Oh, S. S., et al. (2014). Outbreak of enterotoxigenic *Escherichia coli* O169 enteritis in schoolchildren associated with consumption of Kimchi, Republic of Korea, 2012. *Epidemiol. Infect.* 142, 616–623. doi: 10.1017/S0950268813001477

Christieans, S., Picgirard, L., Parafita, E., Lebert, A., and Gregori, T. (2018). Impact of reducing nitrate/nitrite levels on the behavior of *Salmonella* Typhimurium and *Listeria monocytogenes* in French dry fermented sausages. *Meat Sci.* 137, 160–167. doi: 10.1016/j.meatsci.2017.11.028

Cueva, C., García-Ruiz, A., González-Rompinelli, E., Bartolome, B., Martín-Álvarez, P. J., Salazar, O., et al. (2012). Degradation of biogenic amines by vineyard ecosystem fungi. Potential use in winemaking. *J. Appl. Microbiol.* 112, 672–682. doi: 10.1111/j.1365-2672.2012.05243.x

Dadar, M., Shahali, Y., and Whatmore, A. M. (2019). Human brucellosis caused by raw dairy products: a review on the occurrence, major risk factors and prevention. *Int. J. Food Microbiol.* 292, 39–47. doi: 10.1016/j.ijfoodmicro.2018.12.009

Davoren, M. J., Liu, J., Castellanos, J., Rodríguez-Malavé, N. I., and Schiestl, R. H. (2019). A novel probiotic, *Lactobacillus johnsonii* 456, resists acid and can persist in the human gut beyond the initial ingestion period. *Gut Microbes* 10, 458–480. doi: 10.1080/19490976.2018.1547612

De Mey, E., De Klerck, K., De Maere, H., Dewulf, L., Derdelinckx, G., Peeters, M. C., et al. (2014). The occurrence of N-nitrosamines, residual nitrite and biogenic amines in commercial dry fermented sausages and evaluation of their occasional relation. *Meat Sci.* 96(2 Pt A), 821–828. doi: 10.1016/j.meatsci.2013.09.010

Dehkordi, F. S., Yazdani, F., Mozafari, J., and Valizadeh, Y. (2014). Virulence factors, serogroups and antimicrobial resistance properties of *Escherichia coli* strains in fermented dairy products. *BMC Res. Notes* 7:217. doi: 10.1186/1756-0500-7-217

Díez, J. G., and Patarata, L. (2013). Behavior of *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus aureus* in Chouriço de Vinho, a dry fermented sausage made from wine-marinated meat. *J. Food Prot.* 76, 588–594. doi: 10.4315/0362-028X.JFP-12-212

Dimidi, E., Cox, S. R., Rossi, M., and Whelan, K. (2019). Fermented foods: definitions and characteristics, impact on the gut microbiota and effects on gastrointestinal health and disease. *Nutrients* 11:1806. doi: 10.3390/nu11081806

Doeun, D., Davaatseren, M., and Chung, M. S. (2017). Biogenic amines in foods. *Food Sci. Biotechnol.* 26, 1463–1474. doi: 10.1007/s10068-017-0239-3

Ekici, K., and Omer, A. K. (2018). The determination of some biogenic amines in Turkish fermented sausages consumed in Van. *Toxicol. Rep.* 5, 639–643. doi: 10.1016/j.toxrep.2018.05.008

Evans, E., Musa, A., Abubakar, Y., and Mainuna, B. (2013). "Nigerian indigenous fermented foods: processes and prospects," in *Mycotoxin and Food Safety in Developing Countries*, ed. H. A. Makun (Nigeria: Intech), 153–180. doi: 10.5772/52877

Ezekiel, C. N., Sulyok, M., Babalola, D. A., Warth, B., Ezekiel, V. C., and Krska, R. (2013). Incidence and consumer awareness of toxigenic *Aspergillus* section Flavi and Aflatoxin B1 in peanut cake from Nigeria. *Food Control* 30, 596–601. doi: 10.1016/j.foodcont.2012.07.048

Falconer, T. M., Kern, S. E., Brzezinski, J. L., Turner, J. A., Boyd, B. L., and Litzau, J. J. (2017). Identification of the potent toxin bongkrekic acid in a traditional African beverage linked to a fatal outbreak. *Forensic Sci. Int.* 270, e5–e11. doi: 10.1016/j.forsciint.2016.10.015

Falegan, C. (2011). Microbiology profile and biochemical characteristics of commercial "ogiri" samples from South Western Nigeria. *J. Microbiol. Biotechnol. Food Sci.* 1, 187–203.

Gajamer, V. R., and Tiwari, H. K. (2014). Prevalence of gastrointestinal disease and its associated risk factors in Sikkim and Darjeeling Districts. *J. Community Health.* 39, 767–774.

García-Díez, J., and Saraiva, C. (2021). Use of starter cultures in foods from animal origin to improve their safety. *Int. J. Environ. Res. Public Health* 18:2544. doi: 10.3390/ijerph18052544

Gilsdorf, A., Jansen, A., Alpers, K., Dieckmann, H., van Treeck, U., Hauri, A. M., et al. (2005). A nationwide outbreak of *Salmonella* bovismorbificans PT24, Germany, December 2004-March 2005. *Euro Surveill.* 10:E050324.1.

Gómez-Aldapa, C. A., Díaz-Cruz, C. A., Villarruel-López, A., Del Refugio Torres-Vitela, M., Rangel-Vargas, E., and Castro-Rosas, J. (2012). Acid and alcohol tolerance of *Escherichia coli* O157:H7 in pulque, a typical Mexican beverage. *Int. J. Food Micrbiol.* 154, 79–84. doi: 10.1016/j.ijfoodmicro.2011.12.027

Gonzales, L., Ali, Z. B., Nygren, E., Wang, Z., Karlsson, S., Zhu, B., et al. (2013). Alkaline pH Is a signal for optimal production and secretion of the heat labile toxin, LT in enterotoxigenic *Escherichia coli* (ETEC). *PLoS One* 8:e74069. doi: 10.1371/journal.pone.0074069

Gonzalez-Fandos, E., Vazquez de Castro, M., and Martinez-Laorden, A. (2021). Behaviour of *Listeria monocytogenes* and natural microflora during the manufacture of Riojano chorizo (Spanish dry cured sausage). *Microorganisms* 9:1963. doi: 10.3390/microorganisms9091963

Gudo, E. S., Cook, K., Kasper, A. M., Vergara, A., Salomão, C., Oliveira, F., et al. (2018). Description of a mass poisoning in a rural district in Mozambique: the first documented bongkrekic acid poisoning in Africa. *Clin. Infect. Dis.* 66, 1400–1406. doi: 10.1093/cid/cix1005

Guimarães, A., Santiago, A., Teixeira, J. A., Venâncio, A., and Abrunhosa, L. (2018). Anti-aflatoxigenic effect of organic acids produced by *Lactobacillus plantarum*. *Int. J. Food Microbiol.* 264, 31–38. doi: 10.1016/j.ijfoodmicro.2017.10.025

Gungor, O. E., Kirzioglu, Z., and Kivanc, M. (2015). Probiotics: can they be used to improve oral health? *Benef. Microbes* 6, 647–656. doi: 10.3920/BM2014.0167

Hegab, O. W., Abdel-Latif, E. F., and Moawad, A. A. (2020). Isolation of enterotoxigenic *Staphylococcus aureus* harboring *seb* gene and enteropathogenic *Escherichia coli* (serogroups O18, O114, and O125) from soft and hard artisanal cheeses in Egypt. *Open Vet. J.* 10, 297–307. doi: 10.4314/ovj.v10i3.8

Heir, E., Holck, A. L., Omer, M. K., Alvseike, O., Måge, I., Høy, M., et al. (2013). Effects of post-processing treatments on sensory quality and Shiga toxigenic *Escherichia coli* reductions in dry-fermented sausages. *Meat Sci.* 94, 47–54. doi: 10.1016/j.meatsci.2012.12.020

Hesseltine, C. W., and Wang, H. L. (1980). The importance of traditional fermented foods. *Bioscience* 30, 402–404. doi: 10.2307/1308003

Holzapfel, W. H. (1997). Use of starter cultures in fermentation on a household scale. *Food Control* 8, 241–258.

Holzmann, H., Aberle, S. W., Stiasny, K., Werner, P., Mischak, A., Zainer, B., et al. (2009). Tick-borne encephalitis from eating goat cheese in a mountain region of Austria. *Emerg. Infect. Dis.* 15, 1671–1673. doi: 10.3201/eid1510.090743

Horváth, J. K., Mengel, M., Krisztalovics, K., Nogrady, N., Pászti, J., Lenglet, A., et al. (2013). Investigation into an unusual increase of human cases of *Salmonella* Goldcoast infection in Hungary in 2009. *Euro Surveill.* 18:20422. doi: 10.2807/ese.18.11.20422-en

Hospital, X. F., Hierro, E., Stringer, S., and Fernández, M. (2016). A study on the toxigenesis by *Clostridium botulinum* in nitrate and nitrite-reduced dry fermented sausages. *Int. J. Food Microbiol.* 218, I66–I70. doi: 10.1016/j.ijfoodmicro.2015.11.009

Islam, M. S., Sazzad, H. M., Satter, S. M., Sultana, S., Hossain, M. J., Hasan, M., et al. (2016). Nipah virus transmission from bats to humans associated with drinking traditional liquor made from date palm sap, Bangladesh, 2011-2014. *Emerg. Infect. Dis.* 22, 664–670. doi: 10.3201/eid2204.151747

Jeong, H. W., Kim, M. K., Yi, H. J., Kim, D. M., Jeon, S. J., Lee, H. K., et al. (2021). Hepatitis A virus strains identified in jogaejeot associated with outbreaks in Seoul, South Korea. *Lett. Appl. Microbiol.* 73, 107–112. doi: 10.1111/lam.13482

Jung, K. O., Park, S. Y., and Park, K. Y. (2006). Longer aging time increases the anticancer and antimetastatic properties of doenjang. *Nutrition* 22, 539–545. doi: 10.1016/j.nut.2005.11.007

Keisam, S., Tuikhar, N., Ahmed, G., and Jeyaram, K. (2019). Toxigenic and pathogenic potential of enteric bacterial pathogens prevalent in the traditional fermented foods marketed in the Northeast region of India. *Int. J. Food Microbiol.* 296, 21–30. doi: 10.1016/j.ijfoodmicro.2019.02.012

Khattab, A. A., Ibrahim, M. I. M., and El-Kady, A. A. (2018). Ochratoxin A biosorption onto genetically improved of *Lactobacillus delbrueckii* mutants. *Int. Food Res. J.* 25, 515–522.

Kim, C. W., Cho, S. H., Kang, S. H., Park, Y. B., Yoon, M. H., Lee, J. B., et al. (2015). Prevalence, genetic diversity, and antibiotic resistance of *Bacillus cereus* isolated from Korean fermented soybean products. *J. Food Sci.* 80, M123–M128. doi: 10.1111/1750-3841.12720

Kleppen, H. P., Holo, H., Jeon, S. R., Nes, I. F., and Yoon, S. S. (2012). Novel *Podoviridae* family bacteriophage infecting *Weissella cibaria* isolated from Kimchi. *Appl. Environ. Microbiol.* 78, 7299–7308. doi: 10.1128/AEM.00031-12

Kríz, B., Benes, C., and Daniel, M. (2009). Alimentary transmission of tick-borne encephalitis in the Czech Republic (1997-2008). *Epidemiol. Mikrobiol. Imunol.* 58, 98–103.

Kubo, R. (2016). The reason for the preferential use of finger millet (*Eleusine coracana*) in Eastern African Brewing. *J. Inst. Brew.* 122, 175–180.

Ladero, V., Martín, M. C., Redruello, B., Mayo, B., Flórez, A. B., Fernández, M., et al. (2015). Genetic and functional analysis of biogenic amine production capacity among starter and non-starter lactic acid bacteria isolated from artisanal cheeses. *Eur. Food Res. Technol.* 241, 377–383. doi: 10.1007/s00217-015-2469-z

Laranjo, M., Elias, M., and Fraqueza, M. J. (2017). The use of starter cultures in traditional meat products. *J. Food Qual.* 2017:9546026. doi: 10.1155/2017/9546026

Latorre-Moratalla, M. L., Bover-Cid, S., Talon, R., Aymerich, T., Garriga, M., Zanardi, E., et al. (2010). Distribution of aminogenic activity among potential autochthonous starter cultures for dry fermented sausages. *J. Food Prot.* 73, 524–528. doi: 10.4315/0362-028X-73.3.524

Lautié, E., Rozet, E., Hubert, P., Vandelaer, N., Billard, F., Felde, T. Z., et al. (2013). Fast method for the simultaneous quantification of toxic polyphenols applied to the selection of genotypes of yam bean (*Pachyrhizus* sp.) seeds. *Talanta* 117, 94–101. doi: 10.1016/j.talanta.2013.08.038

Le, T. M., Do, T. N., Le, M. H., Tran, T. M. K., and Van, V. Q. (2012). Evaluation of hygiene and safety status of nem chua production in Hanoi. *Vietnam J. Sci. Technol.* 50, 231–237.

Le Guyader, F. S., Bon, F., DeMedici, D., Parnaudeau, S., Bertone, A., Crudeli, S., et al. (2006). Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. *J. Clin. Microbiol.* 44, 3878–3882. doi: 10.1128/JCM.01327-06

Lee, H. M., Lee, J. H., Kim, S. H., Yoon, S. R., Lee, J. Y., and Ha, J. H. (2017). Correlation between changes in microbial/physicochemical properties and persistence of human norovirus during cabbage Kimchi fermentation. *J. Microbiol. Biotechnol.* 27, 2019–2027. doi: 10.4014/jmb.1707.07041

Lee, H. S., Lindahl, J., Nguyen-Viet, H., Khong, N. V., Nghia, V. B., Xuan, H. N., et al. (2017). An investigation into aflatoxin M1in slaughtered fattening pigs and awareness of aflatoxins in Vietnam. *BMC Vet. Res.* 13:363. doi: 10.1186/s12917-017-1297-8

Lee, N., Kim, M. D., Chang, H. J., Choi, S. W., and Chun, H. S. (2017). Genetic diversity, antimicrobial resistance, toxin gene profiles, and toxin production ability of *Bacillus cereus* isolates from doenjang, a Korean fermented soybean paste. *J. Food Saf.* 37:e12363. doi: 10.1111/jfs.12363

Leedom, J. M. (2006). Milk of nonhuman origin and infectious diseases in humans. *Clin. Infect. Dis.* 43, 610–615. doi: 10.1086/507035

Li, L., Wen, X., Wen, Z., Chen, S., Wang, L., and Wei, X. (2018). Evaluation of the biogenic amines formation and degradation abilities of *Lactobacillus curvatus* from Chinese Bacon. *Front. Microbiol.* 9:1015. doi: 10.3389/fmicb.2018.01015

Li, R., Zheng, X., Yang, J., Shen, X., Jiao, L., Yan, Z., et al. (2019). Relation between gut microbiota composition and traditional spontaneous fermented dairy foods among Kazakh nomads in Xinjiang, China. *J. Food Sci.* 84, 3804–3814. doi: 10.1111/1750-3841.14912

Liu, H., Qiu, H., Che, Z., Xu, Q., and Shu, H. (2006). Investigation on a food poisoning case caused by *Bacillus cereus*. *Dis. Surveill.* 21:652.

Lore, T. A., Mbugua, S. K., and Wangoh, J. (2005). Enumeration and identification of microflora in *suusac*, a Kenyan traditional fermented camel milk product. *LWT Food Sci. Technol.* 38, 125–130.

Lorenzo, J. M., Cachaldora, A., Fonseca, S., Gómez, M., Franco, I., and Carballo, J. (2010). Production of biogenic amines "*in vitro*" in relation to the growth phase by *Enterobacteriaceae* species isolated from traditional sausages. *Meat Sci.* 86, 684–691. doi: 10.1016/j.meatsci.2010.06.005

Luz, C., Saladino, F., Luciano, F. B., Mañes, J., and Meca, G. (2017). *In vitro* antifungal activity of bioactive peptides produced by *Lactobacillus plantarum* against *Aspergillus parasiticus* and *Penicillium expansum*. *LWT Food Sci. Technol.* 81, 128–135.

Mah, J. H., Park, Y. K., Jin, Y. H., Lee, J. H., and Hwang, H. J. (2019). Bacterial production and control of biogenic amines in Asian fermented soybean foods. *Foods* 8:85. doi: 10.3390/foods8020085

Maitha, I. M., Kaindi, D. W. M., Wangoh, J., and Mbugua, S. (2019). Microbial quality and safety of traditional fermented camel milk product suusac sampled from different regions in North Eastern, Kenya. *Asian Food Sci. J.* 8, 1–9. doi: 10.9734/afsj/2019/v8i229986

Majou, D., and Christieans, S. (2018). Mechanisms of the bactericidal effects of nitrate and nitrite in cured meats. *Meat Sci.* 145, 273–284. doi: 10.1016/j.meatsci.2018.06.013

Mannaa, M., Han, G., Seo, Y. S., and Park, I. (2021). Evolution of food fermentation processes and the use of multi-omics in deciphering the roles of the microbiota. *Foods* 10:2861. doi: 10.3390/foods10112861

Markovinović, L., Kosanović Ličina, M. L., Tešić, V., Vojvodić, D., Vladušić Lucić, I., Kniewald, T., et al. (2016). An outbreak of tick-borne encephalitis associated with raw goat milk and cheese consumption, Croatia, 2015. *Infection* 44, 661–665. doi: 10.1007/s15010-016-0917-8

Marteau, P., Guyonnet, D., Lafaye de Micheaux, P., and Gelu, S. (2013). A randomized, double-blind, controlled study and pooled analysis of two identical trials of fermented milk containing probiotic *Bifidobacterium lactis* CNCM I-2494 in healthy women reporting minor digestive symptoms. *Neurogastroenterol. Motil.* 25, 331–e252. doi: 10.1111/nmo.12078

Martin, B., Garriga, M., and Aymerich, T. (2011). Prevalence of *Salmonella* spp. and *Listeria monocytogenes* at small-scale Spanish factories producing traditional fermented sausages. *J. Food Prot.* 74, 812–815. doi: 10.4315/0362-028X.JFP-10-437

Maske, B. L., de Melo Pereira, G. V., da Silva Vale, A., Souza, D. S. M., De Dea Lindner, J., and Soccol, C. R. (2021). Viruses in fermented foods: are they good or bad? Two sides of the same coin. *Food Microbiol.* 98:103794. doi: 10.1016/j.fm.2021.103794

Matumba, L., Monjerezi, M., Kankwamba, H., Njoroge, S. M., Ndilowe, P., Kabuli, H., et al. (2016). Knowledge, attitude, and practices concerning presence of molds in foods among members of the general public in Malawi. *Mycotoxin Res.* 32, 27–36. doi: 10.1007/s12550-015-0237-3

McLeod, A., Måge, I., Heir, E., Axelsson, L., and Holck, A. L. (2016). Effect of relevant environmental stresses on survival of enterohemorrhagic *Escherichia coli* in dry-fermented sausage. *Int. J. Food Microbiol.* 229, 15–23. doi: 10.1016/j.ijfoodmicro.2016.04.005

McNulty, N. P., Yatsunenko, T., Hsiao, A., Faith, J. J., Muegge, B. D., Goodman, A. L., et al. (2011). The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. *Sci. Transl. Med.* 3:106ra106. doi: 10.1126/scitranslmed.3002701

Meloni, D. (2015). Presence of *Listeria monocytogenes* in Mediterranean-style dry fermented sausages. *Foods* 4, 34–50. doi: 10.3390/foods4010034

Meloni, D. (2019). High-Hydrostatic-Pressure (HHP) processing technology as a novel control method for *Listeria monocytogenes* occurrence in Mediterranean-style dry-fermented sausages. *Foods* 8:672. doi: 10.3390/foods8120672

Meloni, D., Consolati, S. G., Mazza, R., Mureddu, A., Fois, F., Piras, F., et al. (2014). Presence and molecular characterization of the major serovars of *Listeria monocytogenes* in ten Sardinian fermented sausage processing plants. *Meat Sci.* 97, 443–450. doi: 10.1016/j.meatsci.2014.02.012

Meng, Z., Li, Z., Jin, J., Zhang, Y., Liu, X., Yiang, X., et al. (1988). Studies on fermented corn flour poisoning in rural areas of China. I. Epidemiology, clinical manifestations, and pathology. *Biomed. Environ. Sci.* 1, 101–104.

Michel, A. L., Geoghegan, C., Hlokwe, T., Raseleka, K., Getz, W. M., and Marcotty, T. (2015). Longevity of *Mycobacterium bovis* in raw and traditional souring

milk as a function of storage temperature and dose. *PLoS One* 10:e0129926. doi: 10.1371/journal.pone.0129926

Milani, C., Duranti, S., Napoli, S., Alessandri, G., Mancabelli, L., Anzalone, R., et al. (2019). Colonization of the human gut by bovine bacteria present in Parmesan cheese. *Nat. Commun.* 10:1286. doi: 10.1038/s41467-019-09303-w

Milani, J., and Maleki, G. (2014). Effects of processing on mycotoxin stability in cereals. *J. Sci. Food Agric.* 94, 2372–2375. doi: 10.1002/jsfa.6600

Miranda, C., Contente, D., Igrejas, G., Câmara, S., Dapkevicius, M., and Poeta, P. (2021). Role of exposure to lactic acid bacteria from foods of animal origin in human health. *Foods* 10:2092. doi: 10.3390/foods10092092

Montagnac, J. A., Davis, C. R., and Tanumihardjo, S. A. (2009). Processing techniques to reduce toxicity and antinutrients of cassava for use as a staple food. *Compr. Rev. Food Sci. Food Saf.* 8, 17–27. doi: 10.1111/j.1541-4337.2008.00064.x

Moor, D., Liniger, M., Baumgartner, A., and Felleisen, R. (2018). Screening of ready-to-eat meat products for hepatitis E virus in Switzerland. *Food Environ. Virol.* 10, 263–271. doi: 10.1007/s12560-018-9340-x

Moxley, R. A. (2004). *Escherichia coli* 0157:H7: an update on intestinal colonization and virulence mechanisms. *Anim. Health Res. Rev.* 5, 15–33. doi: 10.1079/ahr200463

Mwangi, L., Matofari, J. W., Muliro, P., and Bebe, B. (2017). Occurrence of *Brucella* and *Mycobacteria* species in raw and fermented camel milk along the value Chain. *Asian J. Agric. Food Sci.* 4, 212–218.

Nahidul-Islam, S. M., Kuda, T., Takahashi, H., and Kimura, B. (2018). Bacterial and fungal microbiota in traditional Bangladeshi fermented milk products analysed by culture-dependent and culture-independent methods. *Food Res. Int.* 111, 431–437. doi: 10.1016/j.foodres.2018.05.048

Nemska, V., Logar, P., Rasheva, T., Sholeva, Z., Georgieva, N., and Danova, S. (2019). Functional characteristics of lactobacilli from traditional Bulgarian fermented milk products. *Turk. J. Biol.* 43, 148–153. doi: 10.3906/biy-1808-34

Nkhata, S. G., Ayua, E., Kamau, E. H., and Shingiro, J. B. (2018). Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes. *Food Sci. Nutr.* 6, 2446–2458. doi: 10.1002/fsn3.846

Odunmbaku, L. A., Sobowale, S. S., Adenekan, M. K., Oloyede, T., Adebiyi, J. A., and Adebo, O. A. (2017). Influence of steeping duration, drying temperature, and duration on the chemical composition of sorghum starch. *Food Sci. Nutr.* 6, 348–355. doi: 10.1002/fsn3.562

Oguntoyinbo, F. A. (2014). Safety challenges associated with traditional foods of West Africa. *Food Rev. Int.* 30, 338–358. doi: 10.1080/87559129.2014.940086

Olea-Popelka, F., Muwonge, A., Perera, A., Dean, A. S., Mumford, E., Erlacher-Vindel, E., et al. (2017). Zoonotic tuberculosis in human beings caused by *Mycobacterium bovis*-a call for action. *Lancet Infect. Dis.* 17, e21–e25. doi: 10.1016/S1473-3099(16)30139-6

Ombarak, R. A., Hinenoya, A., Awasthi, S. P., Iguchi, A., Shima, A., Elbagory, A. M., et al. (2016). Prevalence and pathogenic potential of *Escherichia coli* isolates from raw milk and raw milk cheese in Egypt. *Int. J. Food Microbiol.* 221, 69–76. doi: 10.1016/j.ijfoodmicro.2016.01.009

Ordóñez, J. L., Troncoso, A. M., García-Parrilla, M., and Callejón, R. M. (2016). Recent trends in the determination of biogenic amines in fermented beverages - a review. *Anal. Chim. Acta* 939, 10–25. doi: 10.1016/j.aca.2016.07.045

Ostry, V., Malir, F., Toman, J., and Grosse, Y. (2017). Mycotoxins as human carcinogens-the IARC monographs classification. *Mycotoxin Res.* 33, 65–73. doi: 10.1007/s12550-016-0265-7

Owusu-Kwarteng, J., Akabanda, F., Agyei, D., and Jespersen, L. (2020). Microbial safety of milk production and fermented dairy products in Africa. *Microorganisms* 8:752. doi: 10.3390/microorganisms8050752

Park, J. H., Jung, S., Shin, J., Lee, J. S., Joo, I. S., and Lee, D. Y. (2015). Three gastroenteritis outbreaks in South Korea caused by the consumption of Kimchi tainted by norovirus GI.4. *Foodborne Pathog. Dis.* 12, 221–227. doi: 10.1089/fpd.2014.1879

Park, K. M., Kim, H. J., Jeong, M. C., and Koo, M. (2016). Occurrence of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* in Doenjang, a Korean Fermented Soybean Paste. *J. Food Prot.* 79, 605–612. doi: 10.4315/0362-028X.JFP-15-416

Park, Y. K., Lee, J. H., and Mah, J. H. (2019). Occurrence and reduction of biogenic amines in Kimchi and Korean Fermented seafood products. *Foods* 8:547. doi: 10.3390/foods8110547

Patarata, L., Novais, M., Fraqueza, M. J., and Silva, J. A. (2020). Influence of meat spoilage microbiota initial load on the growth and survival of three pathogens on a naturally fermented sausage. *Foods* 9:676. doi: 10.3390/foods9050676

Pavio, N., Merbah, T., and Thébault, A. (2014). Frequent hepatitis E virus contamination in food containing raw pork liver, France. *Emerg. Infect. Dis.* 20, 1925–1927. doi: 10.3201/eid2011.140891

Pei, X., Zhang, W., Jiang, H., Liu, D., Liu, X., Li, L., et al. (2021). Food-origin mycotoxin-induced neurotoxicity: intend to break the rules of neuroglia cells. *Oxid. Med. Cell. Longev.* 2021:9967334. doi: 10.1155/2021/9967334

Peng, Z., Dottorini, T., Hu, Y., Li, M., Yan, S., Fanning, S., et al. (2021). Comparative genomic analysis of the foodborne pathogen *Burkholderia gladioli* pv. *cocovenenans* harboring a bongkrekic acid biosynthesis gene cluster. *Front. Microbiol.* 12:628538. doi: 10.3389/fmicb.2021.628538

Phukan, R. K., Narain, K., Zomawia, E., Hazarika, N. C., and Mahanta, J. (2006). Dietary habits and stomach cancer in Mizoram, India. *J. Gastroenterol.* 41, 418–424.

Piras, F., Spanu, C., Mocci, A. M., Demontis, M., Santis, E., and Scarano, C. (2019). Occurrence and traceability of *Salmonella* spp. in five Sardinian fermented sausage facilities. *Ital. J. Food Saf.* 8:8011. doi: 10.4081/ijfs.2019.8011

Pringsulaka, O., Patarasinpaiboon, N., Suwannasai, N., Atthakor, W., and Rangsiruji, A. (2011). Isolation and characterisation of a novel Podoviridae-phage infecting *Weissella cibaria* N 22 from Nham, a Thai fermented pork sausage. *Food Microbiol.* 28, 518–525. doi: 10.1016/j.fm.2010.10.011

Pugin, B., Barcik, W., Westermann, P., Heider, A., Wawrzyniak, M., Hellings, P., et al. (2017). A wide diversity of bacteria from the human gut produces and degrades biogenic amines. *Microb. Ecol. Health Dis.* 28:1353881. doi: 10.1080/16512235.2017.1353881

Raiola, A., Tenore, G. C., Manyes, L., Meca, G., and Ritieni, A. (2015). Risk analysis of main mycotoxins occurring in food for children: an overview. *Food Chem. Toxicol.* 84, 169–180.

Rehfeld, I. S., Fraiha, A. L. S., Matos, A. C. D., Guedes, M. I. M. C., Costa, E. A., de Souza, M. R., et al. (2017). Short communication: survival of Vaccinia virus in inoculated cheeses during 60-day ripening. *J. Dairy Sci.* 100, 7051–7054. doi: 10.3168/jds.2016-12560

Renou, C., Roque-Afonso, A. M., and Pavio, N. (2014). Foodborne transmission of hepatitis E virus from raw pork liver sausage, France. *Emerg. Infect. Dis.* 20, 1945–1947. doi: 10.3201/eid2011.140791

Rezac, S., Kok, C. R., Heermann, M., and Hutkins, R. (2018). Fermented foods as a dietary source of live organisms. *Front. Microbiol.* 9:1785. doi: 10.3389/fmicb.2018.01785

Romijn, A. R., Rucklidge, J. J., Kuijer, R. G., and Frampton, C. (2017). A double-blind, randomized, placebo-controlled trial of *Lactobacillus helveticus* and *Bifidobacterium longum* for the symptoms of depression. *Aust. N. Z. J. Psychiatry* 51, 810–821.

Roselli, M., Natella, F., Zinno, P., Guantario, B., Canali, R., Schifano, E., et al. (2021). Colonization ability and impact on human gut microbiota of foodborne microbes from traditional or probiotic-added fermented foods: a systematic review. *Front. Nutr.* 8:689084. doi: 10.3389/fnut.2021.689084

Ruiz-Capillas, C., and Herrero, A. M. (2019). Impact of biogenic amines on food quality and safety. *Foods* 8:62. doi: 10.3390/foods8020062

Sadiq, F. A., Yan, B. W., Tian, F. W., Zhao, J. X., Zhang, H., and Chen, W. (2019). Lactic acid bacteria as antifungal and anti-mycotoxigenic agents: a comprehensive review. *Compr. Rev. Food. Sci. Food Saf.* 10, 1403–1436. doi: 10.1111/1541-4337.12481

Saltanat, H., Li, H., Xu, Y., Wang, J., Liu, F., and Geng, X. H. (2009). The influences of camel milk on the immune response of chronic hepatitis B patients. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 25, 431–433.

Scavia, G., Ciaravino, G., Luzzi, I., Lenglet, A., Ricci, A., Barco, L., et al. (2013). A multistate epidemic outbreak of *Salmonella* Goldcoast infection in humans, June 2009 to March 2010: the investigation in Italy. *Euro Surveill.* 18:20424. doi: 10.2807/ese.18.11.20424-en

Scussel, V. M., Scholten, J. M., Rensen, P. M., Spanjer, M. C., Giordano, B. N. E., and Savi, G. D. (2013). Multitoxin evaluation in fermented beverages and cork stoppers by liquid chromatography-tandem mass spectrometry. *Int. J. Food Sci. Technol.* 48, 96–102. doi: 10.1111/j.1365-2621.2012.03163.x

Seo, D. J., Lee, M. H., Seo, J., Ha, S. D., and Choi, C. (2014). Inactivation of murine norovirus and feline calicivirus during oyster fermentation. *Food Microbiol.* 44, 81–86. doi: 10.1016/j.fm.2014.05.016

Shane, L. E., Porto-Fett, A., Shoyer, B. A., Phebus, R. K., Thippareddi, H., Hallowell, A., et al. (2018). Evaluation of post-fermentation heating times and temperatures for controlling Shiga toxin-producing *Escherichia coli* cells in a non-dried, pepperoni-type sausage. *Ital. J. Food Saf.* 7:7250. doi: 10.4081/ijfs.2018.7250

Sharma, C., and Singh, C. (2014). Therapeutic value of camel milk – a review. *Adv. J. Pharm. Life Sci. Res.* 2, 7–13.

Shen, Q., Yang, Q., and Li, Y. (2005). Investigation of one *Bacillus cereus* food poisoning case. *Pract. Prevent. Med.* 12:713.

Shin, J., Yoon, K. B., Jeon, D. Y., Oh, S. S., Oh, K. H., Chung, G. T., et al. (2016). Consecutive outbreaks of enterotoxigenic *Escherichia coli* O6 in schools in South Korea caused by contamination of fermented vegetable Kimchi. *Foodborne Pathog. Dis.* 13, 535–543. doi: 10.1089/fpd.2016.2147

Shukla, S., Park, H. K., Lee, J. S., Kim, J. K., and Kim, M. (2014). Reduction of biogenic amines and aflatoxins in Doenjang samples fermented with various Meju as starter cultures. *Food Control* 42, 181–187. doi: 10.1016/j.foodcont.2014.02.006

Siegrist, M., and Cvetkovich, G. (2001). Better negative than positive? Evidence of a bias for negative information about possible health dangers. *Risk Anal.* 21, 199–206. doi: 10.1111/0272-4332.211102

Singh, P. P., Rathore, V., and Singh, L. B. (1986). A possible role of fish preparation 'Hentak' in urolithiasis in Manipur–an experimental study. *Indian J. Exp. Biol.* 24, 88–90.

Sivamaruthi, B. S., Kesika, P., and Chaiyasut, C. (2018). Toxins in fermented foods: prevalence and preventions-a mini review. *Toxins* 11:4. doi: 10.3390/toxins11010004

Skåra, T., Axelsson, L., Stefánsson, G., Ekstrand, B., and Hagen, H. (2015). Fermented and ripened fish products in the northern European countries. *J. Ethnic Foods* 2, 18–24.

Smith, T. A. (1980). Amines in food. *Food Chem.* 6, 169–200. doi: 10.1016/0308-8146(81)90008-X

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., et al. (2010). Biogenic amines in fermented foods. *Eur. J. Clin. Nutr.* 64(Suppl. 3), S95–S100. doi: 10.1038/ejcn.2010.218

Sun, Q., Chen, Q., Li, F., Zheng, D., and Kong, B. (2016). Biogenic amine inhibition and quality protection of Harbin dry sausages by inoculation with *Staphylococcus xylosus* and *Lactobacillus plantarum*. *Food Control* 68, 358–366. doi: 10.1016/j.foodcont.2016.04.021

Tamang, J. P., Cotter, P. D., Endo, A., Han, N. S., Kort, R., Liu, S. Q., et al. (2020). Fermented foods in a global age: east meets West. *Compr. Rev. Food Sci. Food Saf.* 19, 184–217. doi: 10.1111/1541-4337.12520

Tamang, J. P., Holzapfel, W. H., and Watanabe, K. (2016). Diversity of microorganisms in global fermented foods and beverages. *Front. Microbiol.* 7:377. doi: 10.3389/fmicb.2016.00377

Tokarz-Deptuła, B., and Deptuła, W. (2017). Probiotics and mammalian gastrointestinal immune system. *Postêpy Mikrobiol.* 56, 157–162. doi: 10.21307/PM-2017.56.2.157

Tóth, A. G., Csabai, I., Judge, M. F., Maróti, G., Becsei, Á., Spisák, S., et al. (2021). Mobile antimicrobial resistance genes in probiotics. *Antibiotics* 10:1287. doi: 10.3390/antibiotics10111287

Tóth, A. G., Csabai, I., Maróti, G., Jerzsele, Á., Dubecz, A., Patai, Á. V., et al. (2020). A glimpse of antimicrobial resistance gene diversity in kefir and yoghurt. *Sci. Rep.* 10:22458. doi: 10.1038/s41598-020-80444-5

Uusitupa, H. M., Rasinkangas, P., Lehtinen, M. J., Mäkelä, S. M., Airaksinen, K., Anglenius, H., et al. (2020). *Bifidobacterium animalis* subsp. *lactis* 420 for metabolic health: review of the research. *Nutrients* 12:892. doi: 10.3390/nu12040892

Uzogara, S. G., Agu, L. N., and Uzogara, E. O. (1990). A review of traditional fermented foods, condiments and beverages in Nigeria: their benefits and possible problems. *Ecol. Food Nutr.* 24, 267–288. doi: 10.1080/03670244.1990.9991145

Valerio, F., Favilla, M., De Bellis, P., Sisto, A., de Candia, S., and Lavermicocca, P. (2009). Antifungal activity of strains of lactic acid bacteria isolated from a semolina ecosystem against *Penicillium roqueforti, Aspergillus niger* and *Endomyces fibuliger* contaminating bakery products. *Syst. Appl. Microbiol.* 32, 438–448. doi: 10.1016/j.syapm.2009.01.004

van Reckem, E., Geeraerts, W., Charmpi, C., Van der Veken, D., De Vuyst, L., and Leroy, F. (2019). Exploring the link between the geographical origin of European fermented foods and the diversity of their bacterial communities: the case of fermented meats. *Front. Microbiol.* 10:2302. doi: 10.3389/fmicb.2019.02302

van Veen, A. G. (1967). "The bongkrek toxins," in *Biochemistry of Some Foodborne Microbial Toxins*, eds E. I. Mateles and G. N. Wogan (Cambridge, MA: MIT Press), 43–50.

Varela-Hernández, J. J., Cabrera-Díaz, E., Cardona-López, M. A., Ibarra-Velázquez, L. M., Rangel-Villalobos, H., Castillo, A., et al. (2007). Isolation and characterization of Shiga toxin-producing *Escherichia coli* O157:H7 and non-O157 from beef carcasses at a slaughter plant in Mexico. *Int. J. Food Microbiol.* 113, 237–241.

Viaro, H. P., da Silva, J. J., de Souza Ferranti, L., Bordini, J. G., Massi, F. P., and Fungaro, M. (2017). The first report of *A. novoparasiticus*, *A. arachidicola* and *A. pseudocaelatus* in Brazilian corn kernels. *Int. J. Food Microbiol.* 21, 46–51. doi: 10.1016/j.ijfoodmicro.2016.12.002

Vignolo, G., Fontana, C., and Fadda, S. (2010). "Semidry and dry fermented sausages," in *Handbook of Meat Processing*, ed. F. Toldrá (Ames, IA: Blackwell Publishing), 379–398.

Vilela, A. (2021). An overview of CRISPR-based technologies in wine yeasts to improve wine flavor and safety. *Fermentation.*7:5. doi: 10.3390/fermentation7010005

Vilela, A., Cosme, F., and Inês, A. (2020). Wine and non-dairy fermented beverages: a novel source of pro- and prebiotics. *Fermentation* 6:113. doi: 10.3390/fermentation6040113

Voidarou, C., Antoniadou, M, Rozos, G., Tzora, A., Skoufos, I., Varzakas, T., et al. (2020). Fermentative foods: microbiology, biochemistry, potential human health benefits and public health issues. *Foods* 10:69. doi: 10.3390/foods10010069

Vu, N. T., and Nguyen, T. V. A. (2016). "Ethnic fermented foods and beverages of Vietnam," in *Ethnic Fermented Foods and Alcoholic Beverages of Asia*, ed. J. P. Tamang (New Delhi: Springer India), 383–409.

Wolff, A., Günther, T., Albert, T., and Johne, R. (2020a). Effect of sodium chloride, sodium nitrite and sodium nitrate on the infectivity of hepatitis E virus. *Food Environ. Virol.* 12, 350–354. doi: 10.1007/s12560-020-09440-2

Wolff, A., Günther, T., Albert, T., Schilling-Loeffler, K., Gadicherla, A. K., and Johne, R. (2020b). Stability of hepatitis E virus at different pH values. *Int. J. Food Microbiol.* 325:108625. doi: 10.1016/j.ijfoodmicro.2020.108625

Wu, D., Xie, W., Li, X., Cai, G., and Lu, J. (2020). Metabolic engineering of *Saccharomyces cerevisiae* using the CRISPR/Cas9 system to minimize ethyl carbamate accumulation during Chinese rice wine fermentation. *Appl. Microbiol. Biotechnol.* 104, 4435–4444. doi: 10.1007/s00253-020-10549-4

Xiang, H., Sun-Waterhouse, D., Waterhouse, G. I. N., Cui, C., and Ruan, Z. (2019). Fermentation-enabled wellness foods: a fresh perspective. *Food Sci. Hum. Wellness* 8, 203–243. doi: 10.1016/j.fshw.2019.08.003

Xu, Y., Zang, J., Regenstein, J. M., and Xia, W. (2021). Technological roles of microorganisms in fish fermentation: a review. *Crit. Rev. Food Sci. Nutr.* 61, 1000–1012. doi: 10.1080/10408398.2020.1750342

Yam, B. A. Z., Khomeiri, M., Mahounak, A. S., and Jafari, S. M. (2014). Hygienic quality of camel milk and fermented camel milk (Chal) in Golestan Province, Iran. *J. Microbiol. Res.* 4, 98–103.

Yakubu, Y., Shuaibu, A. B., Ibrahim, A. M., Hassan, U. L., and Nwachukwu, R. J. (2018). Risk of shiga toxigenic *Escherichia coli* O_{157}:H_7 infection from raw and fermented milk in sokoto metropolis, Nigeria. *J. Pathog* 2018:8938597. doi: 10.1155/2018/8938597

Yim, J. H., Kim, K. Y., Chon, J. W., Kim, D. H., Kim, H. S., Choi, D. S., et al. (2015). Incidence, antibiotic susceptibility, and toxin profiles of *Bacillus cereus* sensu lato isolated from Korean fermented soybean products. *J. Food Sci.* 80, M1266–M1270. doi: 10.1111/1750-3841.12872

Yuan, Y., Gao, R., Liang, Q., Song, L., Huang, J., Lang, N., et al. (2020). A foodborne bongkrekic acid poisoning incident - Heilongjiang Province, 2020. *China CDC Wkly.* 2, 975–978. doi: 10.46234/ccdcw2020.264

Zaman, M. Z., Bakar, F. A., Selamat, J., Bakar, J., Ang, S. S., and Chong, C. Y. (2014). Degradation of histamine by the halotolerant *Staphylococcus carnosus* FS19 isolate obtained from fish sauce. *Food Control* 40, 58–63. doi: 10.1016/j.foodcont.2013.11.031

Zang, J., Xu, Y., Xia, W., and Regenstein, J. M. (2020). Quality, functionality, and microbiology of fermented fish: a review. *Crit. Rev. Food Sci. Nutr.* 60, 1228–1242. doi: 10.1080/10408398.2019.1565491

Zhang, C., Derrien, M., Levenez, F., Brazeilles, R., Ballal, S. A., Kim, J., et al. (2016). Ecological robustness of the gut microbiota in response to ingestion of transient food-borne microbes. *ISME J.* 10, 2235–2245. doi: 10.1038/ismej.2016.13

Zhang, S. H., and Liu, Y. (2000). *Prepare Technology of Seasoning*. Guangzhou: South China University of Technology Press, 85–95.

Zhang, Z., Jin, M., Wang, K., Zhang, N., Zhang, Q., Tao, X., et al. (2021). Short-term intake of *Lactiplantibacillus plantarum* ZDY2013 fermented milk promotes homoeostasis of gut microbiota under enterotoxigenic *Bacillus cereus* challenge. *Food Funct.* 12, 5118–5129. doi: 10.1039/d1fo00162k

Zhao, G., Wang, Y. F., Chen, J., and Yao, Y. (2020). Predominant mycotoxins, pathogenesis, control measures, and detection methods in fermented pastes. *Toxins* 12:78. doi: 10.3390/toxins12020078

Zhou, G., Bester, K., Liao, B., Yang, Z., Jiang, R., and Hendriksen, N. B. (2014). Characterization of three *Bacillus cereus* strains involved in a major outbreak of food poisoning after consumption of fermented black beans (Douchi) in Yunan, China. *Foodborne Pathog. Dis.* 11, 769–774. doi: 10.1089/fpd.2014.1768

Zúñiga Estrada, A., Mota de la Garza, L., Sánchez Mendoza, M., Santos López, E. M., Filardo Kerstupp, S., and López Merino, A. (2005). Survival of *Brucella abortus* in milk fermented with a yoghurt starter culture. *Rev. Latinoam. Microbiol.* 47, 88–91.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 March 2022
doi: 10.3389/fmicb.2022.842390

Microbiota Survey of Sliced Cooked Ham During the Secondary Shelf Life

*Gloria Spampinato[1], Francesco Candeliere[1], Alberto Amaretti[1,2], Fabio Licciardello[1,2], Maddalena Rossi[1,2] and Stefano Raimondi[1]**

[1] Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy, [2] BIOGEST-SITEIA, University of Modena and Reggio Emilia, Modena, Italy

Sliced cooked ham packaged in a modified atmosphere is a popular ready-to-eat product, subjected to abundant microbial contamination throughout its shelf life that can lead to deterioration of both sensorial properties and safety. In this study, the microbial load and the chemical–physical features of cooked ham of five producers were monitored for a period of 12 days after the opening of the packages (i.e., the secondary shelf life), during which the products were stored in a domestic refrigerator at 5.2 ± 0.6°C. The sensorial properties presented a perceivable decay after 8 days and became unacceptable after 12 days. High-performance liquid chromatography analysis and solid-phase microextraction coupled with gas chromatography profiling of volatile metabolites indicated that lactic acid, ethanol, acetic acid, acetoin, 3-methyl-1-butanol, and 2-3 butanediol were the main metabolites that characterized the evolution of the analyzed cooked ham. The microbiota was monitored by 16S ribosomal RNA gene profiling and culture-dependent techniques. Already at the opening of packages, all the products presented high microbial load, generally dominated by lactic acid bacteria, with evident differences among the products. The increase of lactic acid bacteria somehow protected samples from abundant contamination by other bacteria, concurring with the evolution of more safe products. This role was exerted by numerous *Latilactobacillus*, *Leuconostoc*, and *Carnobacterium* species, among which the most frequently detected were *Latilactobacillus sakei*, *Latilactobacillus sakei carnosum*, *Leuconostoc mesenteroides*, and *Carnobacterium divergens.* Some products presented more complex communities that encompassed Proteobacteria such as *Moellerella wisconsensis*, *Proteus hauseri*, *Brochothrix thermosphacta*, and less frequently *Pseudomonas*, *Erwinia*, and *Massilia*. Opportunistic pathogenic bacteria such as *Escherichia coli* and *Vibrio* sp. were found in small quantities. The yeasts *Kazachstania servazzii* and *Debaryomyces hansenii* occurred already at 0 days, whereas various species of *Candida* (*Candida zeylanoides*, *Candida sake*, *Candida norvegica*, and *Candida glaebosa*) were abundant only after 12 days. These results indicated that the microbiological contaminants overgrowing during the secondary shelf life did not derive from environmental cross-contamination at the opening of the tray but were already present when the packages were opened, highlighting the phases of production up to the packaging as those crucial in managing the safety risk associated to this product.

Keywords: cooked ham, secondary shelf life, food waste, *Leuconostoc carnosum*, *Leuconostoc mesenteroides*, *Carnobacterium divergens*

OPEN ACCESS

Edited by:
Laurent Dufossé,
Université de la Réunion, France

Reviewed by:
Giulia Tabanelli,
University of Bologna, Italy
Ioanna Mantzourani,
Democritus University of Thrace, Greece

****Correspondence:***
Stefano Raimondi
stefano.raimondi@unimore.it

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *23 December 2021*
Accepted: *25 January 2022*
Published: *08 March 2022*

Citation:
Spampinato G, Candeliere F, Amaretti A, Licciardello F, Rossi M and Raimondi S (2022) Microbiota Survey of Sliced Cooked Ham During the Secondary Shelf Life. Front. Microbiol. 13:842390. doi: 10.3389/fmicb.2022.842390

INTRODUCTION

According to the Food and Agriculture Organization, food production in the world is higher than the demand and ensues a huge amount of wasted food per year, aggravating CO_2 emissions, land utilization, and blue water consumption (FAO, 2013; Mekonnen and Gerbens-Leenes, 2020). This phenomenon is also reflected in a series of economic, ethical, and social repercussions, considering that 11% of the world's population has no access to food resources (Wohner et al., 2019). Unlike other food losses that may occur along the food production and supply chain, food wastes are recognized to take place in the last phases of the chain, i.e., the distribution, sale, and consumption, especially at the household level (FAO, 2013; Abdelradi, 2018; Salihoglu et al., 2018; UNEP, 2021). The assessment of appropriate secondary shelf life, defined as the time after package opening during which the food product retains a required level of quality, is pivotal for reducing domestic food waste (Nicosia et al., 2021). Indeed, excessively short secondary shelf life suggested by producers, if not relying on scientific data, may mislead consumers to discard foods still suitable for consumption.

The highest cost in wasted resources is associated with animal-based foods, which have the highest value of footprint (FAO, 2013; Mekonnen and Gerbens-Leenes, 2020). Meat waste represents a major contribution to food losses in developed countries (FAO, 2011). The large availability of food, together with the greater awareness of health and hygiene risks associated with the consumption of altered meat, means that meat products are not generally consumed beyond the expiration date or are rejected due to spoilage or minimal undesirable features perceived as defects (Iulietto et al., 2015; Ojha et al., 2020). The changes in consumers' lifestyles have also led to the increasing demand for ready-to-eat products (Bonifacie et al., 2021). Among them, sliced cooked ham in modified atmosphere packaging (MAP) is a main product of the meat processing industry. Although cooked ham undergoes cooking at $T \geq 70°C$, bacterial proliferation occurs during the whole shelf life even after maintaining hygienic precautions and preservative procedures (Raimondi et al., 2019), and a throughout survey on the sources of contamination after the cooking is still lacking.

Sliced cooked ham in MAP is subjected to abundant microbial contamination throughout production and shelf life, which leads to deterioration of both sensorial properties and wholesomeness. The microbiota of this product has been the subject of several studies, consistently indicating lactic acid bacteria (LAB) as the main contaminants that proliferate throughout the shelf life and reach remarkably high counts of 10^7–10^9 cfu/g (Menezes et al., 2018; Raimondi et al., 2019). Temperature, pH, water activity, nutrient availability, redox potential, and gas composition trigger microbial growth (Iulietto et al., 2015), affecting the preservation of food along the cold chain and consequently influencing the wastage (Vasilopoulos et al., 2008). LAB may concur with meat preservation due to the production of organic acids and hydrogen peroxide, which exert an intrinsic antimicrobial effect. Furthermore, selected strains produce bacteriocins specifically inhibiting certain contaminating pathogen bacteria, such as *Listeria* (Raimondi et al., 2021). On the other hand, LAB may contribute to spoilage of MAP cooked ham, inducing discoloration, changes in odor, flavor, and texture, and slime formation, negatively affecting the shelf life (Samelis et al., 2000; Kreyenschmidt et al., 2010; Raimondi et al., 2019). The spoilage of LAB is mainly caused by homofermentative bacteria belonging to the species *Latilactobacillus curvatus* and *Latilactobacillus sakei* or heterofermentative such as *Leuconostoc carnosum*, *Leuconostoc gelidum, Carnobacterium divergens*, and *Carnobacterium maltaromaticum*, whereas the Listeriaceae *Brochothrix thermosphacta* occurs when oxygen is present inside the packages (Vasilopoulos et al., 2008; Hu et al., 2009; Raimondi et al., 2019).

Some information is available on the evolution of microbial and sensorial properties of MAP cooked ham throughout the shelf life, but the evolution of the product after the opening of the package has not been investigated so far (Vasilopoulos et al., 2008; Kreyenschmidt et al., 2010; O'Neill et al., 2018; Raimondi et al., 2019). In this work, we studied the secondary shelf life of MAP cooked hams of five Italian industries, simulating the storage in a domestic fridge. Detailed information on the evolution of the products, including microbiological, sensorial, and chemical features, was collected for a period of 12 days after the opening of packages to provide the scientific basis for a more accurate recommendation of the secondary shelf life of MAP cooked ham.

MATERIALS AND METHODS

Sample Collection and Experimental Design

Samples of MAP sliced cooked ham produced by five selected industries were purchased in local markets (Modena, Italy). For each of the five products, three packages with the same expiration date (±3 days) were purchased. This kind of product available in the Italian market has a shelf life of typically 4 weeks; all the selected packages had at least 2 weeks of remaining shelf life at the purchase time. Where reported, the producers suggest food consumption within 1–3 days after package opening. Ingredients and nutritional composition are reported in **Supplementary Table 1**. Microbiological, chemical, and sensorial analyses were carried out at the opening of packages (0 days), then every 4 days (4, 8, and 12 days), during which the samples were maintained in plastic bags within the same domestic refrigerator set to 4°C. The actual temperature of the domestic refrigerator was monitored and recorded using a probe and data logger (mini TH, XS Instruments, Carpi, Italy).

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, United States) unless otherwise stated.

Sensorial Evaluation and Spoilage Assessment

At each time point, a slice of cooked ham was allowed to reach room temperature for 15 min; then, sensory analyses were carried out by a trained sensory panel. Occurring defects were evaluated through odor, texture, and color descriptors, which were given a score in the range between 0 (no defects, highest sensory quality) and 5 (highest level of defects, lowest sensory quality).

Chemical Analyses

The pH was measured in five distinct points per sample using a puncture electrode (Sension + electrode 5,233, HACH, Switzerland). Organic acids, glucose, and ethanol were determined by high-performance liquid chromatography, according to Vasilopoulos et al. (2008). Volatile organic compounds (VOCs) were tracked by headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry analysis, following the protocol described by Raimondi et al. (2018).

Microbiological Analyses

Ten grams of cooked ham was suspended and homogenized in saline peptone water (8.5 g/L NaCl and 1 g/L peptone) at the concentration of 10% (w/v) utilizing a Lab Blender Stomacher (Seward Medical, London, United Kingdom) for 4 min. Appropriate decimal dilutions in saline peptone water were spread on agar plates for the enumeration and isolation of bacteria and fungi: plate count agar (PCA) for total aerobic bacteria, Lactobacilli MRS Agar for LAB, violet red bile glucose agar (VRBGA) for enterobacteria, and rose bengal chloramphenicol agar (RBCA) for fungi, and Baird–Parker agar (BPA) for putative staphylococci. The plates were incubated in aerobiosis at 30°C for 48 h, except for RBC plates incubated at 25°C for 5–7 days and MRS plates incubated in an anaerobic jar at 30°C for 72 h, with anaerobiosis generated by GasPaK (BD Difco). To detect *Salmonella* sp., the initial suspension was pre-enriched for 24 h at 30°C, and 100 μl was seeded in 10 ml of Rappaport–Vassiliadis soy peptone broth and incubated for 24 h at 41°C. The enrichment culture was then seeded on xylose lysine deoxycholate plates and incubated for 24 h at 37°C. All the media were purchased from BD Difco (Franklin Lake, NJ, United States).

After enumeration, a representative number of colonies (up to 48 colonies per sample per medium) were randomly picked up from PCA, MRSA, RBCA, VRBGA, and BPA and streaked in the same medium. Instagene Matrix (Bio-Rad Laboratories, Redmond, WA, United States) was used for the extraction of polymerase chain reaction (PCR) amplifiable DNA. The clones were amplified with M13-random amplified polymorphic DNA (RAPD) primer (5′-GAGGGTGGCGG TTCT-3′), and the ensuing RAPD-PCR profiles were clustered in biotypes with > 75% similarity by Pearson correlation coefficient (Quartieri et al., 2016). A single strain for each biotype was taxonomically characterized by sequencing the V1–V3 portion of the 16S ribosomal RNA (rRNA) gene for bacteria and the ITS1 for fungi. The couples of primers 16S-500f (5′-TGG AGA GTT TGA TCC TGG CTC AG-3′)/16S-500r (5′-TAC CGC GGC TGC TGG CAC-3′) and ITS1(5′-TCC GTA GGT GAA CCT TGC GG-3′)/ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were utilized to amplify the two target regions, respectively (White et al., 1990; Kolbert et al., 2004). Comparisons with the reference sequences available in the GenBank database were obtained by the National Center for Biotechnology Information BLAST software[1].

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

16S Ribosomal RNA Gene Profiling

Metataxonomic analysis was performed when packages were opened (0 days) and after 8 days, when sensorial properties were still acceptable. Two grams of cooked ham was collected and homogenized in 10 ml of food lysis buffer (DNeasy Mericon Food Kit; Qiagen, Hilden, Germany), and total DNA was extracted following the manufacturer's standard protocol. DNA concentration was adjusted to 5 ng/μl after quantification with a Qubit 3.0 fluorimeter (Thermo Fisher Scientific, Waltham, MA, United States). Partial 16S rRNA gene sequences were performed by a DNA sequencing service provider (Eurofins Genomics, Ebersberg, Germany), which amplified and sequenced the V3–V4 region of the 16S rRNA gene using an Illumina MiSeq instrument (Illumina, San Diego, CA, United States). The sequences are available in the National Center for Biotechnology Information repository with the BioProject ID.

Raw sequences were analyzed with the QIIME 2.0 pipeline, version 2021.4 (Bolyen et al., 2019), with appropriate plugins for trimming (CUTADAPT) and denoising (DADA2) into amplicon sequence variants (Martin, 2011; Callahan, 2016). Taxonomy assignment was carried out with the feature classifier VSEARCH (Rognes et al., 2016), with SILVA SSU database release 138.1 as a reference ([2]note that the database, despite being the latest, is not up to date with the most recent nomenclature changes in the Lactobacillaceae and Leuconostocaceae families), and similarity threshold set at 0.97. The appropriate QIIME2 plugins were utilized to compute the alpha diversity (Chao1 and Pielou's evenness) and beta diversity (Bray–Curtis dissimilarity) and to compare them within and between 0- and 8-day samples (i.e., the Kruskal–Wallis test for alpha diversity; permutational multivariate analysis of variance for beta diversity). Differences were considered significant for $P < 0.05$. Principal coordinate analysis was computed with QIIME2, based on the beta-diversity distance matrix.

Statistical Analysis

Unless otherwise specified, values were reported as means ± SD of triplicate samples. The comparison was performed according to Student's *t*-test or one-way analysis of variance followed by Tukey *post hoc* comparisons, using the MaxStat software (MaxStat Pro, version 3.6).

Kruskal–Wallis test followed by Dunn's non-parametric post-comparison test was performed on sensorial properties. Differences were considered statistically significant for $P < 0.05$.

Principal component analysis (PCA) was used to explore the data matrix of VOCs with size {60, 157}, including the 157 relative areas of VOCs, determined for the three replicates of each product at the four time points. The number of significant principal components (PCs) was defined using the screen plot, which reports the percentage of variance explained by each PC vs. the PC number. The PCA model was calculated using Past version 3.14 (Hammer et al., 2001).

Linear discriminant analysis effect size[3] was utilized to discover distinctive taxonomic features characterizing samples

[2]https://www.arb-silva.de/download/arb-files/

[3]http://huttenhower.sph.harvard.edu/galaxy

at 0 and 8 days (Segata et al., 2011). Taxa presenting a significant differential abundance ($P < 0.05$) and logarithmic, linear discriminant analysis score > 2 were considered as microbial biomarkers of the corresponding timepoint.

RESULTS

Sliced cooked ham trays in MAP of five commercial products were stored for 12 days in a domestic refrigerator, and the temperature profile was reconstructed through a data logger. Values ranged between 4.1 and 6.5°C (**Supplementary Figure 1**), with a mean temperature of 5.2 ± 0.6°C (mean ± SD). The temperature fluctuated in a narrow range that was well representative of possible domestic storage without drastic temperature abuse. It should be noted that the domestic storage temperature suggested on labels is usually 0–4°C: even if the measured temperature falls out of this range, it should be considered as a realistic condition, based on previous data collected in different domestic refrigerators, which ranged from 4.6 to 10°C (Nicosia et al., 2021).

Spoilage Assessment

The sensorial properties of cooked ham samples were evaluated at the time of first opening and after 4, 8, and 12 days of secondary shelf life. Generally, negative changes were recorded after 8 days, and the products became unacceptable after 12 days. The most relevant modifications were the emergence of acid flavor, rotten smell, and discoloration (**Figure 1**). Products behaved differently during this lapse of time. At 8 days, discoloration of D samples was significantly more pronounced than that of C and E ones ($P < 0.05$), and the overall extent of spoilage was higher in D than in other samples. At 12 days, significantly higher scores of rotten smell were registered for samples C, D, and E compared with A and B ($P < 0.05$); furthermore, the whole spoilage was higher in D than in A and B ($P < 0.05$). Samples C and D presented a reduction of drip and an increase of discoloration, rotten smell, and acidity flavor ($P < 0.05$; **Supplementary Table 2**).

Culture-Dependent Enumeration

Viable counts on PCA, MRSA, VRBGA, RBCA, and BPA plates were utilized to monitor total aerobic bacteria, LAB, putative enterobacteria, staphylococci, fungi, and staphylococci, respectively, throughout the secondary shelf life (**Figure 2**).

Total aerobic bacteria ranged between 5.8 and 9.0 Log cfu/g (7.8 ± 0.7 Log cfu/g, mean ± SD) at 0 days, without significant differences among the products ($P > 0.05$). After 4, 8, and 12 days of fridge storage, total aerobic bacteria significantly increased in packages of product D, which progressively became the most contaminated ($P < 0.05$). A significant increase was also observed for products A and C, whereas products B and E did not exhibit any significant change.

The initial charge of LAB widely differed among samples being comprised from 5.7 to 9.1 Log cfu/g (7.7 ± 0.9 Log cfu/g, mean ± SD), with product D samples presenting the lowest values ($P < 0.01$). After 4, 8, and 12 days, the spread became narrower, with LAB charges lying in the range of 7–9 Log cfu/g (mean = 8.3 Log cfu/g). Throughout the secondary shelf life, LAB significantly increased in samples of products C and D, with the latter progressively becoming the richest ($P < 0.05$). In the other products, LAB remained unchanged.

Initially, putative enterobacteria lay below the limit of detection in most of the samples of products A, B, and C, whereas all the samples of products D and E presented contamination in the range of 3.0 and 6.7 Log cfu/g, without significant differences between them ($P > 0.05$). Throughout the secondary shelf life, putative enterobacteria increased up to 9.7 Log cfu/g in D samples ($P < 0.05$), whereas they remained unchanged in E samples ($P > 0.05$). After 12 days, Enterobacteriaceae also appeared in a few samples of products A and B, which presented a load up to 3.3 Log cfu/g, whereas they were never found in product C.

Fungi were initially found only in three samples (A3, B2, and B3). In A samples, the load of fungi increased over time, reaching 5.0 ± 0.6 at T12. After 12 days, fungi were isolated from most of the samples (11 of 15), with a mean charge of 4.9 ± 1.0 Log cfu/g.

Putative staphylococci and *Salmonella* sp. were analyzed after 12 days of secondary shelf life. Putative staphylococci were found in A, D, and E samples, with counts ranging between 2.5 and 6.9 Log cfu/g (**Supplementary Table 3**). *Salmonella* sp. was absent in all the samples.

Identification of the Isolates

Five hundred sixty isolates were selected from plates at 0 and 12 days and were clustered into 68 dominant biotypes, according to RAPD-PCR fingerprinting (**Supplementary Table 4**). One representative of each biotype was subjected to the sequencing of the 16S rRNA gene or ITS to obtain species designation.

The clones isolated in PCA belonged to 25 biotypes belonging to 11 species: *C. divergens* (5), *L. sakei* (5), *Leuconostoc mesenteroides* (5), *L. carnosum* (2), *Bacillus nealsonii* (1), *Candida zeylanoides* (1), *Carnobacterium gallinarum* (1), *C. maltaromaticum* (1), *Carnobacterium* sp. (1), *Massilia aurea* (1), *Moellerella wisconsensis* (1), and *Proteus hauseri* (1). The clones isolated in MRSA belonged to 15 biotypes, attributed to the species *L. sakei* (7), *L. mesenteroides* (3), *L. carnosum* (2), *C. divergens* (1), *L. curvatus* (1), and *Weissella viridescens (*1). Six strains were isolated from VRBGA plates, belonging to the species *Erwinia billingiae* (2), *Massilia arvi* (1), *M. wisconsensis* (1), *P. hauseri* (1), and *Pseudomonas* sp. (1). Thirteen different biotypes were found on RBCA plates, belonging to the species *C. zeylanoides* (5), *Kazachstania servazzii* (3), *Candida glaebosa* (1), *Candida novergica* (1), *Candida sake* (1), *Debaryomyces hansenii* (1), and *Debaryomyces* spp. (1). The nine biotypes were isolated on BPA plates at T12 that belonged to the species *P. hauseri* (5), *Staphylococcus xylosus* (2), *Corynebacterium stationis* (1), and *Kocuria* spp. (1).

Leuconostoc mesenteroides and/or *L. carnosum* abundantly contaminated the samples belonging to products A, B, C, and E, occurring already at 0 days and after 12 days in the magnitude of 7–8 Log cfu/g in PCA or MRSA plates (**Table 1**). In product D, a high load of *L. carnosum* (up to 8.9 Log cfu/g) appeared only after 12 days. *L. sakei* was found only in samples of products A, B, and E, being generally found in the magnitude of 6–8 Log cfu/g MRSA in plates both at 0 and 12 days. In product C, *L. sakei*

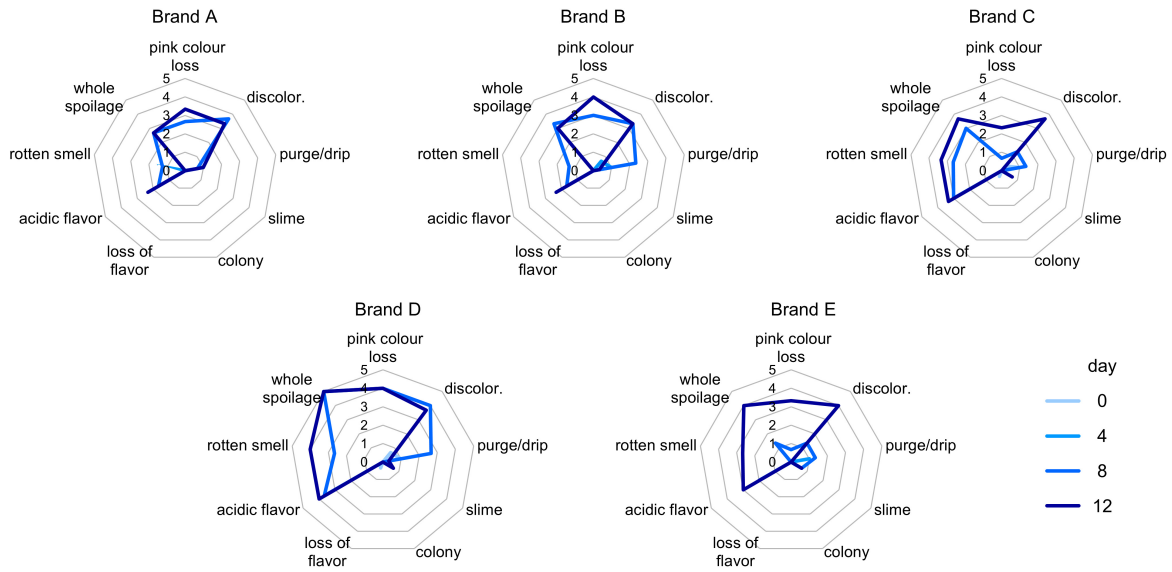


FIGURE 1 | Spider charts for scores (0–5) of sensory defects detected in **(A–E)** samples, with different color lines for each timepoint.

occurred only at 0 days. *C. divergens* characterized only samples of products D and E, being present already at 0 days, generally in the magnitude of 7 Log cfu/g, and reaching up 9 Log cfu/g after 12 days. *C. gallinarum* and other *Carnobacterium* sp. occurred only in samples of product C, at 0 and 12 days, respectively. Interestingly, only for C samples, all the biotypes of the isolates at both 0 and 12 days were LAB. *W. viridescens* occurred only after 12 days in two samples of product D, where it reached the remarkable concentration of 9.1 Log cfu/g.

Contamination with *M. wisconsensis* was observed only in D and E products, both at the opening of packages and after 12 days. At both the time points, the load of *M. wisconsensis* was very variable, ranging from 2.0 to 9.7 Log cfu/g, the highest value being observed in a sample of product D after 12 days. Other Gram-negative species (i.e., *E. billingiae*, *M. arvi*, *M. aurea*, *P. hauseri*, and *Pseudomonas* sp.) appeared sporadically in a single sample of different products and generally in low amounts (2–3 Log cfu/g).

Most of the yeast strains were isolated at 12 days, belonging to different species of the genus *Candida* (*C. glaebosa*, *C. norvegica*, *C. sake, and C. zeylanoides*); an exception was represented by *K. servazzii* that was detected in sample B since T0 reaching up to 5.8 Log cfu/g at the last time point.

Analysis of Microbial Population by 16s Ribosomal RNA Gene Profiling

Metataxonomic analysis was performed at the opening and after 8 days of secondary shelf life when samples were not yet spoiled. The metagenomic survey of 16s rRNA gene profiling yielded 52,964–148,150 reads per sample, with a total of 3,442,935 in 30 samples. The reads were dereplicated into 822 amplicon sequence variants hitting a reference sequence in the Silva database and collapsed at the seventh level of taxonomic annotation into 326 operational taxonomic units. The microbiota of cooked ham was largely dominated by Proteobacteria and Firmicutes phyla (**Figure 3**). Chao1 richness was different among the diverse products, being highest for C samples and lowest for A and D ones (**Supplementary Figure 2A**). Only in D samples, richness significantly decreased after 8 days of conservation. Pielou evenness presented significant differences among the samples and showed similar levels of diversity within the same product, excluding relevant variations during the secondary shelf life (**Supplementary Figure 2B**). In evenness, samples of product D collected at both 0 and 8 days showed the greatest variability.

In the principal coordinate analysis plot computed from the Bray–Curtis beta diversity distance matrix (**Figure 4A**), initial samples could not be clearly separated from those with 8 days of secondary shelf life. On the other hand, samples clustered based on the product, regardless of the timepoint. Samples of products A and D clustered together and shared some biomarkers such as *Lactobacillus* (comprising *Latilactobacillus*) and *L. mesenteroides* that mainly contributed to PCo1 (**Figure 4B**). Accordingly, A and D samples were dominated by Firmicutes, mainly ascribed to the families of Leuconostocaceae and Lactobacillaceae, currently reclassified into a single-family named Lactobacillaceae (**Figure 3**).

Generally, the evolution of the 15 samples during the first 8 days of secondary shelf life did not determine relevant changes, with a few exceptions (B2, C1, and C3). The microbiota composition of A, D, and E products was minimally perturbed over the first 8 days of secondary shelf life.

B samples lay contiguously at low PCo1 and PCo2 values, characterized by *M. wisconsensis* and *C. divergens*. Consistently, in five of six B samples, *M. wisconsensis* outnumbered the other bacteria.

E samples clustered together (high PCo1 and low PCo2 values), with *C. maltoaromaticum* and *Leuconostoc* as major biomarkers. Accordingly, the families Leuconostocaceae

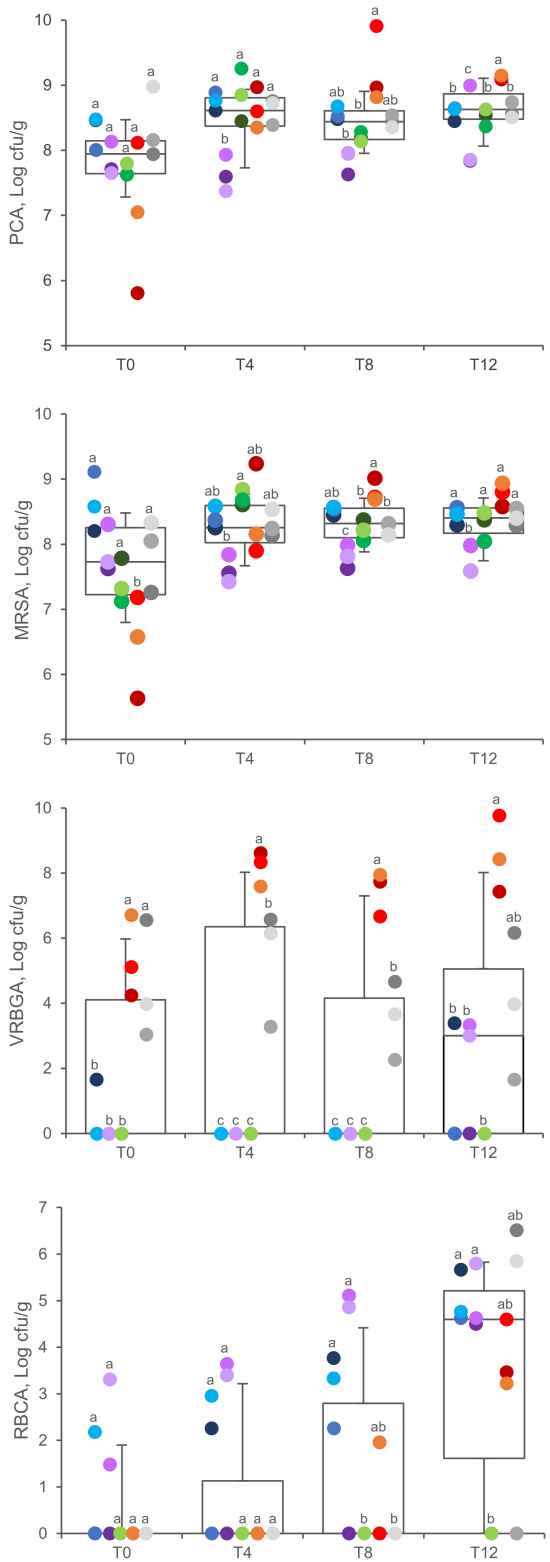


FIGURE 2 | Viable counts in PCA, MRSA, VRBGA, and RBCA plates of A (blue), B (violet), C (green), D (red), and E (gray) samples. Boxes indicate 25th, 50th, and 75th percentiles; whiskers indicate 10th and 90th percentiles. Within each time point, products sharing same letter did not make a significant difference ($p > 0.05$).

(currently comprised in Lactobacillaceae), Carnobacteriaceae, and Morganelaceae dominated E samples (**Figure 3**). Other common bacteria identified in all E samples were *Lactobacillus* (comprising *Latilactobacillus*), *Pseudomonas*, *Psychrobacter, Photobacterium*, *Xanthomonas*, *Proteus*, *Serratia*, *Providencia*, *Citrobacter*, *Aeromonas*, *Shewanella*, *Acinetobacter*, *Vibrio, Hafnia-Obesumbacterium*, *Klebsiella*, *Brochothrix*, and *Halomonas*.

Linear discriminant analysis effect size algorithm was utilized to discover distinctive taxonomic features characterizing samples at 0 or 8 days. Only Bacillaceae, *Geobacillus*, and *Parageobacillus thermoglucosidasius* exhibited significantly higher abundances in samples at 0 days (**Supplementary Figure 3**). The same analysis, applied to identify biomarkers specific for each product, indicated that product B was characterized by *L. mesenteroides*, product D by Enterobacterales and Morganellaceae, and product E by *Photobacterium*.

Glucose and Organic Acids

Glucose initially ranged from 0.09 to 0.68% w/w (g *per* 100 g of ham) (**Figure 5**). Its concentration was significantly higher in D and E samples than in A, B, and C ones. Overall, it significantly decreased during the secondary shelf life ($P < 0.05$). However, after 12 days of refrigeration at 5°C and relevant metabolic activity, it was still present in most samples (11 of 15) although in negligible amounts (0.01 to 0.17% w/w).

Lactic acid was the main metabolite, occurring in all the samples and at all the timepoints throughout the secondary shelf life. The initial concentration ranged from 0.15 to 0.62% w/w, with the highest levels in product A samples ($P < 0.05$). Lactic acid significantly increased during the secondary shelf life in B, C, and E samples ($P < 0.05$), where its concentration almost doubled (from 0.30 ± 0.14 to 0.71 ± 0.01; from 0.26 ± 0.07 to 0.70 ± 0.06; from 0.38 ± 0.02 to 0.68 ± 0.02 in B, C, and E, respectively), whereas it did not significantly increase in A and D samples.

Acetic acid was another major metabolite that accumulated in cooked ham over time. In all the samples, its concentration was under the limit of detection at both 0 and 4 days, whereas it accumulated toward the end of the secondary shelf life. Ethanol was detected in minor amounts, without significant differences among the products, generally tending to accumulate over time.

The pH of cooked ham decreased progressively during the secondary shelf life (5.7 ± 0.3 at T0; 5.5 ± 0.4 at T4; 5.4 ± 0.3 at T8; 5.3 ± 0.3 at T12; overall means ± SD) ($P < 0.01$), with different kinetics depending on the product.

Volatile Organic Compounds

In the headspace of cooked ham samples, solid-phase microextraction coupled with gas chromatography analysis revealed a total of 157 VOCs, including organic acids, ethanol, and other alcohols, ketones, fatty aldehydes, esters, phenols, and a variety of aromatic compounds, furans, linear and branched aliphatic hydrocarbons, terpenoids, and sulfides (**Supplementary Figure 4**).

In the PCA model of VOC profiles, the two most informative dimensions, PC1 and PC2, accounted for 47 and 31% of data variability, respectively (**Figure 6A**). Ethanol, acetic acid, and

TABLE 1 | Dominant microbial contaminants isolated from PCA (P), MRSA (M), VRBGA (V), RBCA (R), and BPA (B) plates at 0 and 12 days of secondary shelf life.

			LAB									**Proteobacteria**						**Other bacteria**				**Fungi**						
Product	**Package**	**Sampling time (d)**	***Carnobacterium divergens* (P, M)**	***Carnobacterium gallinarum* (P)**	***Carnobacterium maltaromaticum* (P)**	***Carnobacterium* sp. (P)**	***Latilactobacillus sakei* (M, P)**	***Latilactobacillus curvatus* (M)**	***Leuconostoc carnosum* (M, P)**	***Leuconostoc mesenteroides* (M, P)**	***Weissella viridescens* (M)**	***Erwinia billingiae* (V)**	***Massilia arvi* (V)**	***Massilia aurea* (P)**	***Moellerella wisconsensis* (V, P)**	***Proteus hauseri* (V, B, P)**	***Pseudomonas* sp. (V)**	***Bacillus nealsonii* (P)**	***Corynebacterium stationis* (B)**	***Kocuria* sp. (B)**	***Staphylococcus xylosus* (B)**	***Candida glaebosa* (R)**	***Candida norvegica* (R)**	***Candida sake* (R)**	***Candida zeylanoides* (R, P)**	***Debaryomyces hansenii* (R)**	***Debaryomyces* sp. (R)**	***Kazachstania servazzii* (R)**
A	1	0					8.2		7.2	8.4																		
		12							8.1	8.5		2.5	2.5			2.5	3.1			3.1	3.3				5.7			
	2	0					7.0		7.8	8.0																		
		12							8.4	8.6											3.0				4.6			
	3	0					8.0			8.5																2.2		
		12								8.6						2.2					2.6				4.8			
B	1	0					7.5		6.9	7.5				7.2														
		12					6.8		7.4	7.7				7.2											3.9			4.4
	2	0					8.3			8.1																		1.5
		12					7.2			8.0		3.3																4.6
	3	0					7.4		7.2	7.7																		3.3
		12					7.2			7.8		3.0																5.8
C	1	0		6.9			7.5		7.8																			
		12				8.2			8.4																			
	2	0		7.3					7.1																			
		12				8.4			8.0																			
	3	0					7.3		7.3																			
		12				8.5			8.5										2.0									
D	1	0	5.5												5.8	5.5												
		12	8.9						7.8		9.5				7.4	6.9						3.5						
	2	0	7.8		7.8											5.1												
		12	9.1						8.7		8.2				9.7	6.1						4.1	4.4					
	3	0								6.6																		
		12	8.9						8.9						8.4	6.4						2.6	3.1					
E	1	0	7.8				6.3		7.1	6.6					6.6													
		12	7.9				8.2		8.2	7.2					6.2	2.8								6.5				
	2	0	7.7						8.0	7.3					2.9	2.3												
		12	8.4				8.1	8.1	7.6	7.9					2.0		2.0	8.4										
	3	0	8.7				8.3		8.3						4.0													
		12					7.7	7.7	8.0	7.4					4.0									5.7			5.2	

Isolates were genotyped by RAPD-PCR fingerprinting and subjected to 16S rRNA gene or ITS sequencing to obtain a taxonomic designation. Concentration, inferred from plate counts, is reported as Log cfu/g.

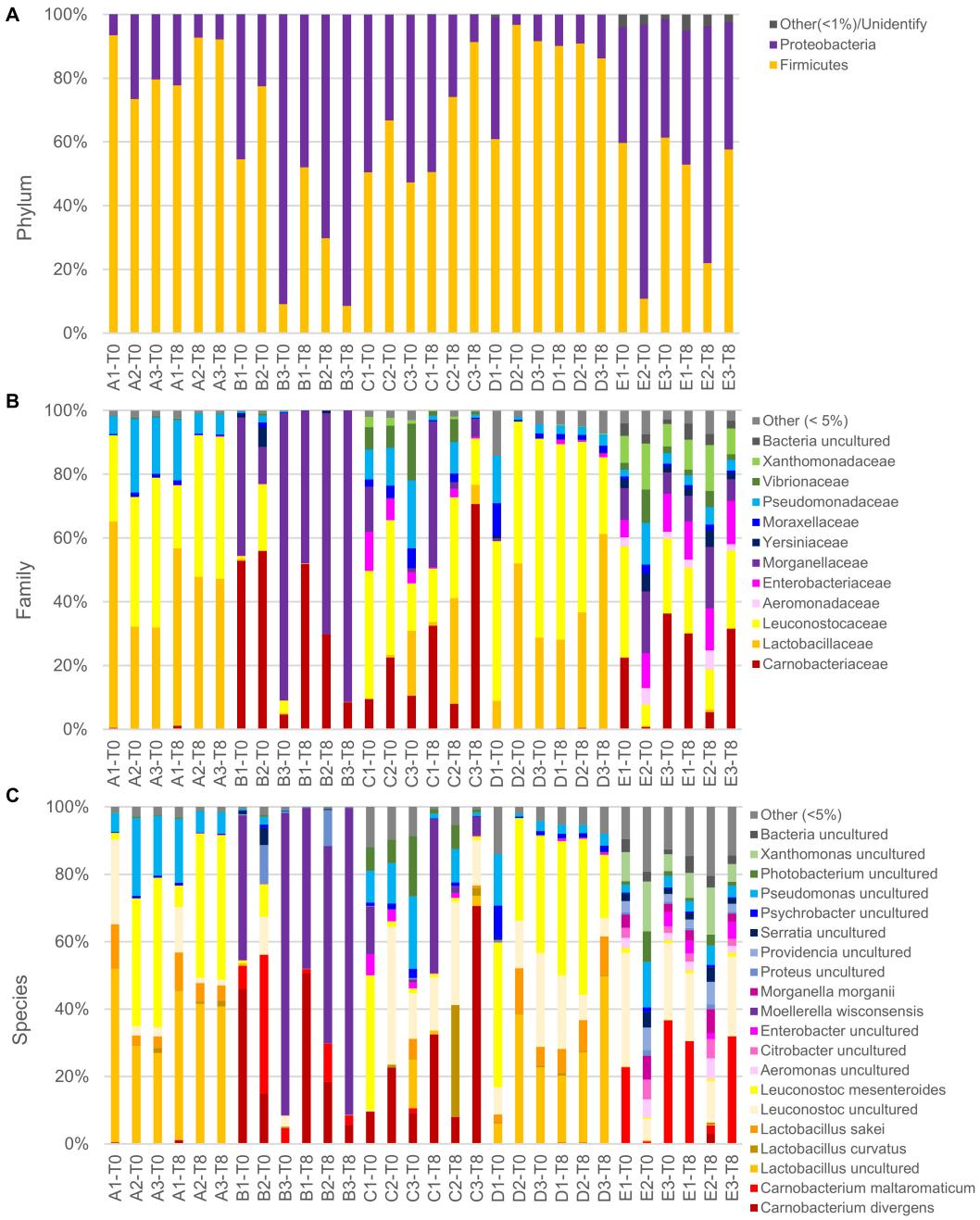


FIGURE 3 | Relative abundance of main phyla **(A)**, families **(B)**, and species **(C)** in slices of cooked ham of five different products at packages opening and after 8 days. Note that in reference database, despite being latest update, Leuconostocaceae and Lactobacillaceae are reported as two separated families, but, accordingly, current nomenclature are included in sole family Lactobacillaceae. Moreover, species *Lactobacillus sakei* and *Lactobacillus curvatus* are not updated to recent name *Latilactobacillus sakei* and *Latilactobacillus curvatus*.

acetoin presented a major loading in determining differences among samples throughout the secondary shelf life. Acetoin, 3-methyl-1-butanol, and 2-3 butanediol presented a positive contribution to PC1, whereas ethanol and acetic acid presented a negative one (**Figure 6B**). Interestingly, only product D was characterized by higher values of PC1 compared with other products, but differences became evident only during the secondary shelf life, with increasing PC1 values over time. Acetic acid was the main positive contributor to PC2. All samples at 0 days were grouped at low values of PC1 and PC2, regardless of the product. A common trend of all products was the accumulation of acetic acid: the distribution of samples along the PC2 clearly showed a correlation between increasing time from the opening of the trays and increasing values of PC2.

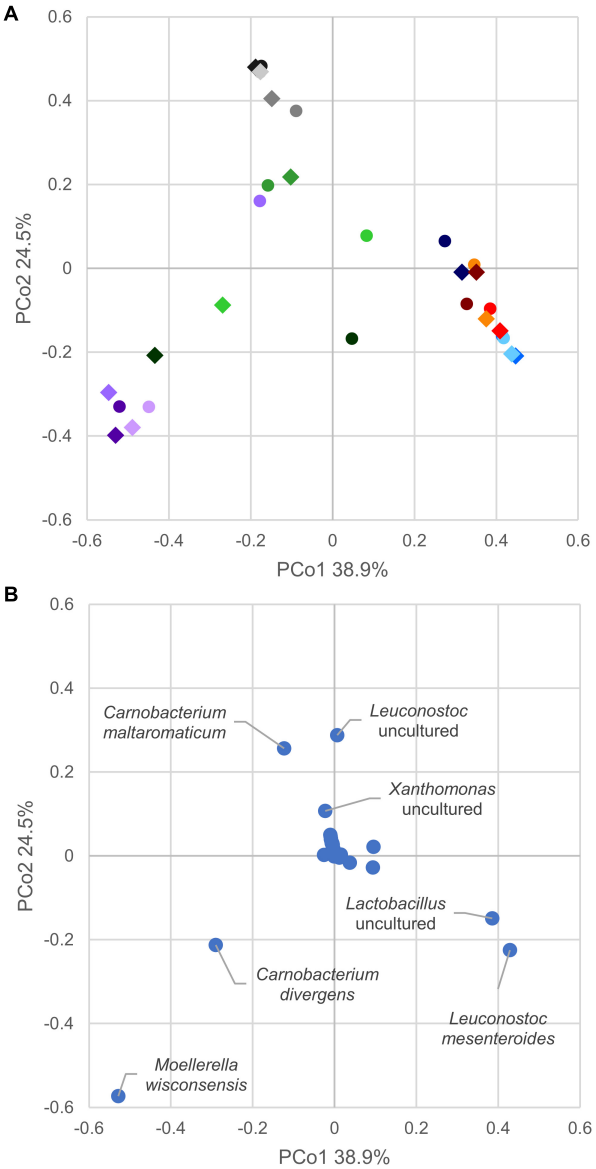


FIGURE 4 | (A) Principal coordinate analysis plot of beta diversity based on Bray–Curtis dissimilarity index of cooked ham microbiome. Different colors indicate products, A (blue), B (violet), C (green), D (red), and E (gray). Circles mark samples at opening, diamond after 8 days. **(B)** Principal coordinate analysis plot of species contribution to sample differentiation.

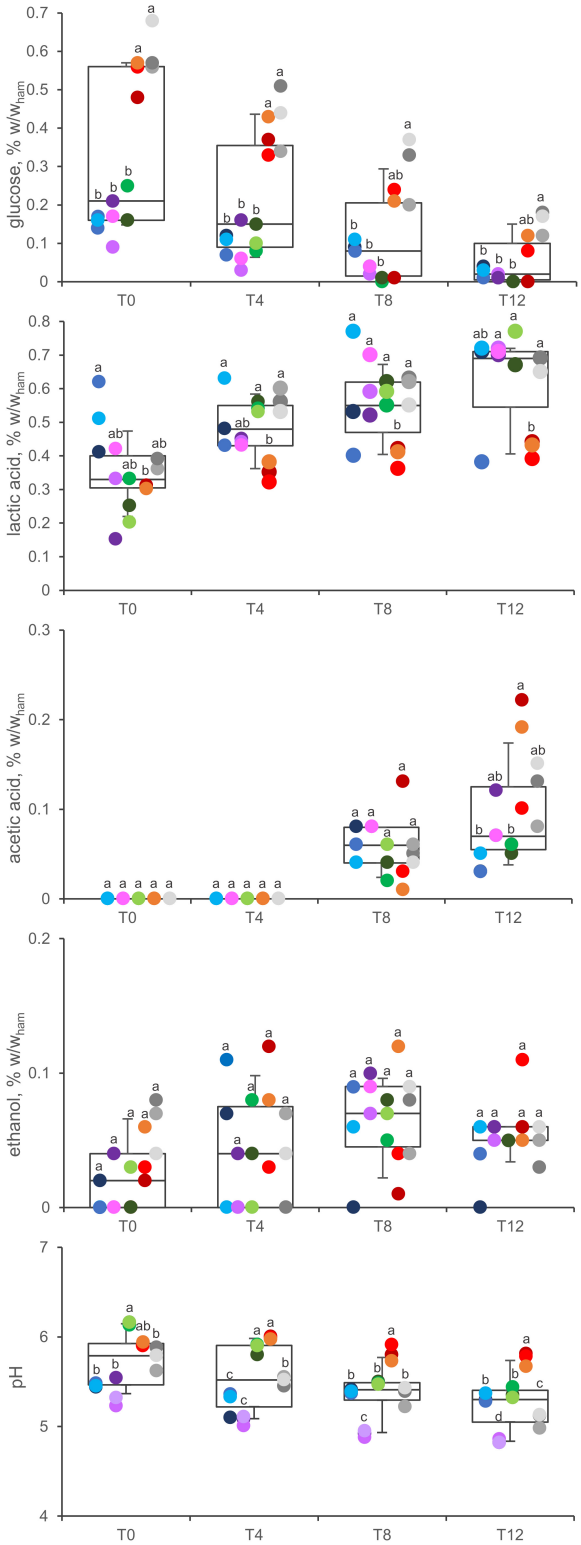


FIGURE 5 | Concentration of glucose, acetic acid, lactic acid, ethanol, and pH variation in A (blue), B (violet), C (green), D (red), and E (gray) samples. Boxes indicate 25th, 50th, and 75th percentiles; whiskers indicate 10th and 90th percentiles. Within each time point, products sharing same letter did not make a significant difference ($p > 0.05$).

DISCUSSION

Modified atmosphere packaging sliced cooked ham is a popular ready-to-eat product that has been the subject of several studies aiming to define its microbiota and the deriving impact on safety, spoilage, and preservation (Vasilopoulos et al., 2010; Raimondi et al., 2019; Zagdoun et al., 2020; Candeliere et al., 2021). In this study, the microbial load and the chemical–physical characteristics of cooked ham were monitored for a period of 12 days after the opening of the packages, during which the products were stored in a domestic refrigerator. All the products

were purchased, opened, and analyzed in a range of 10–15 days before the expiration date. During the secondary shelf life at a mean temperature of 5.2 ± 0.6°C, the sensorial analyses of five products of MAP sliced cooked ham revealed a perceivable decay of quality after 8 days, but products became unacceptable only after 12 days, with the comparison of acid flavor, rotten smell, and discoloration. The organoleptic properties of the samples during the secondary shelf life suggest that cooked ham remains sensorially acceptable far beyond the 1–3 days suggested by the producers (**Supplementary Table 1**). However, the composition of the spoiling microbiota raised some concerns. The absence of viable *Salmonella* sp. in 10 g of the product after 12 days of storage is satisfying; nevertheless, the recurrent or sporadic presence of other opportunistic pathogens, such as *E. coli*, V*ibrio* sp., and *Klebsiella pneumoniae,* was identified by culture-independent methods and had relevant implications on the hygiene and safety of the products. Both metagenomic analysis and identification of the isolates revealed that, even at the opening of packages, all the products presented high microbial load and a quite rich microbiota, generally dominated by LAB. This result is in agreement with data reported in the literature indicating the genera *Leuconostoc*, *Carnobacterium*, and *Latilactobacillus* as among the main colonizers of MAP and vacuum-packed cooked ham (Vasilopoulos et al., 2010; Iskandar et al., 2017; Raimondi et al., 2019; Candeliere et al., 2021).

The 16S rRNA gene profiles were analyzed at the opening of packages and after 8 days of storing before samples became spoiled. The differences among microbiota were evident and mainly ascribed to the product, with LAB representing the prevailing group of bacteria in samples A and D, whereas more complex communities, also encompassing several Proteobacteria, were identified in the others. For instance, the Enterobacteriaceae *M. wisconsensis*, a facultative anaerobic Gram-negative rod, was the major colonizer of B samples already at 0 days. This species is probably an environmental contaminant, as literature reports a variety of isolation sources, including water, foods, and samples of human origin (Leroy et al., 2016). *M. wisconsensis* can be involved in the dissemination of antibiotic resistance, and its pathogenic role is debated (Stock et al., 2003; Leroy et al., 2016; Ahmad et al., 2020).

Culture-based identification revealed the presence of another contaminant, Enterobacteriaceae, *P. hauseri*. It is a Gram-negative motile bacterium, related to the human pathogens *Proteus vulgaris* and *Proteus mirabilis*, which was recurrent within the packages of products A and D, where it increased to remarkably high levels of contamination at 12 days. Other Proteobacteria, belonging to the genera *Pseudomonas*, *Erwinia*, and *Massilia*, occurred less frequently and abundantly. Facultative anaerobic Gram-negatives evidently contaminated the product at some level during the processing and before the packaging and, like LAB, grew in the ham unhindered by the modified atmosphere (Stock et al., 2003; Kreyenschmidt et al., 2010; Drzewiecka, 2016). *S. xylosus* occurred only in product A. It is used as a biocontrol agent to preserve meat products from mycotoxin produced by fungi, can convert nitrates to nitrites, and is responsible for aroma components (Stahnke, 1994; Cebrián et al., 2020).

The metagenomic analysis pointed out the occurrence of other bacterial contaminants that were not retrieved with the culture-dependent method and that could represent a potential concern for sensorial properties and/or food safety if they overgrew. *B. thermosphacta*, which is reported to give cooked ham sour off-flavors, discoloration, and slime (Borch et al., 1996; Vasilopoulos et al., 2015; Dušková et al., 2016; Raimondi et al., 2019), was found in almost all the samples already at the beginning of the secondary shelf life, although in low amounts (up to 1.1%). Opportunistic pathogenic bacteria were also identified, even if in small quantities. *E. coli* and V*ibrio* sp. were other frequent contaminants, being found in most of the samples, reaching up to 3.0 and 1.8% of the microbiota, respectively (means 0.5 and 0.2%, respectively), whereas *Salmonella enterica* and *K. pneumoniae* occurred sporadically in a sole or few samples.

The presence of *K. servazzii* and *D. hansenii* already at 0 days confirms these yeast species as occasional contaminants of cooked meat products (Hernández et al., 2018), which colonized the products before the packaging and thrived during the secondary shelf life; indeed, once the packages have been opened, the protective effect of the modified atmosphere is lost, with only a slight residual inhibitory activity of the CO_2 solubilized in the matrix. Yeast strains belonging to the genus *Candida* (*C. zeylanoides*, *C. sake*, *C. norvegica*, and *C. glaebosa*) lay below the limit of detection at the opening of packages but were abundantly found after 12 days. Although it could not be excluded that *Candida* spp. contaminated the cooked ham during the secondary shelf life, the appearance of *C. sake*, *C. glaebosa*, and *C. zeylanoides* seemed associated with specific products and could originate from the manufacturing. Frequent sources of yeast contamination are the surfaces of machinery entering in contact with meat during the processing (Hernández et al., 2018). The growth of yeasts is undesirable in meat products, as it causes off-flavors, discoloration, and the formation of slime (Nielsen et al., 2008).

Both culture-dependent and independent characterization of the spoiling microbiota suggested that the high load of bacteria already present at the opening of cooked ham packages prevented the bloom of other species and determined the outbreak of dominant bacteria. For example, a further increase of LAB was observed in the samples where they were already abundant at the opening of packages, leading *Leuconostoc*, *Carnobacterium*, *Latilactobacillus*, and, to a lower extent, *Weissella* to dominate these microbial communities. On the other hand, the growth of Gram-negatives and other bacteria occurred in samples initially characterized by a complex community, where LAB was not dominant. The increase of LAB somehow protected the samples from abundant contamination by other bacteria, concurring to the evolution of more safe products. In these samples, a similar role was exerted by several *Latilactobacillus*, *Leuconostoc*, and *Carnobacterium* species, among which the most frequently detected were *L. sakei*, *L. carnosum*, *L. mesenteroides*, and *C. divergens*. Generally, the low temperatures of storage made *L. carnosum* and *L. mesenteroides* the species best adapted to cooked ham (Vasilopoulos et al., 2008; Raimondi et al., 2021). Besides their preservative effect, LAB can also be involved in spoilage (Comi et al., 2016; Raimondi et al., 2019). *L. mesenteroides*, *L. carnosum*, and *C. divergens* were

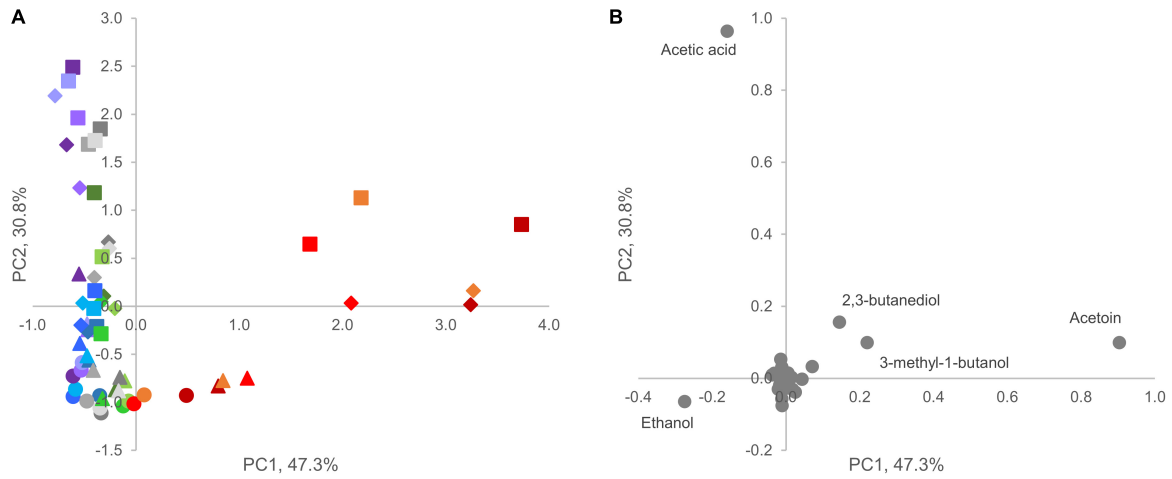


FIGURE 6 | Principal component analysis plot (*PC1* vs. PC2) of VOC profiles in A (blue), B (violet), C (green), D (red), and E (gray) samples. **(A)** Score plot of cooked ham samples; circles, triangles, diamonds, and squares indicate different sampling times (0, 4, 8, and 12 days, respectively). **(B)** Loading plot of volatile molecules. Compounds with highest loading value in PC1 vs. PC2 are labeled.

claimed as responsible for greenish color around the cooked ham slices, discoloration, and off-odor, with *L. carnosum* and *L. mesenteroides* mainly in charge for sour odor (Björkroth et al., 1998; Leisner et al., 2007; Diez et al., 2008; Schirmer et al., 2009; Comi et al., 2016).

The aroma of cooked ham is characterized by molecules obtained by thermally-induced lipid oxidation, such as straight-chain fatty acids, aldehydes, ketones, and alcohols (1-hexanol) (Leroy et al., 2009). The spices added to the product also contributed with terpenoids and sulfides such as 3-carene, gamma-terpinene, humulene, d-limonene, beta-phellandrene, and alpha-pinene (Rux et al., 2019). Moreover, during shelf life, several VOCs were produced from the catabolism of sugars, amino acids, and lipids by the growing microorganisms that dominated the microbiota of the cooked ham during storage (Estévez et al., 2003; Rivas-Cañedo et al., 2009; Holm et al., 2013; Casaburi et al., 2015; Raimondi et al., 2019). Lactic acid, ethanol, acetic acid, acetoin, 3-methyl-1-butanol, and 2-3 butanediol were the main metabolites that characterized the evolution of the analyzed cooked ham during the secondary shelf life, impacting their sensorial properties. Lactic and acetic acids, together with ethanol, were produced by LAB and other bacteria *via* saccharolytic metabolism (Casaburi et al., 2015). The increase of acetic acid as a function of sampling time was justified by the widespread presence of heterofermenting bacteria such as *L. mesenteroides* and *L. carnosum*, which dominated the spoilage microbiota of all samples. The lower amount of acetic acid compared with lactic acid was consistent with the results obtained by Vasilopoulos et al. (2008). Other compounds, such as acetoin, 3-methyl-1-butanol, 2-3 butanediol, 3-methyl-butanal, 2-butanone, 2-methyl-1-propanol, and 1-hexanol, were produced by LAB and enterobacteria. In particular, the alcohols 2-methyl-1-propanol and 3-methyl-1-butanol derive from the catabolism of amino acids of valine and leucine (Ardö, 2006). The esters were principally produced by enterobacteria and microorganisms able to form esterase involved in the formation of ethyl bonds (Argyri et al., 2015). The increased amount of acetoin in the samples was in good agreement with the complexity of isolated bacteria; in particular, the presence of *W. viridescens* and *L. carnosum* was already correlated with acetoin concentration in fermented foods (Diez et al., 2009; Candeliere et al., 2021). In stored sample D, the presence of isolated bacteria ascribable to the genus *Candida* and in particular *C. glaebosa*, *C. norvegica*, and *C. zeylanoides* was peculiar. The latter was previously described as a high-producing species of acetoin among the non-*Saccharomyces* yeast (Tofalo et al., 2014).

CONCLUSION

The main causes of microbial contamination of cooked ham originate from incorrect cooking of meat, improper sanitization practices, and recontamination during slicing and packaging, ensuing the increase of contaminants during the shelf life. Consistently, the analyzed cooked ham presented a more or less rich microbiota already at the opening of packages. Modified atmosphere packaging conditions, usually consisting of 70% N_2 and 30% CO_2, guarantee to some extent the inhibition of chemical and microbial spoilage of the packaged product, thus ensuring a primary shelf life of at least 1 month; the package opening causes a sudden variation of the headspace gas composition and a loss of protection: this event makes so that the secondary shelf life is significantly shorter than primary shelf life. It could be speculated that the problems of microbiological contamination did not derive from conservation during the secondary shelf life but were already present when the packages were opened, 2 weeks before the expiration date of the products, indicating the phases of production up to the packaging as those crucial in managing the safety risk. A high load of LAB already present at the opening of cooked ham packages inhibited the bloom of other species

and likely prevented the outbreak of pathogenic bacteria. The exploitation of specially selected strains belonging to the genera *Latilactobacillus* and *Leuconostoc* with reduced impact on the organoleptic properties of the product could be an effective strategy to control these microbial communities and extend the primary and secondary shelf life of the product, reducing domestic food losses.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA778960.

AUTHOR CONTRIBUTIONS

SR and MR conceived the study. GS and FL carried out chemical analysis. GS and SR carried out the microbiological analysis. AA and FC performed the 16S rRNA gene profiling, bioinformatics, and statistical analysis. SR wrote the manuscript with contributions from GS, FC, AA, FL, and MR. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the Italian Ministry of Agricultural, Food, and Forestry Policies (Ministero delle Politiche Agricole, Alimentari e Forestali) through call no. 6146 of November 20, 2019.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.842390/full#supplementary-material

REFERENCES

Abdelradi, F. (2018). Food waste behaviour at the household level: a conceptual framework. *Waste Manag.* 71, 485–493. doi: 10.1016/j.wasman.2017.10.001

Ahmad, N., Ali, S. M., and Khan, A. U. (2020). Co-existence of blaNDM-1 and blaVIM-1 producing *Moellerella wisconsensis* in NICU of North Indian Hospital. *J. Infect. Dev. Ctries.* 14, 228–231. doi: 10.3855/jidc.10969

Ardö, Y. (2006). Flavour formation by amino acid catabolism. *Biotechnol. Adv.* 24, 238–242. doi: 10.1016/j.biotechadv.2005.11.005

Argyri, A. A., Mallouchos, A., Panagou, E. Z., and Nychas, G. J. (2015). The dynamics of the HS/SPME-GC/MS as a tool to assess the spoilage of minced beef stored under different packaging and temperature conditions. *Int. J. Food Microbiol.* 193, 51–58. doi: 10.1016/j.ijfoodmicro.2014.09.020

Björkroth, K. J., Vandamme, P., and Korkeala, H. J. (1998). Identification and characterization of *Leuconostoc carnosum*, associated with production and spoilage of vacuum-packaged, sliced, cooked ham. *Appl. Environ. Microbiol.* 64, 3313–3319. doi: 10.1128/AEM.64.9.3313-3319.1998

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Author Correction: reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37:1091. doi: 10.1038/s41587-019-0252-6

Bonifacie, A., Promeyrat, A., Nassy, G., Gatellier, P., Santé-Lhoutellier, V., and Théron, L. (2021). Chemical reactivity of nitrite and ascorbate in a cured and cooked meat model implication in nitrosation, nitrosylation and oxidation. *Food Chem.* 348:129073. doi: 10.1016/j.foodchem.2021.129073

Borch, E., Kant-Muermans, M. L., and Blixt, Y. (1996). Bacterial spoilage of meat and cured meat products. *Int. J. Food Microbiol.* 33, 103–120.

Callahan, B. J. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Candeliere, F., Raimondi, S., Spampinato, G., Tay, M. Y. F., Amaretti, A., Schlundt, J., et al. (2021). Comparative Genomics of *Leuconostoc carnosum*. *Front. Microbiol.* 11:605127. doi: 10.3389/fmicb.2020.605127

Casaburi, A., Piombino, P., Nychas, G. J., Villani, F., and Ercolini, D. (2015). Bacterial populations and the volatilome associated to meat spoilage. *Food Microbiol.* 45, 83–102. doi: 10.1016/j.fm.2014.02.002

Cebrián, E., Núñez, F., Gálvez, F. J., Delgado, J., Bermúdez, E., and Rodríguez, M. (2020). Selection and evaluation of *Staphylococcus xylosus* as a biocontrol agent against toxigenic moulds in a dry-cured ham model system. *Microorganisms* 8:793. doi: 10.3390/microorganisms8060793

Comi, G., Andyanto, D., Manzano, M., and Iacumin, L. (2016). *Lactococcus lactis* and *Latilactobacillus sakei* as bio-protective culture to eliminate *Leuconostoc mesenteroides* spoilage and improve the shelf life and sensorial characteristics of commercial cooked bacon. *Food Microbiol.* 58, 16–22. doi: 10.1016/j.fm.2016.03.001

Diez, A. M., Björkroth, J., Jaime, I., and Rovira, J. (2009). Microbial, sensory and volatile changes during the anaerobic cold storage of *morcilla de* Burgos previously inoculated with *Weissella viridescens* and *Leuconostoc mesenteroides*. *Int. J. Food Microbiol.* 131, 168–177. doi: 10.1016/j.ijfoodmicro.2009.02.019

Diez, A. M., Urso, R., Rantsiou, K., Jaime, I., Rovira, J., and Cocolin, L. (2008). Spoilage of blood sausages morcilla de Burgos treated with high hydrostatic pressure. *Int. J. Food Microbiol.* 123, 246–253. doi: 10.1016/j.ijfoodmicro.2008.02.017

Drzewiecka, D. (2016). Significance and roles of *Proteus* spp. bacteria in natural environments. *Microb. Ecol.* 72, 741–758. doi: 10.1007/s00248-015-0720-6

Dušková, M., Kameník, J., Laèanin, I., Šedo, O., and Zdráhal, Z. (2016). Lactic acid bacteria in cooked hams–Sources of contamination and chances of survival in the product. *Food Control* 61, 1–5. doi: 10.1016/j.foodcont.2015.09.019

Estévez, M., Morcuende, D., Ventanas, S., and Cava, R. (2003). Analysis of volatiles in meat from Iberian pigs and lean pigs after refrigeration and cooking by using SPME-GCMS. *J. Agric. Food Chem.* 51, 3429–3435. doi: 10.1021/jf026218h

FAO (2011). *Global food losses and food waste – Extent, causes and prevention*. Rome: Food and Agriculture Organization of the United Nations.

FAO (2013). *Food wastage footprint. Impact on natural resources*. Rome: Food and Agriculture Organization of the United Nations.

Hammer, Ø, Harper, D. A., and Ryan, P. D. (2001). PAST: paleontological statistics software package for education and data analysis. *Palaeontol. Electron.* 4, 1–9.

Hernández, A., Pérez-Nevado, F., Ruiz-Moyano, S., Serradilla, M. J., Villalobos, M. C., Martín, A., et al. (2018). Spoilage yeasts: what are the sources of contamination of foods and beverages? *Int. J. Food Microbiol.* 286, 98–110. doi: 10.1016/j.ijfoodmicro.2018.07.031

Holm, E. S., Adamsen, A. P., Feilberg, A., Schäfer, A., Løkke, M. M., and Petersen, M. A. (2013). Quality changes during storage of cooked and sliced meat products measured with PTR-MS and HS-GC-MS. *Meat Sci.* 95, 302–310. doi: 10.1016/j.meatsci.2013.04.046

Hu, P., Zhou, G., Xu, X., Li, C., and Han, Y. (2009). Characterization of the predominant spoilage bacteria in sliced vacuum-packed cooked ham based on 16S rDNA-DGGE. *Food Control* 20, 99–104. doi: 10.1016/j.foodcont.2008.02.007

Iskandar, C. F., Borges, F., Taminiau, B., Daube, G., Zagorec, M., Remenant, B., et al. (2017). Comparative genomic analysis reveals ecological differentiation in the genus *Carnobacterium*. *Front. Microbiol.* 8:357. doi: 10.3389/fmicb.2017.00357

Iulietto, M. F., Sechi, P., Borgogni, E., and Cenci-Goga, B. T. (2015). Meat spoilage: a critical review of a neglected alteration due to ropy slime producing bacteria. *Ital. J. Anim. Sci.* 14:4011. doi: 10.4081/ijas.2015.4011

Kolbert, C. P., Rys, P. N., Hopkins, M., Lynch, D. T., Germer, J. J., O'Sullivan, C. E., et al. (2004). "16S ribosomal DNA sequence analysis for identification of bacteria in a clinical microbiology laboratory," in *Molecular Microbiology:*

diagnostic Principles and Practice, eds D. H. Persing, F. C. Tenover, J. Versalovic, Y. W. Tang, E. R. Unger, D. A. Relman, et al. (Washington, DC: American Society for Microbiology), 361–378.

Kreyenschmidt, J., Hübner, A., Beierle, E., Chonsch, L., Scherer, A., and Petersen, B. (2010). Determination of the shelf life of sliced cooked ham based on the growth of lactic acid bacteria in different steps of the chain. *J. Appl. Microbiol.* 108, 510–520. doi: 10.1111/j.1365-2672.2009.04451.x

Leisner, J. J., Laursen, B. G., Prévost, H., Drider, D., and Dalgaard, P. (2007). *Carnobacterium*: positive and negative effects in the environment and in foods. *FEMS Microbiol. Rev.* 31, 592–613. doi: 10.1111/j.1574-6976.2007.00080.x

Leroy, A. G., Malandain, D., Duchalais, É, Meurette, G., and Corvec, S. (2016). Accurate MALDI-TOF mass spectrometry identification of a colistin-resistant *Moellerella wisconsensis* strain. *Med. Mal. Infect.* 46, 233–235. doi: 10.1016/j.medmal.2016.01.009

Leroy, F., Vasilopoulos, C., Van Hemelryck, S., Falony, G., and De Vuyst, L. (2009). Volatile analysis of spoiled, artisan-type, modified-atmosphere-packaged cooked ham stored under different temperatures. *Food Microbiol.* 26:94. doi: 10.1016/j.fm.2008.08.005

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17, 10–17. doi: 10.14806/ej.17.1.200

Mekonnen, M. M., and Gerbens-Leenes, W. (2020). The Water Footprint of Global Food Production. *Water* 12:2696. doi: 10.3390/w12102696

Menezes, N. M. C., Martins, W. F., Longhi, D. A., and de Aragão, G. M. F. (2018). Modeling the effect of oregano essential oil on shelf life extension of vacuum-packed cooked sliced ham. *Meat Sci.* 139, 113–119. doi: 10.1016/j.meatsci.2018.01.017

Nicosia, C., Fava, P., Pulvirenti, A., Antonelli, A., and Licciardello, F. (2021). Domestic use simulation and secondary shelf life assessment of industrial *Pesto alla genovese*. *Foods* 10:1948. doi: 10.3390/foods10081948

Nielsen, D. S., Jacobsen, T., Jespersen, L., Koch, A. G., and Arneborg, N. (2008). Occurrence and growth of yeasts in processed meat products–Implications for potential spoilage. *Meat Sci.* 80, 919–926. doi: 10.1016/j.meatsci.2008.04.011

Ojha, S., Bußler, S., and Schlüter, O. K. (2020). Food waste valorisation and circular economy concepts in insect production and processing. *Waste Manag.* 118, 600–609. doi: 10.1016/j.wasman.2020.09.010

O'Neill, C. M., Cruz-Romero, M. C., Duffy, G., and Kerry, J. P. (2018). Shelf life extension of vacuum-packed salt reduced frankfurters and cooked ham through the combined application of high pressure processing and organic acids. *Food Packag. Shelf Life* 17, 120–128. doi: 10.1016/j.fpsl.2018.06.008

Quartieri, A., Simone, M., Gozzoli, C., Popovic, M., D'Auria, G., Amaretti, A., et al. (2016). Comparison of culture-dependent and independent approaches to characterize fecal bifidobacteria and lactobacilli. *Anaerobe* 38, 130–137. doi: 10.1016/j.anaerobe.2015.10.006

Raimondi, S., Luciani, R., Sirangelo, T. M., Amaretti, A., Leonardi, A., Ulrici, A., et al. (2019). Microbiota of sliced cooked ham packaged in modified atmosphere throughout the shelf life: microbiota of sliced cooked ham in MAP. *Int. J. Food Microbiol.* 289, 200–208. doi: 10.1016/j.ijfoodmicro.2018.09.017

Raimondi, S., Nappi, M. R., Sirangelo, T. M., Leonardi, A., Amaretti, A., Ulrici, A., et al. (2018). Bacterial community of industrial raw sausage packaged in modified atmosphere throughout the shelf life. *Int. J. Food Microbiol.* 280, 78–86. doi: 10.1016/j.ijfoodmicro.2018.04.041

Raimondi, S., Spampinato, G., Candeliere, F., Amaretti, A., Brun, P., Castagliuolo, I., et al. (2021). Phenotypic traits and immunomodulatory properties of *Leuconostoc carnosum* isolated from meat products. *Front. Microbiol.* 12:730827. doi: 10.3389/fmicb.2021.730827

Rivas-Cañedo, A., Fernández-García, E., and Nuñez, M. (2009). Volatile compounds in fresh meats subjected to high pressure processing: effect of the packaging material. *Meat Sci.* 81, 321–328. doi: 10.1016/j.meatsci.2008.08.008

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ.* 4:e2584. doi: 10.7717/peerj.2584

Rux, G., Luca, A., and Mahajan, P. V. (2019). Changes in volatile organic compounds in the headspace of modified atmosphere packed and unpacked white sausages. *Food Packag. Shelf Life* 19, 167–173. doi: 10.1016/j.fpsl.2018.12.010

Salihoglu, G., Salihoglu, N. K., Ucaroglu, S., and Banar, M. (2018). Food loss and waste management in Turkey. *Bioresour. Technol.* 248, 88–99. doi: 10.1016/j.biortech.2017.06.083

Samelis, J., Kakouri, A., and Rementzis, J. (2000). Selective effect of the product type and the packaging conditions on the species of lactic acid bacteria dominating the spoilage microbial association of cooked meats at 4 °C. *Food Microbiol.* 17, 329–340. doi: 10.1006/fmic.1999.0316

Schirmer, B. C., Heir, E., and Langsrud, S. (2009). Characterization of the bacterial spoilage flora in marinated pork products. *J. Appl. Microbiol.* 106:2106. doi: 10.1111/j.1365-2672.2009.04183.x

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Stahnke, L. H. (1994). Aroma components from dried sausages fermented with *Staphylococcus xylosus*. *Meat Sci.* 38, 39–53. doi: 10.1016/0309-1740(94)90094-9

Stock, I., Falsen, E., and Wiedemann, B. (2003). *Moellerella wisconsensis*: identification, natural antibiotic susceptibility and its dependency on the medium applied. *Diagn. Microbiol. Infect. Dis.* 45, 1–11. doi: 10.1016/s0732-8893(02)00483-2

Tofalo, R., Fasoli, G., Schirone, M., Perpetuini, G., Pepe, A., Corsetti, A., et al. (2014). The predominance, biodiversity and biotechnological properties of *Kluyveromyces marxianus* in the production of Pecorino di Farindola cheese. *Int. J. Food Microbiol.* 187, 41–49. doi: 10.1016/j.ijfoodmicro.2014.06.029

UNEP (2021). *Food Waste Index Report 2021*. Nairobi: United Nations Environment Programme.

Vasilopoulos, C., De Maere, H., De Mey, E., Paelinck, H., De Vuyst, L., and Leroy, F. (2010). Technology-induced selection towards the spoilage microbiota of artisan-type cooked ham packed under modified atmosphere. *Food Microbiol.* 27, 77–84. doi: 10.1016/j.fm.2009.08.008

Vasilopoulos, C., De Vuyst, L., and Leroy, F. (2015). Shelf life reduction as an emerging problem in cooked hams underlines the need for improved preservation strategies. *Crit. Rev. Food Sci. Nutr.* 55, 1425–1443. doi: 10.1080/10408398.2012.695413

Vasilopoulos, C., Ravyts, F., De Maere, H., De Mey, E., Paelinck, H., De Vuyst, L., et al. (2008). Evaluation of the spoilage lactic acid bacteria in modified-atmosphere-packaged artisan-type cooked ham using culture-dependent and culture-independent approaches. *J. Appl. Microbiol.* 104, 1341–1353. doi: 10.1111/j.1365-2672.2007.03664.x

White, T. J., Bruns, T., Lee, S., and Taylor, J. W. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols: a Guide to Methods and Applications*, eds M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (New York, NY: Academic Press), 315–322. doi: 10.1016/b978-0-12-372180-8.50042-1

Wohner, B., Pauer, E., Heinrich, V., and Tacker, M. (2019). Packaging-Related Food Losses and Waste: an Overview of Drivers and Issues. *Sustainability* 11:264. doi: 10.3390/su11010264

Zagdoun, M., Coeuret, G., N'Dione, M., Champomier-Vergès, M. C., and Chaillou, S. (2020). Large microbiota survey reveals how the microbial ecology of cooked ham is shaped by different processing steps. *Food Microbiol.* 91:103547. doi: 10.1016/j.fm.2020.103547

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 10 March 2022
doi: 10.3389/fmicb.2022.841841

Prudent Antimicrobial Use Is Essential to Prevent the Emergence of Antimicrobial Resistance in *Yersinia enterocolitica* 4/O:3 Strains in Pigs

Juho Koskinen*, Pilar Ortiz-Martínez, Riikka Keto-Timonen, Suvi Joutsen, Maria Fredriksson-Ahomaa and Hannu Korkeala

Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland

OPEN ACCESS

Edited by:
Dario De Medici, National Institute of Health (ISS), Italy

Reviewed by:
Thilo M. Fuchs, Friedrich-Loeffler-Institute, Germany
Huaiqi Jing, Chinese Center for Disease Control and Prevention, China

***Correspondence:**
Juho Koskinen
juho.koskinen@helsinki.fi

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *22 December 2021*
Accepted: *17 February 2022*
Published: *10 March 2022*

Citation:
Koskinen J, Ortiz-Martínez P, Keto-Timonen R, Joutsen S, Fredriksson-Ahomaa M and Korkeala H (2022) Prudent Antimicrobial Use Is Essential to Prevent the Emergence of Antimicrobial Resistance in Yersinia enterocolitica 4/O:3 Strains in Pigs. Front. Microbiol. 13:841841. doi: 10.3389/fmicb.2022.841841

Yersinia enterocolitica is a psychrotrophic zoonotic foodborne pathogen. Pigs are considered the main reservoir of *Y. enterocolitica* 4/O:3, which is the most commonly isolated bioserotype in many European countries. Consuming pork contaminated with *Y. enterocolitica* can be a health threat, and antimicrobial-resistant strains may complicate the treatment of the most severe forms of yersiniosis. We analyzed the antimicrobial resistance of 1,016 pathogenic porcine *Y. enterocolitica* 4/O:3 strains originating from Belgium, Estonia, Finland, Germany, Italy, Latvia, Russia, Spain, and the United Kingdom. Based on available reports, we also compared antimicrobial sales for food production animals in these countries, excluding Russia. Antimicrobial resistance profiles were determined using a broth microdilution method with VetMIC plates for 13 antimicrobial agents: ampicillin, cefotaxime, ceftiofur (CTF), chloramphenicol (CHL), ciprofloxacin, florfenicol, gentamicin, kanamycin, nalidixic acid (NAL), streptomycin (STR), sulfamethoxazole (SME), tetracycline (TET), and trimethoprim (TMP). The antimicrobial resistance of *Y. enterocolitica* 4/O:3 strains varied widely between the countries. Strains resistant to antimicrobial agents other than ampicillin were rare in Estonia, Finland, Latvia, and Russia, with prevalence of 0.7, 0.4, 0, and 8.3%, respectively. The highest prevalence of antimicrobial resistance was found in Spanish and Italian strains, with 98 and 61% of the strains being resistant to at least two antimicrobial agents, respectively. Resistance to at least four antimicrobial agents was found in 34% of Spanish, 19% of Italian, and 7.1% of English strains. Antimicrobial resistance was more common in countries where the total sales of antimicrobials for food production animals are high and orally administered medications are common. Our results indicate that antimicrobials should be used responsibly to treat infections, and parenteral medications should be preferred to orally administered mass medications.

Keywords: antibiotic, antimicrobial, antimicrobial policy, control, foodborne pathogen, pig, resistance, *Yersinia enterocolitica*

INTRODUCTION

Yersinia enterocolitica is a foodborne pathogen capable of causing yersiniosis, the fourth most reported bacterial zoonosis in the European Union [European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC), 2021]. Pigs are the main reservoir of pathogenic *Y. enterocolitica*, especially bioserotype 4/O:3, and these bacteria have frequently been isolated from the tonsils and feces of clinically healthy pigs (Gürtler et al., 2005; Fredriksson-Ahomaa et al., 2007; Laukkanen et al., 2009; Ortiz Martínez et al., 2009, 2011; Virtanen et al., 2014; Koskinen et al., 2019). Consequently, pork products are important sources of human yersiniosis (Tauxe et al., 1987; Ostroff et al., 1994; Fredriksson-Ahomaa et al., 2006, 2010a; Virtanen et al., 2013).

Yersiniosis usually manifests as gastroenteritis, but the infection may, for example, cause pseudoappendicitis or sepsis or lead to immunological sequelae such as reactive arthritis or erythema nodosum (Bottone, 1999; Fredriksson-Ahomaa et al., 2010a). Most infections are self-limiting, and antimicrobial therapy is therefore not needed. Antimicrobial agents, such as fluoroquinolones, trimethoprim-sulfamethoxazole (SME), tetracycline (TET), and third-generation cephalosporins, are warranted for more severe infections, in which mortality can rise to 50%, and for severe postinfectious complications (Crowe et al., 1996; Jiménez-Valera et al., 1998; Hoogkamp-Korstanje et al., 2000; Guinet et al., 2011; Fàbrega and Vila, 2012). *Y. enterocolitica* has intrinsic resistance to many β-lactam antibiotics, such as penicillin, ampicillin, and first-generation cephalosporins, due to the presence of two β-lactamase genes *blaA* and *blaB* (Cornelis and Abraham, 1975; Bent and Young, 2010; Bonke et al., 2011).

Antimicrobial resistance is a concerning global health threat [World Health Organization (WHO), 2015]. If necessary actions are not taken, an estimated 10 million people could die in 2050 due to antimicrobial resistance with massive consequences for patients, healthcare systems, and economies (Dadgostar, 2019). Using antimicrobial agents for livestock is a significant part of this multifactorial problem and may increase the antimicrobial resistance of foodborne pathogens. These pathogens may transmit from production animals to humans via food products, water, or by direct contact (Collignon, 2012). The transmission of antimicrobial-resistant porcine *Y. enterocolitica* strains to humans may complicate the treatment of the most severe forms of yersiniosis or other bacterial infections. To control this public health threat, regular and comprehensive monitoring of antimicrobial resistance is required worldwide, so that necessary actions can be taken.

The aim of our study was to assess the antimicrobial resistance of 1,016 *Y. enterocolitica* bioserotype 4/O:3 strains isolated from porcine origin in nine European countries, and to compare the use of antimicrobial agents in these countries. We observed wide variation in antimicrobial resistance. Concerning levels of antimicrobial resistance were found in countries where the total use of antimicrobial agents is high, especially Spain and Italy. Our results indicate that prudent use of antimicrobials is essential to control antimicrobial resistance already at the farm level.

MATERIALS AND METHODS

Strains

A total of 1,016 strains of *Y. enterocolitica* pathogenic bioserotype 4/O:3 from nine European countries (Belgium, Estonia, Finland, Germany, Italy, Latvia, Russia, Spain, and the United Kingdom) were studied. *Yersinia enterocolitica* strains originated from pork and were chosen from the culture collection of the Department of Food Hygiene and Environmental Health (University of Helsinki, Helsinki, Finland). The strains had been isolated between 1999 and 2007 (**Supplementary Table 1**) from pig tonsils, except for eight of the German strains that were isolated from the tongue ($n = 4$), surface samples of pig carcasses ($n = 3$), and head meat ($n = 1$).

Antimicrobial Resistance Testing

Antimicrobial resistance was tested using a broth microdilution method according to the standards of the Clinical and Laboratory Standards Institute (CLSI) (2017). The following 13 antimicrobials were tested: ampicillin (concentration range 0.25–32 mg/L), cefotaxime (0.06–2 mg/L), ceftiofur (CTF; 0.12–16 mg/L), chloramphenicol (CHL; 1–128 mg/L), ciprofloxacin (0.008–1 mg/L), florfenicol (4–32 mg/L), gentamicin (0.5–64 mg/L), kanamycin (2–16 mg/L), nalidixic acid (NAL; 1–128 mg/L), streptomycin (STR; 2–256 mg/L), sulfamethoxazole (16–2,048 mg/L), tetracycline (0.5–64 mg/L), and trimethoprim (TMP; 0.25–32 mg/L). Susceptibility monitoring was performed on VetMIC plates (SVA, Uppsala, Sweden). The strains were grown on *Yersinia* selective agar base (Oxoid Ltd., Basingstoke, New Hampshire, United Kingdom) at 30°C for 24 h. Four or five colonies were transferred into 5 ml of Müller-Hinton II broth (BBL, Müller-Hinton II broth, cation adjusted; Beckton, Dickinson and Company, Sparks, MD, United States) and incubated at 30°C until the absorbance of the broth was 0.08–0.1, to obtain an inoculum size of 10^8 CFU/ml. The inoculums for broth microdilution were prepared by mixing 10 µl of the inoculum to 10 ml of Müller-Hinton II broth. Each well of the VetMIC plate was filled with 50 µl of the inoculum and sealed with covering tape. The plates were incubated for 1 h in a shaker (150 rpm) and 16–18 h at 30°C. *Escherichia coli* ATCC 25922 was used as a control strain and incubated at 37°C.

The plates were evaluated with visual examination using a magnifying mirror. The minimum inhibitory concentration (MIC) was defined as the lowest antimicrobial concentration that inhibited bacterial growth. The strains were categorized as susceptible (S), intermediately resistant (I), or resistant (R). Clinical breakpoints for *Enterobacteriaceae* from the Clinical and Laboratory Standards Institute (CLSI) (2017) were used, except for ceftiofur, florfenicol, and streptomycin, for which we used the breakpoints for *Salmonella* spp. of Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) (2015). The results were analyzed using WHONET 5.6 software (Stelling and O'Brien, 1997). In our study, a strain

was classified as multiresistant if it was resistant to at least two of the antimicrobial agents tested, excluding ampicillin, which shows frequent intrinsic resistance.

Estimations of Antimicrobial Use in European Countries

We evaluated the antimicrobial use and policies of eight countries (Belgium, Estonia, Finland, Germany, Italy, Latvia, Spain, and the United Kingdom) based on the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) sales report by the European Medicines Agency (EMA) for year 2011 [European Medicines Agency (EMA), 2013], as no direct data are available for the actual use of antimicrobial agents. This report was the first to cover all eight countries. Respective data were not available from Russia.

In the ESVAC reports, sales are measured in relation to the estimated quantity of animal biomass, i.e., the antimicrobials used for food production animals in milligrams per population correction unit (mg/PCU). The PCU is a technical unit of measurement, which is used to estimate the mass of treated livestock and slaughtered animals during a year, and animals exported or imported for slaughter or fattening in another member country are also taken into account [European Medicines Agency (EMA), 2013]. In addition, we compared the use of orally administered and injectable veterinary antimicrobials based on the corresponding proportions of the sales in each country. Data from the first Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) report by the European Centre for Disease Prevention and Control, European Food Safety Authority, and European Medicines Agency (ECDC, EFSA, and EMA) (2015) were also used to measure antimicrobial use for food production animals and as a baseline for humans.

A report by the European Agency for the Evaluation of Medicinal Products (EMEA) (1999) was used to estimate the use of all antimicrobials, therapeutic antimicrobials, and growth promoter antimicrobials for animals in the mid-1990s. Antimicrobial use in Belgium and Luxemburg, Finland, Germany, Italy, Spain, and the United Kingdom in 1997 was made proportional to the number of animals slaughtered (mg/kg) in these countries in 1996. These data represent antimicrobial use in the countries in the mid-1990s, a decade before antimicrobial growth promoters were banned in the European Union (EU) in 2006 [European Union (EU), 2005]. The data regarding Belgium include Luxemburg, and in Finland, growth promoter antimicrobial levels were less than 2 mg/kg, but 2 mg/kg was used for the analyses.

Statistical Analyses

Statistical analyses were performed in IBM SPSS Statistics 27 (IBM, Armonk, NY, United States). One-tailed Pearson's correlation was used to measure correlations between the observed prevalence of antimicrobial resistance and the different estimations of antimicrobial use, that is, total, oral, and injectable antimicrobial sales in mg/PCU, growth promoter sales, as well as proportions of antimicrobial sales by administration route. In addition, countries were categorized into two groups based on whether more than two thirds of antimicrobial sales were oral medications instead of parenteral medications such as injections, and antimicrobial resistance between the two groups was compared with the Student's *t* test with unequal variances.

RESULTS

Antimicrobial Resistance of *Yersinia enterocolitica*

All strains were susceptible to cefotaxime, ceftiofur, ciprofloxacin, florfenicol, gentamicin, and kanamycin (**Table 1**). The MICs are shown in **Supplementary Table 2**. Estonian, Finnish, and Latvian strains were susceptible to all other antimicrobials than ampicillin, excluding one Estonian strain resistant to trimethoprim and one Finnish strain resistant to sulfamethoxazole. Resistance to streptomycin was found frequently, with highest resistance levels in Spain (98%), Italy (62%), and Belgium (55%). Resistance to sulfamethoxazole was also common, with highest prevalence of resistance in Spanish (99%), English (82%), and Italian (61%) strains. Tetracycline resistance was found in 49% of Italian and 27% of Spanish strains. Trimethoprim resistance was most common among Italian (30%) and English (21%) strains. Resistance to chloramphenicol and nalidixic acid was found in Spanish strains only.

Antimicrobial Resistance to at Least Two Antimicrobial Agents

Resistance to multiple antimicrobials was found especially in Spanish, Italian, and English *Y. enterocolitica* 4/O:3 strains, and in a few Belgian strains (**Table 2**). The majority of the Spanish strains (98%) were multiresistant, and the most common antimicrobial resistance pattern was CHL-STR-SME (**Table 3**). Multiresistance patterns CHL-STR-SME-TET and CHL-NAL-STR-SME were found in 23 and 6.0% of Spanish strains, respectively. More than half of the Italian strains (61%) were multiresistant, while 21, and 5.3% of the English, and Belgian strains were resistant to at least two antimicrobials, respectively. Resistance to four or five antimicrobials was observed in Spanish ($n = 62$), Italian ($n = 20$), and English ($n = 2$) strains.

Use of Antimicrobial Agents and Its Correlation With Antimicrobial Resistance

Based on sales data, we observed vast differences in the use of antimicrobial agents in the eight countries (**Table 4**). In general, higher levels of antimicrobials were used in countries, where antimicrobial resistance and multiresistance were frequent (**Figures 1, 2**). The prevalence of antimicrobial resistance of *Y. enterocolitica* in the countries significantly correlated with antimicrobial multiresistance (**Table 5**).

Correlations between estimated antimicrobial use and observed antimicrobial resistance are summarized in **Table 5**. Total sales of antimicrobial agents and oral antimicrobial agents in the countries in 2011, calculated from the ESVAC report (European Medicines Agency (EMA), 2013), positively correlated with antimicrobial multiresistance of the *Y. enterocolitica* strains.

TABLE 1 | Number of porcine *Yersinia enterocolitica* 4/O:3 strains resistant to antimicrobials. All strains were susceptible to cefotaxime[a], ceftiofur[b], ciprofloxacin[c], florfenicol[d], gentamicin[e], and kanamycin[f].

Country (number of strains)	Ampicillin[g]				Chloramphenicol[h]				Nalidixic acid[i]			Streptomycin[j]			Sulfamethoxazole[k]			Tetracycline[l]				Trimethoprim[m]		
	S	I	R	% (CI)[n]	S	I	R	% (CI)[n]	S	R	% (CI)[n]	S	R	% (CI)[n]	S	R	% (CI)[n]	S	I	R	% (CI)[n]	S	R	% (CI)[n]
Belgium (94)	0	9	85	90 (82–95)	94	0	0	0 (0–4.9)	94	0	0 (0–4.9)	42	52	55 (45–65)	92	2	2.1 (0.4–8.2)	94	0	4	4.3 (1.4–11)	94	0	0 (0–4.9)
Estonia (143)	0	0	143	100 (97–100)	143	0	0	0 (0–3.3)	143	0	0 (0–3.3)	143	0	0 (0–3.3)	143	0	0 (0–3.3)	143	0	0	0 (0–3.3)	142	1	0.7 (0–4.4)
Finland (233)	0	17	216	93 (88–96)	233	0	0	0 (0–2.0)	233	0	0 (0–2.0)	233	0	0 (0–2.0)	232	1	0.4 (0–2.7)	233	0	0	0 (0–2.0)	233	0	0 (0–2.0)
Germany (98)	0	0	98	100 (95–100)	98	0	0	0 (0–4.7)	98	0	0 (0–4.7)	92	6	6.1 (2.5–13)	94	4	4.1 (1.3–11)	98	0	0	0 (0–4.7)	98	0	0 (0–4.7)
Italy (105)	0	24	81	77 (68–85)	104	1	0	0 (0–4.4)	105	0	0 (0–4.4)	40	65	62 (52–71)	41	64	61 (51–70)	42	12	51	49 (39–59)	73	32	30 (22–40)
Latvia (70)	0	0	70	100 (94–100)	70	0	0	0 (0–6.5)	70	0	0 (0–6.5)	70	0	0 (0–6.5)	70	0	0 (0–6.5)	70	0	0	0 (0–6.5)	70	0	0 (0–6.5)
Russia (60)	0	5	55	92 (81–97)	60	0	0	0 (0–7.5)	60	0	0 (0–7.5)	57	3	5.0 (1.3–15)	59	1	1.7 (0.1–10)	59	0	1	1.7 (0.1–10)	60	0	0 (0–7.5)
Spain (185)	0	0	185	100 (98–100)	20	0	165	89 (84–93)	166	19	10 (6.5–16)	4	181	98 (94–99)	2	183	99 (97–100)	134	1	50	27 (21–34)	185	0	0 (0–2.5)
UK (28)	0	1	27	96 (80–100)	28	0	0	0 (0–15)	28	0	0 (0–15)	26	2	7.1 (1.2–25)	5	23	82 (62–93)	26	0	2	7.1 (1.2–25)	22	6	21 (9–41)

[a]*MIC < 2 mg/L susceptible (S), 2 mg/L intermediately resistant (I), and >2 mg/L resistant (R); tested concentration range 0.06–2 mg/L.*
[b]*MIC < 4 mg/L susceptible (S), and ≥4 mg/L resistant (R); tested concentration range 0.12–16 mg/L.*
[c]*MIC < 2 mg/L susceptible (S), 2 mg/L intermediately resistant (I), and >2 mg/L resistant (R); the breakpoints were higher than the tested concentration range 0.008–1 mg/L.*
[d]*MIC < 32 mg/L susceptible (S), and ≥32 mg/L resistant (R); tested concentration range 4–32 mg/L.*
[e]*MIC < 8 mg/L susceptible (S), 8 mg/L intermediately resistant (I), and >8 mg/L resistant (R); tested concentration range 0.5–64 mg/L.*
[f]*MIC < 32 mg/L susceptible (S), 32 mg/L intermediately resistant (I), and >32 mg/L resistant (R); the breakpoints were higher than the tested concentration range 2–16 mg/L.*
[g]*MIC < 16 mg/L susceptible (S), 16 mg/L intermediately resistant (I) and >16 mg/L resistant (R); tested concentration range 0.25–32 mg/L.*
[h]*MIC < 16 mg/L susceptible (S), 16 mg/L intermediately resistant (I), and > 16 mg/L resistant (R); tested concentration range 1–128 mg/L.*
[i]*MIC < 32 mg/L susceptible (S), and ≥32 mg/L resistant (R); tested concentration range 1–128 mg/L.*
[j]*MIC < 32 mg/L susceptible (S), and ≥32 mg/L resistant (R); tested concentration range 2–256 mg/L.*
[k]*MIC < 512 mg/L susceptible (S), and ≥512 mg/L resistant (R); tested concentration range 16–2048 mg/L.*
[l]*MIC < 8 mg/L susceptible (S), 8 mg/L intermediately resistant (I), and >8 mg/L resistant (R); tested concentration range 0.5–64 mg/L.*
[m]*MIC < 16 mg/L susceptible (S), and ≥16 mg/L resistant (R); tested concentration range 0.25–32 mg/L.*
[n]*Percentage of resistant strains for an antimicrobial agent and CIs (95% confidence level).*

TABLE 2 | Antimicrobial resistance of porcine *Yersinia enterocolitica* 4/O:3 strains in nine European countries.

Country (number of strains)	Total number of resistant[a] strains (%)	Number of strains *n* (%) resistant[a] to					Total number of multiresistant[b] strains (%)
		One antimicrobial	Two antimicrobials	Three antimicrobials	Four antimicrobials	Five antimicrobials	
Belgium (94)	53 (56%)	48 (51%)	5 (5.3%)	0 (0%)	0 (0%)	0 (0%)	5 (5.3%)
Estonia (143)	1 (0.70%)	1 (0.70%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Finland (233)	1 (0.43%)	1 (0.43%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Germany (98)	10 (10%)	10 (10%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Italy (105)	65 (62%)	1 (0.9%)	1 (0.9%)	43 (41%)	20 (19%)	0 (0%)	64 (61%)
Latvia (70)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Russia (60)	5 (8.3%)	5 (8.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Spain (185)	184 (99%)	3 (1.6%)	16 (8.6%)	103 (56%)	55 (30%)	7 (3.8%)	181 (98%)
United Kingdom (28)	23 (82%)	17 (61%)	4 (14%)	0 (0%)	2 (7.1%)	0 (0%)	6 (21%)
Total	342 (34%)	86 (8.5%)	26 (2.6%)	146 (14.4%)	77 (7.6%)	7 (0.7%)	256 (25%)

[a]*Ampicillin excluded.*
[b]*Strains resistant to at least two antimicrobials, excluding ampicillin.*

TABLE 3 | Antimicrobial multi-resistance patterns of porcine *Yersinia enterocolitica* 4/O:3 strains.

Antimicrobial multi-resistance pattern[a]	Number of strains (%)	Countries[b] showing pattern (*n*)
STR-SME	18 (1.8%)	BE (1), ES (16), IT (1)
STR-TET	4 (0.4%)	BE (4)
SME-TMP	4 (0.4%)	UK (4)
CHL-STR-SME	103 (10%)	ES (103)
STR-SME-TET	31 (3.1%)	IT (31)
STR-SME-TMP	12 (1.2%)	IT (12)
CHL-STR-SME-TET	43 (4.2%)	ES (43)
STR-SME-TET-TMP	22 (2.2%)	IT (20), UK (2)
CHL-NAL-STR-SME	11 (1.1%)	ES (11)
CHL-CTF-STR-SME	1 (0.1%)	ES (1)
CHL-NAL-STR-SME-TET	7 (0.7%)	ES (7)

[a]*Ampicillin (AMP), cefotaxime (CTX), ceftiofur (CTF), chloramphenicol (CHL), ciprofloxacin (CIP), florfenicol (FLO), gentamicin (GEN), kanamycin (KAN), Nalidixic acid (NAL), streptomycin (STR), sulfamethoxazole (SME), tetracycline (TET), and trimethoprim (TMP).*
[b]*BE, Belgium; ES, Spain; IT, Italy; and UK, United Kingdom.*

By contrast, the proportion of injectable antimicrobial agents sold in the countries correlated negatively with antimicrobial resistance. In addition, resistance levels were statistically significantly higher in countries where more than two thirds of antimicrobial sales were orally administered products than in countries where less than two thirds were oral antimicrobials ($p = 0.015$). Total use of antimicrobial agents in 2012, obtained from the JIACRA I report [European Centre for Disease Prevention and Control, European Food Safety Authority, and European Medicines Agency (ECDC, EFSA, and EMA), 2015], did not statistically significantly correlate with antimicrobial resistance but did correlate with antimicrobial multiresistance.

Prevalence of antimicrobial resistance in the studied strains positively correlated with the total sales of antimicrobials and the sales of therapeutic antimicrobials in the mid-1990s, calculated from the report of European Agency for the Evaluation of Medicinal Products (EMEA) (1999). However, the use of growth promoter antimicrobials in the mid-1990s did not significantly correlate with either antimicrobial resistance or multiresistance (**Table 5**).

DISCUSSION

We observed wide variation in the antimicrobial resistance profiles of *Y. enterocolitica* 4/O:3 strains in the nine European countries. Alarming antimicrobial resistance levels were found in Spain and Italy, where most strains were resistant to two or more antimicrobials. Similar concerning levels of antimicrobial resistance of *Y. enterocolitica*, including multiresistant strains, have also previously been reported in Italy (Bonardi et al., 2013, 2014, 2016) and Greece (Gousia et al., 2011). Gkouletsos et al. (2019) studied the antimicrobial resistance of Greek *Y. enterocolitica* O:3 strains isolated from production animals, companion animals, and humans, and found no statistically significant differences in the overall levels of antimicrobial resistance between the three groups. As *Y. enterocolitica* strains can spread between production animals, companion animals, and humans, antimicrobial-resistant strains may also spread between these groups. In Spain, trends of increasing antimicrobial resistance to various antimicrobials, including streptomycin, sulfonamides, trimethoprime-sulfamethoxazole, chloramphenicol, and nalidixic acid, have been observed in *Y. enterocolitica* strains isolated from human patients (Prats et al., 2000; Marimon et al., 2017).

According to the ESVAC report [European Medicines Agency (EMA), 2013], tetracyclines were the most common veterinary antimicrobial agents sold for treating food production animals in both Italy and Spain in 2011. The common tetracycline use is also likely to explain the high tetracycline resistance observed in our present study. Tetracyclines were the most sold antimicrobials in Italy in 2018 and, bypassed only by penicillin preparations, the second most sold antimicrobial agents in Spain [European Medicines Agency (EMA), 2020]. Of our study countries, Spain and Italy showed the highest antimicrobial sales for treating food production animals per population correction unit (mg/CFU). Multiresistant strains were frequent in these countries and the observed antimicrobial resistance levels the highest in the present study.

The English *Y. enterocolitica* strains were mainly resistant to sulfamethoxazole, but resistance to trimethoprim, streptomycin,

TABLE 4 | Estimated antimicrobial use for food production animals, and for humans as a baseline, in eight European countries based on available reports.

	Country							
	Belgium	**Estonia**	**Finland**	**Germany**	**Italy**	**Latvia**	**Spain**	**UK**
Antimicrobial sales in 2011[a]								
Total (mg/PCU[b])	175.2	66.0	23.8	211.5	369.7	35.0	249.4	51.2
Oral powders, oral solutions, and premixes								
Sales (mg/PCU)	157.0	43.6	8.4	203.2	349.9	22.4	222.9	43.6
Proportion of total sales	90%	66%	35%	96%	95%	64%	89%	85%
Injections								
Sales (mg/PCU)	17.3	20.0	13.9	6.7	18.7	9.9	10.8	6.3
Proportion of total sales	9.9%	30%	58%	3.2%	5.1%	28%	4.3%	12%
Proportion of pigs in PCU	55%	30%	35%	47%	22%	19%	47%	11%
Antimicrobial consumption in 2012[c]								
Consumption in hospitals included	Yes	Yes	Yes	No	Yes	Yes	No	No
Consumption (mg/kg biomass)								
Humans	162.6	70.1	140.1	66.9	167.1	88.8	108.6	104.2
Production animals	161.1	56.0	23.8	204.8	341.0	44.1	242.0	66.3
Antimicrobials sold in 1997 (mg) divided by production animals slaughtered in 1996 (kg)[d]								
Total (mg/kg)	92.2[e]	no data	24.1	83.7	81.3	no data	135.8	183.5
Therapeutic antimicrobials (mg/kg)	49.1	no data	24.1	55.1	64.5	no data	102.6	147.7
Growth promoter antimicrobials	43.2	no data	< 2	28.8	16.6	no data	33.0	35,8

[a]*Data from ESVAC report [European Medicines Agency (EMA), 2013].*
[b]*PCU, population correction unit.*
[c]*Data from JIACRA report [European Centre for Disease Prevention and Control, European Food Safety Authority, and European Medicines Agency (ECDC, EFSA, and EMA), 2015].*
[d]*Data from European Agency for the Evaluation of Medicinal Products (EMEA) (1999).*
[e]*Data from Belgium includes Luxemburg.*

and tetracycline was also observed. According to the ESVAC reports [European Medicines Agency (EMA), 2013, 2020], sulfonamides, following tetracyclines and penicillins, were the third most common veterinary antimicrobial agents sold in the United Kingdom in 2011 and 2018, which may partly explain the relatively high resistance rates to sulfamethoxazole. Sulfamethoxazole is commonly used with trimethoprim, against which antimicrobial resistance was also observed.

Over half of the Belgian strains were resistant to streptomycin, but otherwise antimicrobial resistance levels were moderate in Belgium and Germany. Interestingly, aminoglycosides are not particularly commonly used in Belgium or Germany, yet streptomycin resistance was frequent in Belgium but not in Germany. Low or moderate resistance levels of *Y. enterocolitica* have been reported in Austria, Germany, and Switzerland (Mayrhofer et al., 2004; Baumgartner et al., 2007; Fredriksson-Ahomaa et al., 2007, 2010b; Bucher et al., 2008; von Altrock et al., 2010; Bonke et al., 2011; Meyer et al., 2011; Schneeberger et al., 2015).

The low antimicrobial resistance levels of *Y. enterocolitica* 4/O:3 strains detected in Latvia, Estonia, Finland, and Russia, are in accordance with earlier studies available from these countries (Kontiainen et al., 1994; Terentjeva and Bērziņs, 2010; Bonke et al., 2011). However, Terentjeva and Bērziņs (2010) found high resistance (100%) to sulfamethoxazole in Latvian *Y. enterocolitica* strains and concluded that extensive sulfonamide use for livestock during the time when Latvia belonged to the Soviet Union could have affected the development of such resistance. In our study, resistance to sulfamethoxazole was not observed in the Latvian strains. However, we used the broth microdilution method while Terentjeva and Bērziņs (2010) utilized the disc diffusion method. Different breakpoints and methods complicate the comparison of antimicrobial susceptibility results gained from various studies, and disagreement between different tests is relatively frequent, especially with sulfamethoxazole (Meyer et al., 2011), which may partly explain this difference in results.

The multiresistant *Y. enterocolitica* 4/O:3 strains were most commonly resistant to streptomycin, sulfamethoxazole, and chloramphenicol. This pattern appears to be common in *Y. enterocolitica* strains in several European countries (Baumgartner et al., 2007; Sihvonen et al., 2011; Bonardi et al., 2014, 2016). Karlsson et al. (2021) reported a chromosomally encoded multi-drug resistance cassette containing resistance genes to these three types of antimicrobials and mercury.

Antimicrobial resistance in our study positively and statistically significantly correlated with the total sales of antimicrobials and the sales of therapeutic antimicrobials in the mid-1990s, but no statistically significant correlation was found with growth promoter sales. However, the EU banned the use of antimicrobials as growth promoters in 2006 [European Union (EU), 2005], and most of the antimicrobial growth promoters, such as vancomycin and avoparcin, used in the EU in the past are mainly active against Gram-positive bacteria (Wegener et al., 1999). Despite veterinary antimicrobials currently being prescription-only medicines in the member countries of the ESVAC reports [European Medicines Agency (EMA), 2013, 2020], preventative medications are still commonly used. For example, prophylactic medications are given to healthy animals with no clinical symptoms but with a high risk of disease, while metaphylactic medications are given to healthy animals living in the same group as symptomatic animals. Callens et al. (2012) studied 50 Belgian herds of fattening pigs and found that antimicrobials, including

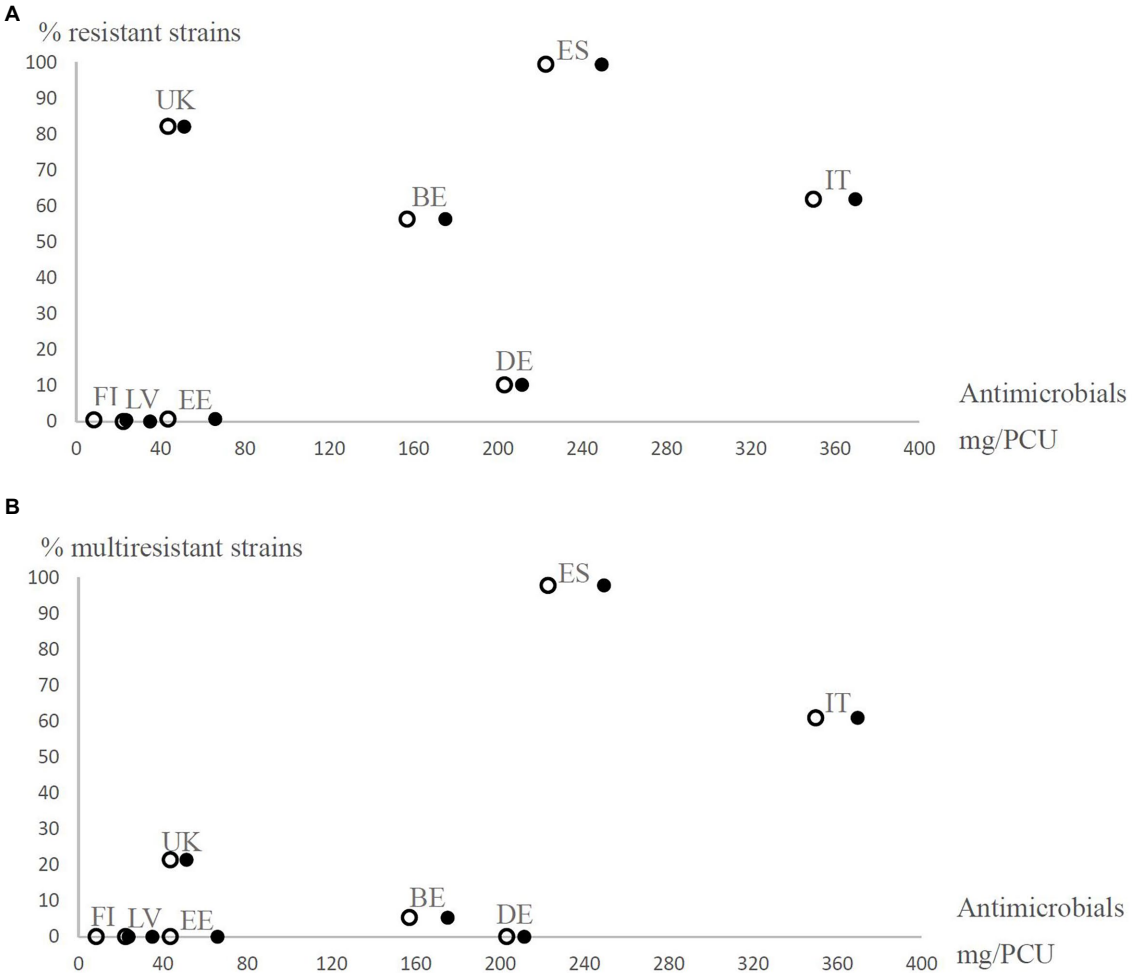


FIGURE 1 | Prevalence of resistant (resistance to at least one antimicrobial agent excluding ampicillin; **A**) and multiresistant (resistance to at least two antimicrobial agents excluding ampicillin; **B**) *Yersinia enterocolitica* 4/O:3 strains from different European countries (BE, Belgium; DE, Germany; EE, Estonia; ES, Spain; FI, Finland; IT, Italy; LV, Latvia; and UK=United Kingdom) observed in the present study in relation to the estimated number of antimicrobials sold for treating production animals in mg per population correction unit (mg/PCU) in 2011. Open circles represent orally administered antimicrobials and closed circles represent all antimicrobials. The proportions of oral solutions, oral powders, and premixes as percentages of total sales of antimicrobial agents in each country [European Medicines Agency (EMA), 2013] were used as coefficients to estimate how many mg/PCU of antimicrobials were administered orally for production animals.

critical and broad-spectrum ones, had been used preventatively in 98% of the herds, and 93% of the group treatments were prophylactic while only 7% were metaphylactic. Along with the therapeutic use of antimicrobials, prophylaxis, and metaphylaxis may be needed in certain situations, for example, if a serious disease is threatening an entire group of animals. However, the benefits of preventative medications should always be considered in relation to the risk of developing antimicrobial resistance.

In the present study, the antimicrobial resistance levels were higher in countries where more than two thirds of antimicrobials were sold in enteral forms, which reflect the importance of using parenteral medications for individual animals rather than enteral mass medications *via* feed. The overall sales data collected by EMA and summarized in **Table 4** show that most veterinary antimicrobials in Belgium, Germany, Italy, Spain, and the United Kingdom were sold in enteral forms, such as premixes, oral powders, and oral solutions, while parenteral medications were preferred in Finland, and both enteral and parenteral forms were commonly used in Estonia and Latvia. This finding is supported by Sjölund et al. (2016), who compared antimicrobial use in Belgium, France, Germany, and Sweden and found that the overall use of antimicrobials was highest in German pig herds and lowest in Swedish herds, and antimicrobials were usually given in enteral forms, except in Sweden, where parenteral forms were preferred. According to European Medicines Agency (EMA) (2013), the sales of orally administered antimicrobials are a reasonable estimate of group treatments, because premixes and the majority of oral powders and oral solutions are applicable for group treatment while the sales of small packages of oral powders and oral solutions sufficient for treatment of only a single or a few animals are very low. Frequent use of oral antimicrobials indicates that mass medications are common in certain countries. By contrast, parenteral forms are preferred in Northern Europe [European Medicines Agency (EMA), 2013],

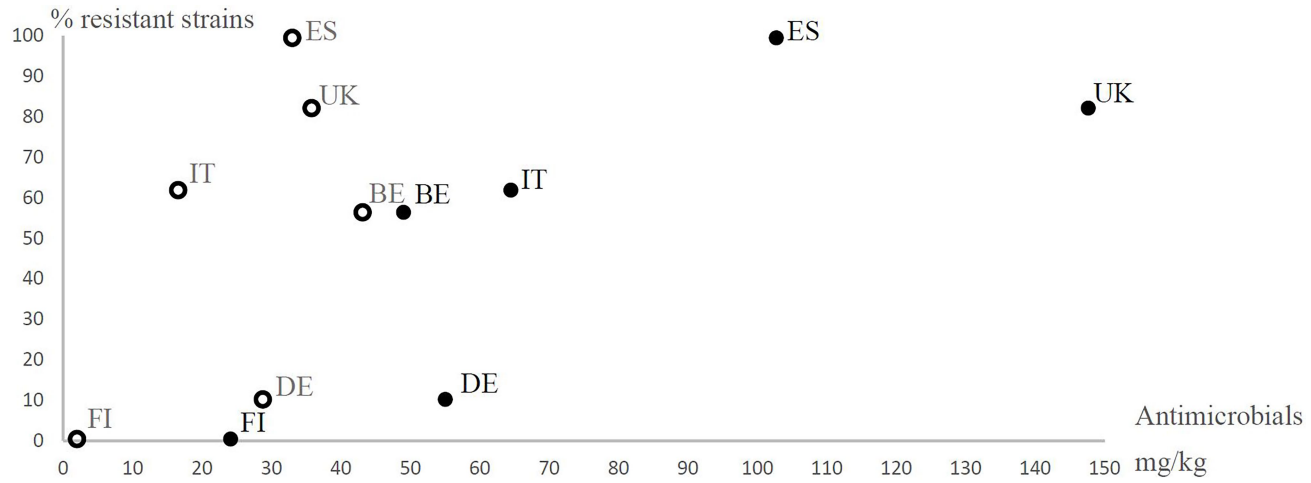


FIGURE 2 | Prevalence of antimicrobial resistant (resistant to at least one antimicrobial agent excluding ampicillin) *Yersinia enterocolitica* 4/O:3 strains in different European countries (BE, Belgium; DE, Germany; ES, Spain; FI, Finland; IT, Italy; and UK, United Kingdom) observed in the present study in relation to the estimated number of growth promoter antimicrobials used (open circles) and the therapeutic antimicrobials used (closed circles) for animals (mg/kg) in six European countries in the mid-1990s. The use of antimicrobial agents for animals in mg per kg was calculated from the estimated numbers of antimicrobial agents sold for the treatment of animals in 1997 and the estimated weights of slaughtered animals in 1996 in each country. The data are from European Agency for the Evaluation of Medicinal Products (EMEA) (1999). The data regarding Belgium include Luxemburg. In Finland, growth promoter antimicrobial levels were less than 2 mg/kg, but 2 mg/kg was used for the analyses.

which indicates more prudent use of antimicrobial agents mostly targeting individual animals rather than groups of animals.

We observed major differences in veterinary antimicrobial use between the countries. This variation cannot solely be explained by the different proportions of production animal species in European countries, because the antimicrobial use also depends on several other factors, such as the infectious disease situation, economic incentives, and the culture of prescribing antimicrobials [Grave et al., 2012; European Medicines Agency (EMA), 2013, 2020; Speksnijder et al., 2015]. For example, veterinarians in Finland are not allowed to financially profit from selling antimicrobials or other prescription medications. According to guidelines by the European Commission (2015), one key factor in prudent antimicrobial use is to avoid any financial or material benefits for the suppliers or prescribers of medicines.

Some limitations of our study should be considered when interpreting the results. Sales data are an indirect way to simulate the use of antimicrobial agents, as there are no available data on actual antimicrobial use. Dosages vary between and within the classes of antimicrobial agents and between animal species, the proportions of domestic animal species differ by country, and the population correction unit represents all animals, not only pigs. In addition, the population correction unit is a mathematical unit of measurement only and does not represent any actual animal population possibly treated with antimicrobials. Hence, a detailed comparison is difficult, and ESVAC reports should be interpreted carefully [European Medicines Agency (EMA), 2013, 2020]. Despite the limitations of our study and the multifactorial nature of antimicrobial resistance as a phenomenon, the present study shows that the use of antimicrobial agents is a key factor in the emergence of antimicrobial resistance. Our study also shows differences in general antimicrobial policies between the countries. Similarly, Chantziaras et al. (2013) found that antimicrobial use positively correlated with antimicrobial resistance of *E. coli* isolates.

According to the first JIACRA report by European Centre for Disease Prevention and Control, European Food Safety Authority, and European Medicines Agency (ECDC, EFSA, and EMA) (2015), antimicrobials were used more for production animals than for humans in 2011 and 2012 in Europe. However, according to the newest JIACRA report, the situation was reversed in 2016 [European Centre for Disease Prevention and Control, European Food Safety Authority, and European Medicines Agency (ECDC, EFSA, and EMA), 2021]. Despite increasing efforts to reduce antimicrobial use in the EU, the global trends are concerning. For example, Van Boeckel et al. (2015) estimated that antimicrobial consumption in livestock will increase by 67% from 2010 to 2030, mainly because intensive farming is becoming more common in middle-income countries.

To conclude, the antimicrobial resistance of *Y. enterocolitica* 4/O:3 strains of porcine origin varied widely between European countries. Resistance was most frequent in countries where antimicrobials, especially enteral medications, are used in large quantities. The antimicrobial resistance of numerous pathogens, including *Y. enterocolitica*, is considered one of the most severe global health threats. Despite encouraging news that antimicrobial use has generally decreased in Europe during recent years, much work is required globally. We recommend that antimicrobial resistance control should begin already at the farm level. This can be achieved through the strict control of prescriptions, sales, and use of antimicrobial agents. When antimicrobial agents are needed, treating individual animals should be preferred to mass medications whenever possible. Regular antimicrobial susceptibility monitoring and data on actual antimicrobial use are also needed in the battle against antimicrobial resistance.

TABLE 5 | Correlation between antimicrobial use and resistance of porcine *Yersinia enterocolitica* 4/O:3 strains in Europe.

Antimicrobial use	Prevalence of antimicrobial resistance of *Y. enterocolitica* 4/O:3							
	Resistance[a] to at least one antimicrobial				Resistance[a] to at least two antimicrobials			
	Pearson's correlation coefficient	*p*-value (one-tailed)	Number of strains	Number of countries	Pearson's correlation coefficient	*p*-value (one-tailed)	Number of strains	Number of countries
Estimated total use of antimicrobials (mg/PCU) in 2011[b]	0.507	0.100	956	8	0.672*[c]	0.034	956	8
Use of oral antimicrobials (mg/CPU) in 2011	0.501	0.103	956	8	0.653*	0.040	956	8
Use of injectable antimicrobials (mg/CPU) in 2011	−0.142	0.368	956	8	0.015	0.486	956	8
Proportion of oral antimicrobials (%) in 2011	0.619	0.051	956	8	0.450	0.132	956	8
Proportion of injectable antimicrobials (%) in 2011	−0.644*	0.042	956	8	−0.503	0.102	956	8
Estimated total use of antimicrobials (mg/kg) in 2012[d]	0.540	0.084	956	8	0.694*	0.028	956	8
Estimated total use of antimicrobials (mg/kg) in the mid-1990s[e]	0.808*	0.026	743	6	0.375	0.232	743	6
Use of therapeutic antimicrobials (mg/kg) in the mid-1990s	0.777*	0.035	743	6	0.417	0.205	743	6
Use of growth promoters (mg/kg) in the mid-1990s	0.593	0.107	743	6	0.104	0.422	743	6
Resistance to multiple antimicrobial agents	0.795*	0.009	956	8	1	-	956	8

[a]*Ampicillin resistance is excluded.*
[b]*Data from ESVAC report [European Medicines Agency (EMA), 2013].*
[c]*Statistically significant ($p < 0.05$) correlations are marked with * symbols.*
[d]*Data from JIACRA I report [European Centre for Disease Prevention and Control, European Food Safety Authority, and European Medicines Agency (ECDC, EFSA, and EMA), 2015].*
[e]*Data from European Agency for the Evaluation of Medicinal Products (EMEA) (1999).*

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

HK and RK-T designed the study. PO-M, SJ, JK, and MF-A performed the laboratory work and analyzed the antimicrobial resistance data. JK performed the analysis on antimicrobial use. JK and SJ drafted the manuscript. HK, RK-T, MF-A, and PO-M contributed to manuscript revision. All authors contributed to the article and approved the submitted version.

FUNDING

This work was partially supported by the Finnish Ministry of Agriculture and Forestry (4877/501/2005) and by the Walter Ehrström Foundation and performed in the Finnish Centre of Excellence in Microbial Food Safety, Academy of Finland (grant numbers 118602 and 141140).

ACKNOWLEDGMENTS

We thank Sonja Alaskewicz, Vilja Kajanti, Erika Pitkänen, and Johanna Ranta for laboratory assistance. We also thank Anna-Liisa Myllyniemi and Riikka Laukkanen-Ninios for methodological discussions, and Henriette Helin-Soilevaara and Katariina Kivilahti-Mäntylä for discussions regarding the interpretation of antimicrobial sales data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.841841/full#supplementary-material

REFERENCES

Baumgartner, A., Küffer, M., Suter, D., Jemmi, T., and Rohner, P. (2007). Antimicrobial resistance of *Yersinia enterocolitica* strains from human patients, pigs and retail pork in Switzerland. *Int. J. Food Microbiol.* 115, 110–114. doi: 10.1016/j.ijfoodmicro.2006.10.008

Bent, Z. W., and Young, G. M. (2010). Contribution of BlaA and BlaB beta-lactamases to antibiotic susceptibility of *Yersinia enterocolitica* biovar 1B. *Antimicrob. Agents Chemother.* 54, 4000–4002. doi: 10.1128/AAC.01754-09

Bonardi, S., Alpigiani, I., Pongolini, S., Morganti, M., Tagliabue, S., Bacci, C., et al. (2014). Detection, enumeration and characterization of *Yersinia enterocolitica* 4/O:3 in pig tonsils at slaughter in Northern Italy. *Int. J. Food Microbiol.* 177, 9–15. doi: 10.1016/j.ijfoodmicro.2014.02.005

Bonardi, S., Bassi, L., Brindani, F., D'Incau, M., Barco, L., Carra, E., et al. (2013). Prevalence, characterization and antimicrobial susceptibility of *Salmonella enterica* and *Yersinia enterocolitica* in pigs at slaughter in Italy. *Int. J. Food Microbiol.* 163, 248–257. doi: 10.1016/j.ijfoodmicro.2013.02.012

Bonardi, S., Bruini, I., D'Incau, M., Van Damme, I., Carniel, E., Brémont, S., et al. (2016). Detection, seroprevalence and antimicrobial resistance of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in pig tonsils in northern Italy. *Int. J. Food Microbiol.* 235, 125–132. doi: 10.1016/j.ijfoodmicro.2016.07.033

Bonke, R., Wacheck, S., Stüber, E., Meyer, C., Märtlbauer, E., and Fredriksson-Ahomaa, M. (2011). Antimicrobial susceptibility and distribution of β-lactamase A (*blaA*) and β-lactamase B (*blaB*) genes in enteropathogenic *Yersinia* species. *Microb. Drug Resist.* 17, 575–581. doi: 10.1089/mdr.2011.0098

Bottone, E. J. (1999). *Yersinia enterocolitica*: overview and epidemiologic correlates. *Microbes Infect.* 1, 323–333. doi: 10.1016/s1286-4579(99)80028-8

Bucher, M., Meyer, C., Grötzbach, B., Wacheck, S., Stolle, A., and Fredriksson-Ahomaa, M. (2008). Epidemiological data on pathogenic *Yersinia enterocolitica* in southern Germany during 2000-2006. *Foodborne Pathog. Dis.* 5, 273–280. doi: 10.1089/fpd.2007.0076

Callens, B., Persoons, D., Maes, D., Laanen, M., Postma, M., Boyen, F., et al. (2012). Prophylactic and metaphylactic antimicrobial use in Belgian fattening pig herds. *Prev. Vet. Med.* 106, 53–62. doi: 10.1016/j.prevetmed.2012.03.001

Chantziaras, I., Boyen, F., Callens, B., and Dewulf, J. (2013). Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *J. Antimicrob. Chemother.* 69, 827–834. doi: 10.1093/jac/dkt443

Clinical and Laboratory Standards Institute (CLSI) (2017). *Performance Standards for Antimicrobial Susceptibility Testing; 27th Edn. CLSI Supplement M100*. Wayne, PA: Clinical and Laboratory Standards Institute.

Collignon, P. (2012). Clinical impact of antimicrobial resistance in humans. *Rev. Sci. Tech.* 31, 211–220. doi: 10.20506/rst.31.1.2111

Cornelis, G., and Abraham, E. P. (1975). ß-lactamases from *Yersinia enterocolitica*. *J. Gen. Microbiol.* 87, 273–284. doi: 10.1099/00221287-87-2-273

Crowe, M., Ashford, K., and Ispahani, P. (1996). Clinical features and antibiotic treatment of septic arthritis and osteomyelitis due to *Yersinia enterocolitica*. *J. Med. Microbiol.* 45, 302–309. doi: 10.1099/00222615-45-4-302

Dadgostar, P. (2019). Antimicrobial resistance: implications and costs. *Infect. Drug Resist.* 12, 3903–3910. doi: 10.2147/IDR.S234610

Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) (2015). DANMAP 2014—Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. ISSN 1600-2032.

European Agency for the Evaluation of Medicinal Products (EMEA) (1999). Final report: Antibiotic resistance in the European Union associated with therapeutic use of veterinary medicines – report and qualitative risk assessment by the Committee for Veterinary Medicinal Products. EMEA/CVMP/342/99.

European Centre for Disease Prevention and Control, European Food Safety Authority, and European Medicines Agency (ECDC, EFSA, and EMA) (2015). First joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. *EFSA J.* 13:4006. doi: 10.2903/j.efsa.2015.4006

European Centre for Disease Prevention and Control, European Food Safety Authority, and European Medicines Agency (ECDC, EFSA, and EMA) (2021). Third joint inter-agency report on integrated analysis of consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals in the EU/EEA, JIACRA III. 2016–2018. *EFSA J.* 19:6712. doi: 10.2903/j.efsa.2021.6712

European Commission (2015). Commission notice: guidelines for the prudent use of antimicrobials in veterinary medicine (2015/C 299/04). *Off. J. Eur. Union* 58, 7–26.

European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC) (2021). The European Union one health 2019 zoonoses report. *EFSA J.* 19:6406. doi: 10.2903/j.efsa.2021.6406

European Medicines Agency (EMA) (2013). European Surveillance of Veterinary Antimicrobial Consumption (ESVAC). Sales of veterinary antimicrobial agents in 25 European countries in 2011. EMA/236501/2013.

European Medicines Agency (EMA) (2020). European Surveillance of Veterinary Antimicrobial Consumption (ESVAC). Sales of veterinary antimicrobial agents in 31 European countries in 2018. EMA/24309/2020.

European Union (EU) (2005). Ban on Antibiotics as Growth Promoters in Animal Feed Enters Into Effect. European Union. Available at: http://europa.eu/rapid/press-release_IP-05-1687_en.htm (Accessed October 12, 2021).

Fàbrega, A., and Vila, J. (2012). *Yersinia enterocolitica*: pathogenesis, virulence and antimicrobial resistance. *Enferm. Infecc. Microbiol. Clin.* 30, 24–32. doi: 10.1016/j.eimc.2011.07.017

Fredriksson-Ahomaa, M., Lindström, M., and Korkeala, H. (2010a). "*Yersinia enterocolitica* and *Yersinia pseudotuberculosis*," in *Pathogens and Toxins in Foods: Challenges and Interventions*. eds. V. K. Juneja and J. N. Sofos (Washington, DC: ASM Press), 164–180.

Fredriksson-Ahomaa, M., Meyer, C., Bonke, R., Stűber, E., and Wacheck, S. (2010b). Characterization of *Yersinia enterocolitica* 4/O:3 isolates from tonsils of Bavarian slaughter pigs. *Lett. Appl. Microbiol.* 50, 412–418. doi: 10.1111/j.1472-765X.2010.02816.x

Fredriksson-Ahomaa, M., Stolle, A., Siitonen, A., and Korkeala, H. (2006). Sporadic human *Yersinia enterocolitica* infections by bioserotype 4/O:3 originate mainly from pigs. *J. Med. Microbiol.* 55, 747–749. doi: 10.1099/jmm.0.46523-0

Fredriksson-Ahomaa, M., Stolle, A., and Stephan, R. (2007). Prevalence of pathogenic *Yersinia enterocolitica* in pigs slaughtered at a Swiss abattoir. *Int. J. Food Microbiol.* 119, 207–212. doi: 10.1016/j.ijfoodmicro.2007.07.050

Gkouletsos, T., Patas, K., Lambrinidis, G., Neubauer, H., Sprague, L. D., Ioannidis, A., et al. (2019). Antimicrobial resistance of *Yersinia enterocolitica* and presence of plasmid pYV virulence genes in human and animal isolates. *New Microbe. New Infect.* 32:100604. doi: 10.1016/j.nmni.2019.100604

Gousia, P., Economou, V., Sakkas, H., Leveidiotou, S., and Papadopoulou, C. (2011). Antimicrobial resistance of major foodborne pathogens from major meat products. *Foodborne Pathog. Dis.* 8, 27–38. doi: 10.1089/fpd.2010.0577

Grave, K., Greko, C., Kvaale, M. K., Torren-Edo, J., Mackay, D., Muller, A., et al. (2012). Sales of veterinary antibacterial agents in nine European countries during 2005–09: trends and patterns. *J. Antimicrob. Chemother.* 67, 3001–3008. doi: 10.1093/jac/dks298

Guinet, F., Carniel, E., and Leclercq, A. (2011). Transfusion-transmitted *Yersinia enterocolitica* sepsis. *Clin. Infect. Dis.* 53, 583–591. doi: 10.1093/cid/cir452

Gürtler, M., Alter, T., Kasimir, S., Linnebur, M., and Fehlhaber, K. (2005). Prevalence of *Yersinia enterocolitica* in fattening pigs. *J. Food Prot.* 68, 850–854. doi: 10.4315/0362-028x-68.4.850

Hoogkamp-Korstanje, J. A., Moesker, H., and Bruyn, G. A. (2000). Ciprofloxacin v placebo for treatment of *Yersinia enterocolitica* triggered reactive arthritis. *Ann. Rheum. Dis.* 59, 914–917. doi: 10.1136/ard.59.11.914

Jiménez-Valera, M., Gonzalez-Torres, C., Moreno, E., and Ruiz-Bravo, A. (1998). Comparison of ceftriaxone, amikacin, and ciprofloxacin in treatment of experimental *Yersinia enterocolitica* O9 infection in mice. *Antimicrob. Agents Chemother.* 42, 3009–3011. doi: 10.1128/AAC.42.11.3009

Karlsson, P. A., Tano, E., Jernbergm, C., Hickman, R. A., Guy, L., Järhult, J. D., et al. (2021). Molecular characterization of multidrug-resistant *Yersinia enterocolitica* from foodborne outbreaks in Sweden. *Front. Microbiol.* 12:664665. doi: 10.3389/fmicb.2021.664665

Kontiainen, S., Sivonen, A., and Renkonen, O.-V. (1994). Increased yields of pathogenic *Yersinia enterocolitica* strains by cold enrichment. *Scand. J. Infect. Dis.* 26, 685–691. doi: 10.3109/00365549409008636

Koskinen, J., Keto-Timonen, R., Virtanen, S., Vilar, M. J., and Korkeala, H. (2019). Prevalence and dynamics of pathogenic *Yersinia enterocolitica* 4/O:3 among Finnish piglets, fattening pigs, and sows. *Foodborne Pathog. Dis.* 16, 831–839. doi: 10.1089/fpd.2019.2632

Laukkanen, R., Ortiz Martínez, P., Siekkinen, K.-M., Ranta, J., Maijala, R., and Korkeala, H. (2009). Contamination of carcasses with human pathogenic *Yersinia enterocolitica* 4/O:3 originates from pigs infected on farms. *Foodborne Pathog. Dis.* 6, 681–688. doi: 10.1089/fpd.2009.0265

Marimon, J. M., Figueroa, R., Idigoras, P., Gomariz, M., Alkorta, M., Cilla, G., et al. (2017). Thirty years of human infections caused by *Yersinia enterocolitica* in northern Spain: 1985-2014. *Epidemiol. Infect.* 145, 2197–2203. doi: 10.1017/S095026881700108X

Mayrhofer, S., Paulsen, P., Smulders, F. J., and Hilbert, F. (2004). Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. *Int. J. Food Microbiol.* 97, 23–29. doi: 10.1016/j.ijfoodmicro.2004.04.006

Meyer, C., Stolle, A., and Fredriksson-Ahomaa, M. (2011). Comparison of broth microdilution and disk diffusion test for antimicrobial resistance testing in *Yersinia enterocolitica* 4/O:3 strains. *Microbial. Drug. Res.* 17, 479–484. doi: 10.1089/mdr.2011.0012

Ortiz Martínez, P., Fredriksson-Ahomaa, M., Pallotti, A., Rosmini, R., Houf, K., and Korkeala, H. (2011). Variation in the prevalence of enteropathogenic *Yersinia* in slaughter pigs from Belgium, Italy, and Spain. *Foodborne Pathog. Dis.* 8, 445–450. doi: 10.1089/fpd.2009.0461

Ortiz Martínez, P., Fredriksson-Ahomaa, M., Sokolova, J., Roasto, M., Bérziņš, A., and Korkeala, H. (2009). Prevalence of enteropathogenic *Yersinia* in Estonian, Latvian and Russian (Leningrad region) pigs. *Foodborne Pathog. Dis.* 6, 719–724. doi: 10.1089/fpd.2008.0251

Ostroff, S. M., Kapperud, G., Hutwagner, L. C., Nesbakken, T., Bean, N. H., Lassen, J., et al. (1994). Sources of sporadic *Yersinia enterocolitica* infections in Norway: a prospective case-control study. *Epidemiol. Infect.* 112, 133–141. doi: 10.1017/s0950268800057496

Prats, G., Mirelis, B., Llovet, T., Muñoz, C., Miró, E., and Navarro, F. (2000). Antibiotic resistance trends in enteropathogenic bacteria isolated in 1985-1987 and 1995-1998 in Barcelona. *Antimicrob. Agents Chemother.* 44, 1140–1145. doi: 10.1128/AAC.44.5.1140-1145.2000

Schneeberger, M., Brodard, I., and Overesch, G. (2015). Virulence-associated gene pattern of porcine and human *Yersinia enterocolitica* biotype 4 isolates. *Int. J. Food Microbiol.* 198, 70–74. doi: 10.1016/j.ijfoodmicro.2014.12.029

Sihvonen, L. M., Toivonen, S., Haukka, K., Kuusi, M., Skurnik, M., and Siitonen, A. (2011). Multilocus variable-number tandem-repeat analysis, pulsed-field gel electrophoresis, and antimicrobial susceptibility patterns in discrimination of sporadic and outbreak-related strains of *Yersinia enterocolitica*. *BMC Microbiol.* 11:42. doi: 10.1186/1471-2180-11-42

Sjölund, M., Postma, M., Collineau, L., Lösken, S., Backhans, A., Belloc, C., et al. (2016). Quantitative and qualitative antimicrobial usage patterns in farrow-to-finish pig herds in Belgium France, Germany and Sweden. *Prev. Vet. Med.* 130, 41–50. doi: 10.1016/j.prevetmed.2016.06.003

Speksnijder, D. C., Jaarsma, A. D. C., van der Gugten, A. C., Verheij, T. J. M., and Wagenaar, J. A. (2015). Determinants associated with veterinary antimicrobial prescribing in farm animals in the Netherlands: a qualitative study. *Zoonoses Public Health* 62, 39–51. doi: 10.1111/zph.12168

Stelling, J. M., and O'Brien, T. F. (1997). Surveillance of antimicrobial resistance: the WHONET program. *Clin. Infect. Dis.* 24, S157–S168. doi: 10.1093/clinids/24.Supplement_1.S157

Tauxe, R. V., Vandepitte, J., Wauters, G., Martin, S. M., Goossens, V., De Mol, P., et al. (1987). *Yersinia enterocolitica* infections and pork: the missing link. *Lancet* 329, 1129–1132. doi: 10.1016/s0140-6736(87)91683-7

Terentjeva, M., and Bērziņs, A. (2010). Prevalence and antimicrobial resistance of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in slaughter pigs in Latvia. *J. Food Prot.* 73, 1335–1338. doi: 10.4315/0362-028x-73.7.1335

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., et al. (2015). Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci. U. S. A.* 112, 5649–5654. doi: 10.1073/pnas.1503141112

Virtanen, S., Laukkanen-Ninios, R., Ortiz Martínez, P., Siitonen, A., Fredriksson-Ahomaa, M., and Korkeala, H. (2013). Multiple-locus variable-number tandem-repeat analysis in genotyping *Yersinia enterocolitica* strains from human and porcine origins. *J. Clin. Microbiol.* 51, 2154–2159. doi: 10.1128/JCM.00710-13

Virtanen, S., Nikunen, S., and Korkeala, H. (2014). Introduction of infected animals to herds is an important route for the spread of *Yersinia enterocolitica* infection between pig farms. *J. Food Prot.* 77, 116–121. doi: 10.4315/0362-028X.JFP-13-144

von Altrock, A., Roesler, U., Merle, R., and Waldmann, K. H. (2010). Prevalence of pathogenic *Yersinia enterocolitica* strains on liver surfaces of pigs and their antimicrobial susceptibility. *J. Food Prot.* 73, 1680–1683. doi: 10.4315/0362-028x-73.9.1680

Wegener, H. C., Aarestrup, F. M., Jensen, L. B., Hammerum, A. M., and Bager, F. (1999). Use of antimicrobial growth promoters in food animals and *Enterococcus faecium* resistance to therapeutic antimicrobial drugs in Europe. *Emerg. Infect. Dis.* 5, 329–335. doi: 10.3201/eid0503.990303

World Health Organization (WHO) (2015). Global Action Plan on Antimicrobial Resistance. Geneva, Switzerland.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 22 March 2022
doi: 10.3389/fmicb.2022.846653

Prevalence and Diversity of *Staphylococcus aureus* and Staphylococcal Enterotoxins in Raw Milk From Northern Portugal

Ricardo Oliveira[1,2,3], *Eva Pinho*[1,2,3], *Gonçalo Almeida*[1,4], *Nuno F. Azevedo*[2,3] *and Carina Almeida*[1,2,3,5]*

[1] I.P – National Institute for Agrarian and Veterinarian Research (INIAV), Vairão, Portugal, [2] Laboratory for Process Engineering, Environment, Biotechnology and Energy (LEPABE), Faculty of Engineering, University of Porto, Porto, Portugal, [3] Associate Laboratory in Chemical Engineering (ALiCE), Faculty of Engineering, University of Porto, Porto, Portugal, [4] Center for Study in Animal Science (CECA), ICETA, University of Porto, Porto, Portugal, [5] Centre of Biological Engineering (CEB), University of Minho, Braga, Portugal

Staphylococcus aureus and staphylococcal enterotoxins are a serious public health concern associated with hospital and community-acquired illnesses. Dairy animals frequently shed *S. aureus* into the milk supply which can lead to food poisoning in humans. This study aims to investigate the prevalence and genetic diversity of *S. aureus* and staphylococcal enterotoxins in raw milk from the main dairy region of mainland Portugal. *S. aureus* was found in 53.0% (95% CI: 40.6–65.4%) of 100 raw cow's milk samples collected from bulk cooling tanks. The highest contamination level was 3.4 log10 CFU.mL^{-1}, and in some samples more than one *S. aureus* strain was identified. Staphylococcal enterotoxins (SEA-SEE) were detected in one sample. Spa typing revealed 62 distinct *S. aureus* isolates, being t529 (17.7%, 95% CI: 8.2–27.3%) and t1403 (16.1%, 95% CI: 7.0–25.3%) the predominant types, commonly associated with livestock infection or carriage. The antimicrobial susceptibility test showed that 35.5% of the *S. aureus* isolates were resistant to at least one antimicrobial agent, with resistance to penicillin being the highest (32.3%, 95% CI: 20.6–43.9%) followed by tetracycline (24.2%, 95% CI: 13.5–34.9%), ciprofloxacin (16.1%, 95% CI: 7.0–25.3%) and chloramphenicol (16.1%, 95% CI: 7.0–25.3%). Moreover, five isolates (8.1%, 95% CI: 1.3–14.8%) were identified as methicillin-resistant *S. aureus* (MRSA, cefoxitin resistant). Regarding virulence/resistance genes, 46,8% (95% CI: 34.4–59.2%) isolates harbored at least one enterotoxin-encoding gene, and the *seg* gene was the most frequently detected (41.9%, 95% CI: 29.7–54.2%) followed by the *sei* (40.3%, 95% CI: 28.1–52.5%), *sec* (6.5%, 95% CI: 0.3–12.6%), *seh* (4.8%, 95% CI: 0.0–10.2%), and *sea* (1.6%, 95% CI: 0.0–4.7%) genes. Five (8.1%, 95% CI: 1.3–14.8%) non-enterotoxigenic isolates carried the *mecA* gene (corresponding to isolates phenotypically classified as MRSA), and 4.8% (95% CI: 0.0–10.2%) enterotoxigenic strains also had the *tsst-1* gene. Our study confirm that raw milk can be a zoonotic source of *S. aureus*, including enterotoxigenic

OPEN ACCESS

Edited by:
Laurent Dufossé,
Université de la Réunion, France

Reviewed by:
Nathalia Silva,
State University of Campinas, Brazil
Teresa Conceicao,
Universidade Nova de Lisboa, Portugal

***Correspondence:**
Carina Almeida
carina.almeida@iniav.pt

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *31 December 2021*
Accepted: *16 February 2022*
Published: *22 March 2022*

Citation:
Oliveira R, Pinho E, Almeida G, Azevedo NF and Almeida C (2022) Prevalence and Diversity of Staphylococcus aureus and Staphylococcal Enterotoxins in Raw Milk From Northern Portugal. Front. Microbiol. 13:846653. doi: 10.3389/fmicb.2022.846653

and MRSA strains. Furthermore, the majority of enterotoxigenic isolates were found to contain genes encoding SEs (SEG, SEH and SEI) not routinely screened. This shows the need for a broader SE screening in food safety control, as well as the relevance of risk mitigation measures to control *S. aureus* transmission along the food chain in Portugal.

Keywords: *Staphylococcus aureus*, raw milk, staphylococcal enterotoxins, MRSA, spa typing

INTRODUCTION

Food and water are well-known vectors for the dissemination of zoonotic microorganisms, some of them can be extremely harmful to human health (Gallo et al., 2020). Foodborne diseases are a serious public health concern, associated with losses in productivity and high medical expenses every year (Garcia et al., 2020). Milk, as a central food in the human diet is a critical vehicle of both beneficial and pathogenic microorganisms. Several pathogens including *Brucella* spp., *Campylobacter* spp., Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium* spp., *Salmonella* spp., and also bacterial toxins have been associated with milk-borne diseases (Dhanashekar et al., 2012). Furthermore, there has been a drastic increase in antimicrobial resistance among zoonotic pathogens. Thus, food surveillance is a major concern for the food industry and for public health (Pérez-Rodríguez and Mercanoglu Taban, 2019).

Staphylococcus aureus is ubiquitous in the environment and a major cause of bovine mastitis. Thus, milk is a common source of contamination for the dairy supply chain, the environment, as well as for final consumers (Rola et al., 2016). In the European Union, 43 foodborne outbreaks (FBO), 402 cases and 32 hospitalizations associated to this pathogen were reported in 2020, according to the recent European Food Safety Authority report (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021b). Typically, staphylococcal food poisoning (SFP) are caused by the ingestion of food contaminated with preformed staphylococcal enterotoxins (SEs) produced by coagulase-positive *staphylococci* (CPS) (Argudín et al., 2010). There are 24 SEs and enterotoxin like (SE*l*-) toxins currently identified, including the classical (SEA-SEE) and the newer (SEG-SElY), which are encoded on different pathogenicity islands (Fisher et al., 2018). Most SFP outbreaks are reported in countries where consumption of unpasteurized milk cheeses is common, such as France and Italy. Additionally, raw milk vending machines and traditionally made food products, such as cheese manufactured at local dairy farms, are becoming more popular throughout Europe, increasing the risk of SFP (Rola et al., 2016).

Staphylococcus aureus can also produce several other extracellular virulent proteins such as toxic shock syndrome toxin 1 (TSST-1), Panton-Valentine Leukocidin (PVL), hemolysins, and coagulase. These proteins can contribute to a broad spectrum of pathologies beyond food poisoning that can range from toxin-mediated syndromes to fatal systemic diseases (Chambers and DeLeo, 2009). Moreover, this pathogen is a well-known example of acquired resistance to multiple antibiotics. Methicillin-resistant *Staphylococcus aureus* (MRSA) are of particular concern to human health because it is virtually resistant to all available β-lactam antibiotics and represent a significant cause of morbidity and mortality throughout the world. These have often been found outside the health environment, including in farm animals (van Duijkeren et al., 2014; Wu et al., 2018). Surveillance of raw milk is therefore essential for a better understanding of the risk factors along the milk food chain and to guarantee public health safety.

In Portugal, data on *S. aureus* circulating in raw milk are scarce. Pereira et al. (2009) characterized several *S. aureus* isolates from different foods in Portugal, including a limited number of raw milk samples, and detected the presence of enterotoxigenic *S. aureus*. Molecular typing can be a powerful tool for improve such epidemiological studies with data about clonal relatedness, genetic diversity, and tracking the spread of pathogens. *S. aureus* protein A (spa) typing is a rapid, affordable, and easy molecular typing method that assigns a classification to *S. aureus* strains from the number/sequence variation in repeats at a specific region of the *spa* gene. It offers excellent discriminatory results to the study of *S. aureus* diversity (Sabat et al., 2013). Molecular typing and characterization of virulence factors are thus an important tool in the control of zoonotic diseases.

The aim of this study was to determine the prevalence and diversity of *S. aureus* and staphylococcal enterotoxins in raw cow's milk collected from bulk cooling tanks on dairy farms from the main dairy region of mainland Portugal. Genetic determinants associated with enterotoxinogenicity (i.e., SE-encoding genes), antimicrobial resistance (i.e., *mecA* and *mecC* genes) and severe human infections (i.e., *pvl* and *tsst-1* genes) were investigated.

MATERIALS AND METHODS

Study Design and Sampling

Between November 2020 and August 2021, 100 raw cow's milk samples were collected from bulk cooling tanks of 100 dairy farms located in the "Bacia Leiteira Primária de Entre Douro e Minho." Only dairy cow farms were included in this study. One-liter samples were collected in sterile labeled screwed top bottles, quickly stored at 4°C and analyzed within 24 h. Farms participation was voluntary and anonymous. Information on sampling and number of milking cows of each dairy farm can be found in **Supplementary Table 1**.

Detection of Staphylococcal Enterotoxins

The presence of staphylococcal enterotoxins A, B, C, D, and E on raw milk samples was analyzed by a two-step

method: extraction/concentration and toxin detection by enzyme linked fluorescent assay (ELFA) with the VIDAS SET2 test (bioMerieux, Marcy-I'Etoile, France) according to ISO 19020:2017 (International Organization for Standardization [ISO], 2017). Confidence Interval (CI), for proportions (Wald method), were determined considering a 95% CI (critical z value of 1.96), prevalence/frequency values and the sample size.

Isolation and Identification of *Staphylococcus aureus*

Bulk tank milk samples were analyzed according to ISO 6888–2 (International Organization for Standardization [ISO], 2021) method for the enumeration of coagulase-positive *staphylococci* (CPS). Briefly, 1 mL of serial dilutions of raw milk were plated on Baird-Parker agar with rabbit plasma fibrinogen (bioMerieux). After 48 h of incubation at 37 ± 1°C, the number of colonies displaying a phenotype characteristic for CPS were counted and morphologically different colonies were subcultured on tryptic soya agar (TSA) for further identification.

Then, colonies identified as CPS were confirmed as *S. aureus* by the presence of the *nuc* gene. For this, total DNA of each colony was extracted using the boiling method (95°C for 15 min). The suspensions were centrifuged at 12,000 g for 5 min and supernatants employed as DNA template. The primers used to amplify the *nuc* gene had the sequences forward: 5′-GCGATTGATGGTGATACGGTT-3 and reverse: 5′- AGCCAAGCCTTGACGAACTAAAGC-3, as described by Brakstad et al. (1992). The PCR amplification consisted of a reaction mixture containing 1X Supreme NZYTaq II 2 × Green Master Mix (NZYTech, Portugal), 0.25 μM of each primer and 2 μL of DNA. Thermal cycling reaction conditions used were: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 56°C and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. *S. aureus* ATCC 25923 and *S. epidermidis* CECT 231 were used as positive control and negative control, respectively. The PCR products were subjected to electrophoresis at 100 V for 1 h in a 1.5% (w/v) agarose gel, previous stained with GreenSafe Premium (NZYTech), and finally analyzed under UV light. CIs for prevalence of CPS were determined by the Wald method as mentioned in "Detection of Staphylococcal Enterotoxins" section.

Spa Typing

Spa typing was performed as described by Shopsin et al. (1999) and Roussel et al. (2015). Briefly, amplification and sequencing of the *spa* gene were performed using the total DNA of each *S. aureus* isolate. The PCR amplification consisted of a reaction mixture containing 1X NZYProof Green Master Mix (NZYTech), 0.2 μM of primers spa-1113f (5′-TAA AGA CGA TCC TTC GGT GAG C-3′) and spa-1514r (5′-CAG CAG TAG TGC CGT TTG CTT-3′) and 2 μL of DNA template (Shopsin et al., 1999). Thermal cycling reaction conditions used were: 95°C for 5 min for the initial denaturation, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 56°C and extension at 72°C for 30 s. A final extension was set at 72°C for 5 min. Amplification was confirmed by electrophoresis on a 2% (w/v) agarose gel at 100 V for 1 h, previous stained with GreenSafe Premium (NZYTech). Both strands were then purified and sequenced by I3S genomics platform (Porto, Portugal). The sequences were analyzed using automated workflow provided by BioNumerics software (bioMérieux) which analyze raw sequencer trace files and assign the repeat codes and spa types in connection to SeqNet/Ridom Spa Server1. CIs for prevalence of spa types were determined by the Wald method as mentioned in "Detection of Staphylococcal Enterotoxins" section.

Antimicrobial Susceptibility Test

Antimicrobial susceptibility profile for the *S. aureus* isolates was determined by the Kirby–Bauer disk diffusion method and interpreted according to the criteria of the Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute [CLSI], 2020, 2021). Briefly, a colony suspension of each *S. aureus* isolate was resuspended in saline solution at 0.5 McFarland standard. The suspension was streaked on Muller-Hinton Agar (Oxoid, Basingstoke, United Kingdom) and allowed to dry. Then, the antibiotic disks were placed on the medium and incubated at 37°C for 16–18h. The incubation time was extended to 24 h for cefoxitin, which was used as a surrogate test for methicillin resistance. After the appropriate incubation time, the zones of inhibition were measured and interpreted as sensitive (S), intermediate (I), and resistant (R). The following antimicrobials agents were used: penicillin G (PG, 10 IU), cefoxitin (FOX, 30 μg), ceftaroline (CPT, 5 μg), cefoperazone (CFP, 30 μg), ceftiofur (EFT, 30 μg), tetracycline (TE, 30 μg), chloramphenicol (C, 30 μg), gentamicin (CN, 10 μg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 μg), trimethoprim (TM, 5 μg), sulfonamides (S, 300 μg), erythromycin (E, 15 μg), ciprofloxacin (CIP, 5 μg), clindamycin (DA, 2 μg), quinupristin-dalfopristin (QD, 15 μg), and linezolid (LZD, 30 μg). *S. aureus* strains ATCC 25923 was used as quality control. CIs for antimicrobial susceptibility ratios were determined by the Wald method as mentioned in "Detection of Staphylococcal Enterotoxins" section.

Detection of Staphylococcal Enterotoxins Genes

The isolates identified as *S. aureus* were screened for the presence of 11 main SE-encoding genes suspected to cause SFP outbreaks (*sea, seb, sec, sed, see, ser, seg, seh, sei, sej*, and *sep*) using two multiplex PCR assays according to the European Reference Laboratory (EURL) official method (Roussel et al., 2015). For *sea, seb, sec, sed, see*, and *ser* genes, the PCR amplification consisted of a reaction mixture containing 1X Supreme NZYTaq II 2 × Green Master Mix (NZYTech), 0.2 μM of each primer for *sea, seb, sec, ser*, 0.8 μM of each primer for *sed*, 0.6 μM of each primer for *see* and 2 μL of DNA template. Regarding the *seg, seh, sei, sej* and *sep* genes, the PCR amplification consisted of a reaction mixture containing 1X Supreme NZYTaq II 2 × Green Master Mix (NZYTech), 0.4 μM of each primer for *seh*, 0.6 μM of each primer for *seg*, 0.8 μM of each primer for *sei* and *sep*, 1.0 μM of each primer for *sej* and 2 μL of DNA template. Thermal cycling reaction conditions used were an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 s,

annealing for 30 s at 58°C and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. Five reference *S. aureus* strains supplied by EURL CPS were used as positive controls (FRI137 for *sec*, *seg, seh, sei*; FRI361 for *seg, sei, sej, sec, sed, ser*; FRIS6 for *sea, seb*; FRI326 for *see*; and A900322 for *sei, seg, sep*). The PCR products were subjected to electrophoresis at 100 V for 1 h in a 2.5% (w/v) agarose gel, previous stained with GreenSafe Premium (NZYTech), and finally analyzed under UV light. CIs for prevalence of SE genes were determined by the Wald method as mentioned in "Detection of Staphylococcal Enterotoxins" section.

Detection of Resistance Genes (*mecA* and *mecC*) and Other Virulence Genes (*pvl* and *tsst-1*)

S. aureus isolates were, also, characterized regarding the presence of methicillin resistance genes (*mecA* and *mecC),* as well as *pvl* and *tsst1* virulence genes. For the detection of *mecA*, *mecC* and *pvl* genes, a multiplex reaction was used as described by EURL protocol (Stegger et al., 2012). The PCR amplification consisted of a reaction mixture containing 1X Supreme NZYTaq II 2 × Green Master Mix (NZYTech), 0.2 μM of each primer for *mecA*, *mecC* and *pvl* and 2 μL of DNA template. The detection of the *tsst1* gene was performed using the primers described by Johnson et al. (1991) and a reaction mixture containing 1X Supreme NZYTaq II 2 × Green Master Mix (NZYTech), 0.5 μM of each primer for *tsst1* and 2 μL of DNA template. The thermal cycling reaction conditions used for both PCR were an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 56°C for *mecA*, *mecC* and *pvl* genes or 54°C for *tsst1* gene, extension at 72°C for 30 s, and a final extension 72°C for 5 min. The PCR products were subjected to electrophoresis at 100 V for 1 h in a 2.0% (w/v) agarose gel, previous stained with GreenSafe Premium (NZYTech), and finally analyzed under UV light. All PCR reactions were performed in 20 μL and using MJ Mini Personal Thermal Cycler (Bio-Rad). CIs for the prevalence of virulence/resistance genes were determined by the Wald method as mentioned in "Detection of Staphylococcal Enterotoxins" section.

RESULTS

Prevalence of *Staphylococcus aureus* and Staphylococcal Enterotoxins in Raw Milk

CPS were identified in 54.0% (95% CI: 41.6–66.4%) of raw milk samples and the number of colonies forming units (CFU) ranged between 0 and 3.4 log10 CFU.mL^{-1} (**Figure 1A**). One hundred and ten morphologically different CPS colonies were isolated, which means that in some cases more than one colony per raw milk sample was CPS characteristic and catalase positive. All isolates were tested for the *nuc* gene, and from the 110 CPS isolated, 104 isolates were confirmed as *S. aureus*. The remaining six CPS isolates were identified as *S. hyicus* (API® Staph, bioMérieux). Of the 54 samples positive for CPS, *S. aureus* strains were identified in 53 (53.0%, 95% CI: 40.6–65.4%) of the samples.

From 100 bulk tank milk samples, only one was positive for the presence of SEA-SEE (**Figure 1B**). Interestingly, the SEs-positive sample was negative for the presence of CPS and, consequently, no *S. aureus* was isolated from that sample.

Spa Typing Characterization

Among the 104 *S. aureus* isolates, 25 different *spa* types were detected. As the same *S. aureus* type was identified in different colonies from the same raw milk sample, only 62 *S. aureus* strains from different raw milk samples were considered as distinct and used for further characterization. In terms of prevalence, the most common spa type was t529 (17.7%, 95% CI: 8.2–27.3%) followed by t1403 (16.1%, 95% CI: 7.0–25.3%), t337 (9.7%, 95% CI: 2.3–17.0%), t543 (9.7%, 95% CI: 2.3–17.0%), and t011 (8.1%, 95% CI: 1.3–14.8%). Other spa types, such as t528, t571, t2802, and t2873 were associated with two (3.2%, 95% CI: 0.0–7.6%) distinct isolates, while t002, t108, t117, t127, t189, t208, t267, t843, t899, t1200, t1207, t1334, t2383, t3585, t9216, and t19272, were associated to one (1.6%, 95% CI: 0.0–4.7%) *S. aureus* isolate (**Figure 2**).

Antimicrobial Susceptibility Test

The antimicrobial susceptibility of the 62 *S. aureus* isolates to 16 antimicrobial agents are shown in **Figure 3**. The highest resistance was observed for penicillin G (32.3%, 95% CI: 20.6–43.9%) followed by tetracycline (24.2%, 95% CI: 13.5–34.9%), chloramphenicol (16.1%, 95% CI: 7.0–25.3%) and ciprofloxacin (16.1%, 95% CI: 7.0–25.3%), and to a lesser extent clindamycin (14.5%, 95% CI: 5.7–23.3%), erythromycin (12.9%, 95% CI: 4.6–21.2%), trimethoprim (12.9%, 95% CI: 4.6–21.2%), gentamicin (9.7%, 95% CI: 2.3–17.0%), cefoperazone (8.1%, 95% CI: 1.3–14.8%), ceftiofur (8.1%, 95% CI: 1.3–14.8%), sulfonamides (4.8%, 95% CI: 0–10.2%), and trimethoprim–sulfamethoxazole (1.6%, 95% CI: 0–4.7%). No resistance was observed for ceftaroline, quinupristin-dalfopristin and linezolid. In addition, five isolates (8.1%, 95% CI: 1.3–14.8%) were identified as methicillin-resistant strains (cefoxitin resistance) and showed complete resistance to all other β-lactams tested, except to ceftaroline. Overall, 64.5% (95% CI: 52.6–76.4%) of the *S. aureus* isolates were susceptible to all antimicrobial agents tested and 35.5% (95% CI: 23.6–47.4%) of the *S. aureus* isolates were resistant to at least one of the antimicrobial agents tested, being that 9.7% (95% CI: 2.3–17.0%) were resistant to only one, 3.2% (95% CI: 0–7.6%) to two, and 22.6% (95% CI: 12.2–33.0%) were multi-drug resistant (resistant to three or more antimicrobial agents of different classes). Individual resistance profiles can be found in the **Supplementary Table 2**.

Distribution of Genes Encoding Staphylococcal Enterotoxins

Among the 62 *S. aureus* isolates, 46.8% (95% CI: 34.4–59.2%) harbored at least one of the SEs gene analyzed, 35.5% (95% CI: 23.6–47.4%) isolates had two SE genes and 4.8% (95% CI: 0.0–10.2%) isolates carried three SE genes (**Figure 4A**). From the 11

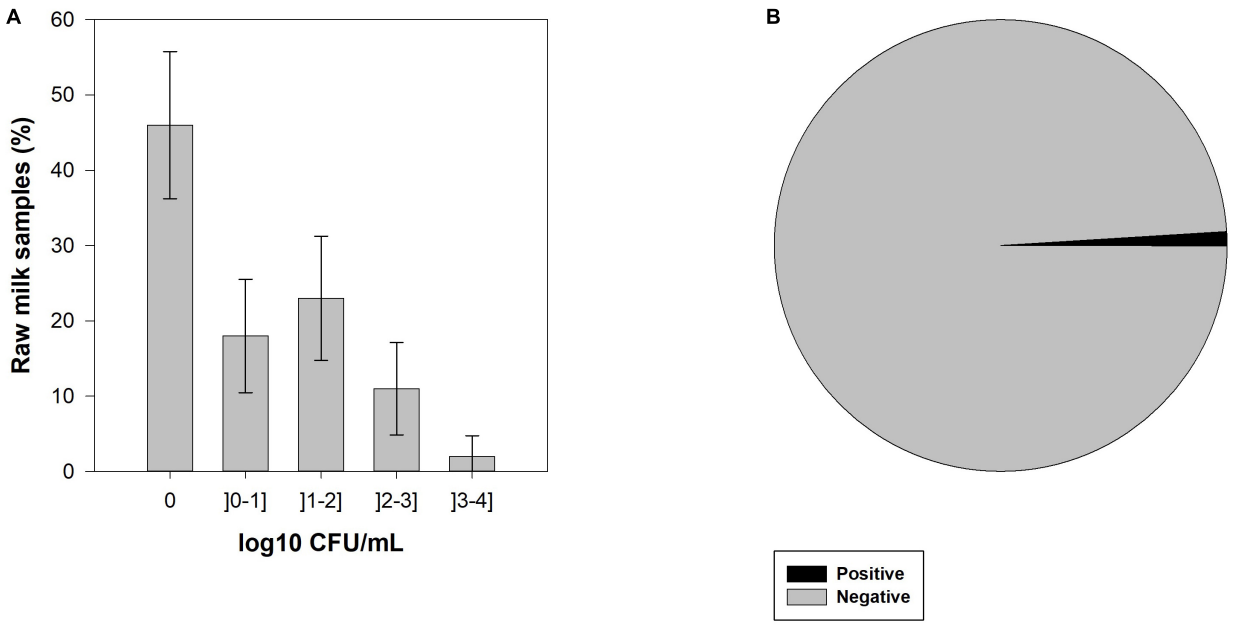


FIGURE 1 | (A) Enumeration of bulk tank milk samples with coagulase-positive staphylococci (CPS) by colony forming units (CFU) categories and its 95% confidence interval (CI), and **(B)** prevalence of staphylococcal enterotoxins (SEs) in raw cow's milk samples from bulk cooling tanks of 100 dairy farms located in the main dairy basin of mainland Portugal.

investigated SE genes, the *seg* was the most frequently detected (41.9%, 95% CI: 29.7–59.2%) followed by the *sei* (40.3%, 95% CI: 28.1–52.5%), *sec* (6.4%, 95% CI: 0.3–12.6%), *seh* (4.8%, 95% CI: 0–10.2%), and *sea* (1.6%, 95% CI: 0.0–4.7%). The genes *seg* and *sei* were always detected together (i.e., 40.3%, 95% CI: 28. 1–52.5%) of the *S. aureus* isolates, except one *S. aureus* isolate that was positive only for the *seg* gene. The gene *sec* was always detected in combination with *seg* and *sei*, and the isolate positive for *sea* was also positive for the *seh* gene. Moreover, *seh* gene was found alone in two isolates.

Distribution of the Resistance Genes (*mecA* and *mecC*) and Virulence Genes (*pvl* and *tsst-1*)

From the 62 *S. aureus* isolates, 8.1% (95% CI: 1.3–14.8%), the same isolates that showed a cefoxitin resistance phenotype were confirmed as MRSA since they harbored the *mecA* gene. The *tsst1* gene was identified in 4.8% (95% CI: 0.0–10.2%) of the *S. aureus* isolates. No *pvl* or *mecC* genes were detected among the *S. aureus* analyzed (**Figure 4B**). MRSA isolates did not contain any genes encoding SEs or TSST-1, but all *tsst1*-positive isolates were enterotoxigenic *S. aureus* with the same SE gene pattern, *sec/seg/sei*.

Genetic Patterns of *Staphylococcus aureus*

Based on the *spa* characterization and distribution of SEs-, resistance-, and other virulence-encoding genes, an analysis of the molecular patterns of *S. aureus* disseminated in raw cow's milk was performed. Individual data for each isolate can be found in the **Supplementary Table 3**.

Seven genetic patterns can be identified: (1) *seg;* (2) *seh;* (3) *sea/seh;* (4) *seg/sei;* (5) *sec/seg/sei;* (6) *tsst-1/sec/seg/sei;* and (7) *mecA*. Furthermore, 45.2% (95% CI: 32.8–57.5%) of the *S. aureus* isolates did not present any of the virulence or resistance genes analyzed. Combining spa types and virulence/resistance determinants, 32 *S. aureus* patterns were detected among 62 isolates in raw milk samples from northern Portugal. Enterotoxigenic *S. aureus* are associated to spa types t002, t117, t127, t337, t528, t529, t543, t899, t2873, and t9216. Out of the five *mecA*-MRSA isolates, four were found to be t011 and one t2383. *S. aureus* t1403, t2802, t571, t108, t189. t208, t267, t843, t1200, t1207, t1334, and t19272 were exclusively associated with strains that did not contain any of the virulence/resistance genes evaluated. In total, *S. aureus* t1403-none (16.1%, 95% CI: 7.0–25.3%) is the predominant molecular pattern, followed by t529-*seg/sei* and t543-*seg/sei* (9.8%, 95% CI: 2.3–17.0%) (**Figure 5**).

DISCUSSION

The presence of *S. aureus* and its enterotoxins in raw milk can be a serious threat to public health given the importance of dairy products in human diet. Therefore, an estimation of the prevalence and genetic determinants of *S. aureus* is always important to implement rational mitigation strategies and avoid the dissemination of this pathogen through the food chain (Kadariya et al., 2014).

In this study, a prevalence of 53.0% of *S. aureus* was established in raw cow's milk collected from the bulk cooling tanks of

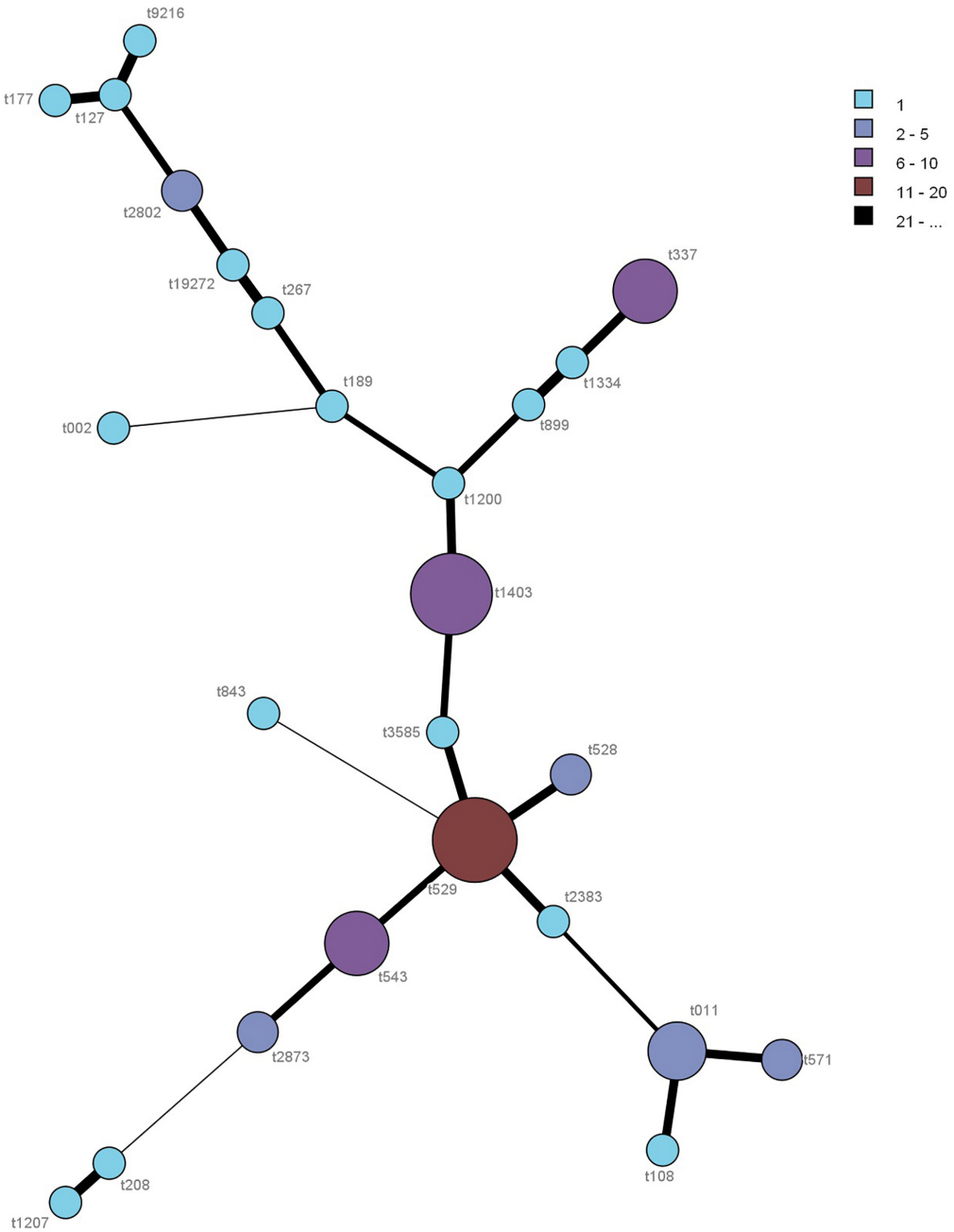


FIGURE 2 | Minimum spanning tree of the spa typing for the 62 *S. aureus* isolates. Each spa type is depicted by a single node, with the size of the node representing the number of isolates associated with this spa type.

100 dairy farms located in the main dairy region of mainland Portugal. To date, this is the first significant study of *S. aureus* in raw milk carried out in Portugal. A similar level of contamination (approximately 40–70%) in raw cow's milk has been found in other countries such as Poland, Italy, New Zealand, Norway, India and China (Jørgensen et al., 2005b; Jakobsen et al., 2011; Hill et al., 2012; Bianchi et al., 2014; Sudhanthiramani et al., 2015; Rola et al., 2016; Liu et al., 2017; Wang et al., 2018; Kou et al., 2021). In fact, the presence of *S. aureus* in bulk tank milk is not surprising given that this species is ubiquitous in nature and one of the main causes of bovine mastitis. Even so, the EU regulation established a criterion for CPS in cheeses made from raw milk (m = 10^4 CFU/g – M = 10^5 CFU/g) during the manufacturing process. If values are higher than 10^5 CFU/g cheese, the batch has to be tested for staphylococcal enterotoxin (EU Commission, 2007). In the present study, the CFU counts obtained on the CPS positive samples were all below the imposed limits. Low levels of CPS at the farms tanks are expected since milk storage is

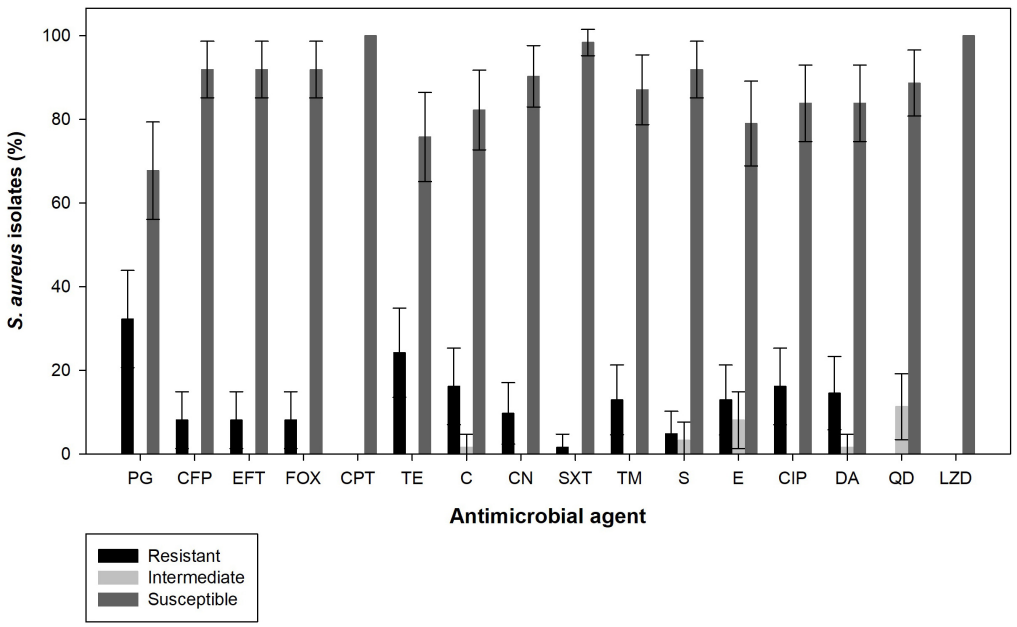


FIGURE 3 | Antimicrobial susceptibility of *S. aureus* isolated from raw cow's milk samples collected from bulk cooling tanks of 100 dairy farms located in the from main dairy basin in of mainland Portugal. PG, Penicillin G; FOX, cefoxitin; CPT, ceftaroline; CFP, cefoperazone; EFT, ceftiofur; TE, tetracycline; C, chloramphenicol; CN, gentamicin; SXT, trimethoprim-sulphamethoxazole; TM, trimethoprim; S, sulfonamides; E, erythromycin; CIP, ciprofloxacin; DA, clindamycin; QD, quinupristin-dalfopristin; LZD, linezolid.

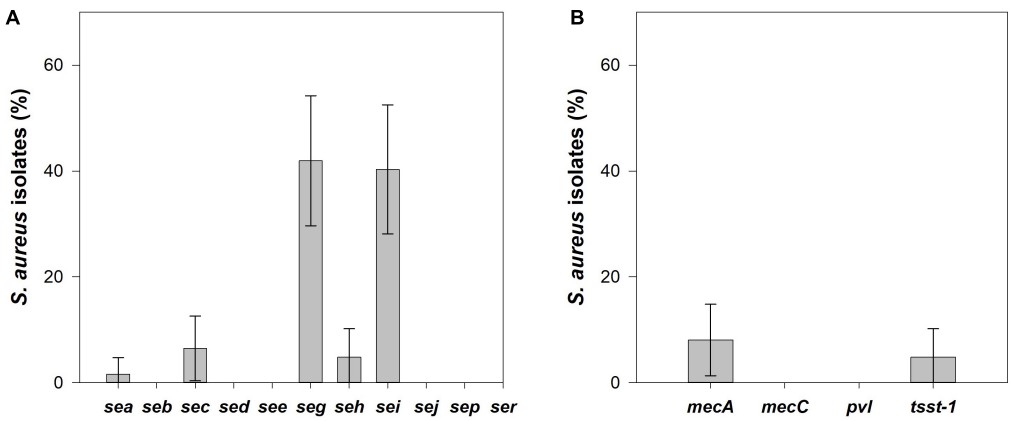


FIGURE 4 | (A) Prevalence and its 95% confidence interval (CI) of enterotoxin coding genes, and **(B)** methicillin resistance genes, *mecA* and *mecC*, toxic shock syndrome toxin-1 (*tsst-1*) and Panton-Valentine leukocidin (*pvl*) genes in *S. aureus* isolated from raw cow's milk samples collected from bulk cooling tanks of 100 dairy farms located in the main dairy basin of mainland Portugal.

controlled at low temperatures (≈4°C) and for a short time after milking (12 – 24 h), as a way to prevent the growth of pathogens, including *S. aureus* (Owusu-Kwarteng et al., 2020).

Still, *S. aureus* presence in foods can represent a risk for human health since this pathogen produces an array of exoproteins with toxicological effects that are highly stable. Although the conditions used during the pasteurization process are suitable to destroy harmful microorganisms, they are not capable of eliminating some proteins, such as toxins. They will keep their activity in pasteurized milk, even at few micrograms, and be a serious threat to end consumers. From those toxins, SEs are the most relevant in the context of foodstuff. In fact, SEs are responsible for several SFP outbreaks reported every year worldwide (Argudín et al., 2010). Thus, their presence in foods is strictly prohibited in Europe (EU Commission, 2007; European Food Safety Authority and European Centre for Disease Prevention and Control, 2021a). In this study, one raw milk sample was positive for staphylococcal enterotoxins (SEA-SEE). Data on SEs in raw milk are scarce, as direct analysis on the raw material is out of scope of the European

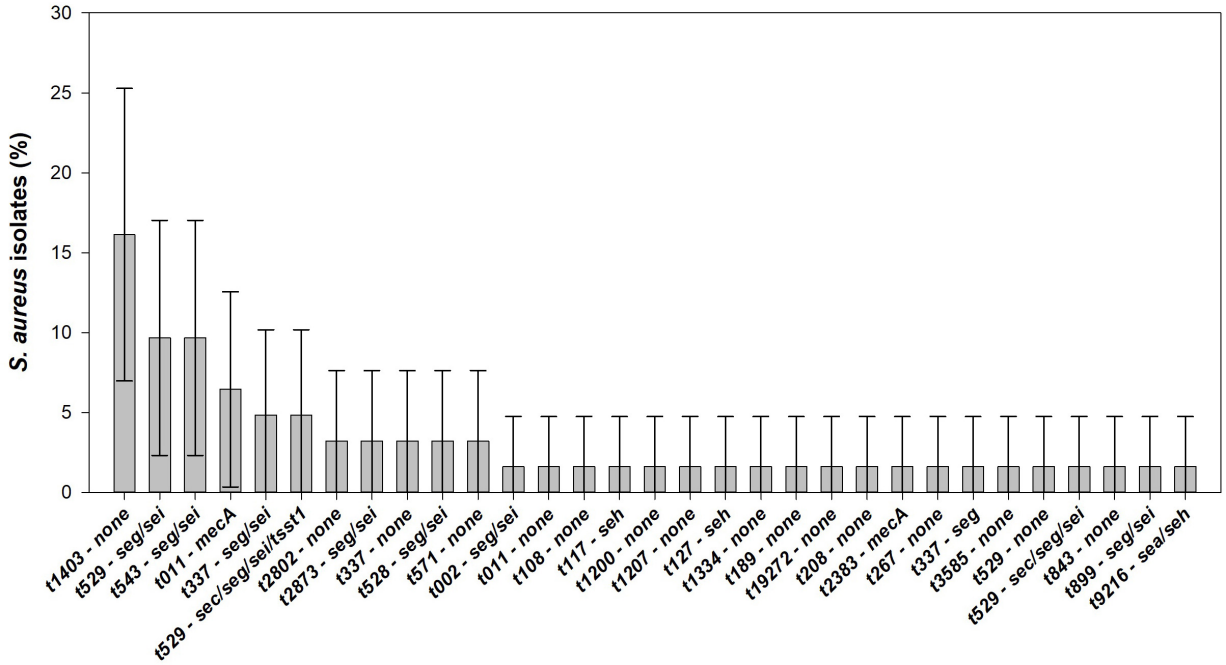


FIGURE 5 | Distribution and its 95% confidence interval (CI) of virulence factors analyzed in *S. aureus* isolated from raw cow's milk samples collected from bulk cooling tanks of 100 dairy farms located in the main dairy basin of mainland Portugal.

regulations. Although the presence of enterotoxins in raw milk has never been studied in Portugal, it has been found in cheeses during official controls, and also an outbreak affecting 13 persons due to an unknown food source (European Food Safety Authority [EFSA], 2019; European Food Safety Authority and European Centre for Disease Prevention and Control, 2021a). In Poland, the presence of SEs in raw milk was assessed without any positive result (Rola et al., 2016). Furthermore, SEs production usually requires the presence of *S. aureus* in high amounts, at least 5–6 log10 CFU.mL^{-1} (Argudín et al., 2010). None of the bulk tank milk samples analyzed had a level of contamination near or above this value, contributing to a lower probability of detection of SEs in the analyzed samples. Surprisingly, in the SE-positive sample found in this study no CPS was detected. Several hypotheses can be made to explain this result. Contamination of milk directly with enterotoxin from the udder of an animal is one of them. In fact, if *S. aureus* was in the udder of dairy cows with enough number of cells able to produce SEs, then enterotoxins could be transferred to the milk during the milking process, even after the bacteria have been eliminated (Rola et al., 2016). However, neither clinical nor subclinical mastitis were indicated in the animals from the dairy farm. Another possible explanation might be related with a dilution effect in the bulk milk, if only one or a few caws are infected. This dilution effect might affect more significantly culture techniques than SE determination, considering that the detection limit reported for VIDAS SET 2 is as low as 0.25 ng toxin per gram of food (Schultz et al., 2004). Enterotoxigenic coagulase-negative staphylococci (CNS) cannot be ruled out as several virulence determinants, usually associated with *S. aureus*, such as SE-encoding genes, have been detected in CNS genomes (Park et al., 2011; Podkowik et al., 2013) and different studies have identified several enterotoxigenic CNS species in dairy animals, capable of producing enterotoxins, predominantly SEC (Bergonier et al., 2003; Taponen et al., 2006; Ünal and Çinar, 2012). Since the methodology followed, in this study, for the isolation of *S. aureus* reject CNS strains, it is not possible to evaluate if CNS are responsible for the SE-positive sample. Finally, we should consider the possibility of a false positive result. Nonetheless, replicates were tested to confirm the sample result.

In recent years, the emergence of antimicrobial resistant strains, particularly MRSA, in livestock animals that is readily transferable to humans, has also become a growing public health concern (Sharma et al., 2018). In the present study, only 35.5% of the *S. aureus* isolates showed resistance to at least one antimicrobial agent. Similar results were reported on studies carry out in Italy (39.4%), Poland (23.0%) and China (38.5%) (Rola et al., 2015; Giacinti et al., 2017; Wang et al., 2018). Penicillin resistance was the most prevalent (32.3%) among the antimicrobial agents tested in accordance with previous studies in raw milk (Rola et al., 2015; Wang et al., 2018). Tetracycline, a broad-spectrum antimicrobial agent frequently used in the treatment of infections in cattle, was the second most prevalent antimicrobial resistance (24.2%), a level similar found to other studies (Liu et al., 2017). It was notable that ciprofloxacin- and chloramphenicol-resistant *S. aureus* were the third most frequently detected antimicrobial resistance. Ciprofloxacin is a

third generation fluoroquinolone used at clinical level, while chloramphenicol is an antibiotic not authorized for use in food-producing animals in the European Union (Benford et al., 2014; Wang et al., 2018). Moreover, five isolates (8.1%) were identified as MRSA by antimicrobial susceptibility, revealing resistance to cefoxitin and all other β-lactams, except ceftaroline (Clinical and Laboratory Standards Institute [CLSI], 2020). Despite the low percentage of resistance observed, antibiotics should be used with caution in dairy animals because they may compromise the treatment of future infections. The presence of isolates with the MRSA phenotype is of major concern for human public health.

Although the presence of SEs was not detected in samples carrying *S. aureus*, enterotoxins' production is still possible under appropriate environmental conditions (e.g., temperature) if the SE-encoding genes are part of the *S. aureus* genome. In our study, we have identified the presence of five different SE-encoding genes among 46.8% of *S. aureus* isolates, including two classical SE genes (*sea* and *sec*) and three non-classical SE genes (*seh, seg* and *sei)*. Only five (8.1%) *S. aureus* isolates had classical SE genes (*sea-see*), although the literature suggested that about 95% of SFP outbreaks are caused by classical SEs (Argudín et al., 2010). Based on our results, the official method recommended for the detection of SEs in foodstuff does not reflect the complete diversity of enterotoxins found in nature. In fact, three of the SEs genes identified in this study, including the two most prevalent SEs (*seg* and *sei*), are not covered by ISO 19020:2017 methodology. The low prevalence of *sea-see* genes explains the low prevalence of SEA-SEE found in the bulk tank raw milk samples. Moreover, since the presence of non-classic SEs is not covered by the recommended methodologies, their prevalence may be underestimated. In Portugal, the characterization of *S. aureus* from a small number of raw milk samples had already verified the predominance of the *seg* and *sei* genes (Pereira et al., 2009). High prevalence of *seg* and *sei* was also observed in raw milk in Italy, and its co-existence in most isolates is also consistent with previous reports (Blaiotta et al., 2006; Bianchi et al., 2014; Johler et al., 2018). In fact, these two SE genes are typically located in tandem on the *ecg* locus and have already been implicated in scarlet fever, toxic shock and neonatal intractable diarrhea cases (Naik et al., 2008). However, these genes do not exist strictly together, as verified in one isolate of this study (only *seg)* and suggested by Jørgensen et al. (2005b). Other studies on raw milk have observed a higher prevalence of *sea-see* genes in Italy, Poland and China, yet most of them are in agreement with our study on the predominance of non-classic SE genes (Bianchi et al., 2014; Rola et al., 2016; Wu et al., 2018). Classical SE genes seems to be more prevalent in isolates of human and non-animal food origin than in animal food origin, such as *S. aureus* from raw milk (Chao et al., 2015). Furthermore, it should be mentioned that despite the prevalence of certain enterotoxin genes, they may have different genomic localization that may differentiate the virulence potential of the strains. Enterotoxins can be encoded in prophages, pathogenicity islands, genomic islands or plasmids, which can have different levels of regulation and expression of these virulence factors (Malachowa and Deleo, 2010).

Regarding the presence of resistance genes, the detection of *mecA* (8.1%) shows that raw cow's milk can also be an antimicrobial resistance vehicle. The positive *mecA*-MRSA isolates correspond with the isolates classified as MRSA by the antimicrobial susceptibility test, demonstrating a complete correspondence between the genomic and phenotype results. The presence of MRSA strains have been reported in raw milk and dairy cattle in Europe (Tenhagen et al., 2014, 2018; Cortimiglia et al., 2016; Hansen et al., 2019; Schnitt et al., 2020; Lienen et al., 2021), but also in Algeria, Uganda, Brazil, and China (Asiimwe et al., 2017; Guimarães et al., 2017; Dai et al., 2019; Titouche et al., 2019). In Europe, MRSA prevalence in raw milk was 3–10%, which is in accordance with our results (Schnitt et al., 2020). In Portugal, *mecA*-MRSA strains have also been reported in bovine mastitis (Pereira et al., 2009), while *mecA* is also the predominant gene in most MRSA isolates worldwide. No association between MRSA strains and enterotoxigenic determinants was found in our analysis. In contrast, TSST-1 genetic determinant was found in enterotoxigenic isolates (*sec/sec/sei*-positive). Such relation may result in an increase in the toxigenic consequences of these strains. No *pvl* or *mecC* genes were detected, consistent with previous studies demonstrating the low prevalence of these genes among *S. aureus* isolated from dairy products (van Duijkeren et al., 2014; Johler et al., 2018). However, three isolates were identified as spa types (t843 and t528) commonly associated to CC130, the frequent genetic background of *mecC* (Bortolami et al., 2017). Given the epidemiological relevance of this genetic determinant of MRSA, the negative result for the presence of the *mecC* gene was confirmed. Furthermore, these isolates revealed a complete susceptibility to the tested antimicrobials, including cefoxitin (*mecC*-positive strains are typically cefoxitin resistant).

Regarding the diversity of *S. aureus*, we have identified 25 spa types among the 62 *S. aureus* isolates from the 100 bulk tank milk samples. Wang et al. (2018) found seven *spa* types of *S. aureus* in 96 isolates from raw milk collected on 2 dairy farms in China. The lower diversity obtained on that study might be due to the limited number of farms enrolled. Furthermore, the most predominant types observed in our study, t529 and t1403, differed from the t127 observed in China. A better correspondence is observed when compared to studies in European countries. Higher diversity and t529 and t1403 types were also predominant in raw milk and bovine isolates in Denmark and Switzerland (Johler et al., 2011; Ronco et al., 2018). Most of the other spa types detected in our study were also associated with bovine *S. aureus* from different European countries (Boss et al., 2016). Thus, the diversity of *S. aureus* found in raw milk in Portugal seems to be in line with that found in the rest of Europe, suggesting a geographic predominance of some spa types. In terms of human health, spa types t002 and t127, detected in one isolate each, are frequently observed in human invasive infections (Grundmann et al., 2010). Combining spa typing with virulence factors, 32 distinct *S. aureus* types can be identified in this study. Some spa types were exclusively associated to enterotoxigenic *S. aureus,* mainly t002, t117, t127, t528, t543, t899, t2873, and t9216. In contrast, *S. aureus* t1403 was also exclusively associated with the absence of virulence/resistance factors. The MRSA-t011 type have recently emerged in European countries, such as Germany and Denmark, associated with livestock-associated MRSA (LA-MRSA) strains including from raw milk isolates (van Duijkeren et al., 2014; Tenhagen et al., 2018; Hansen et al., 2019; Schnitt et al., 2020; Lienen et al., 2021). Accordingly, our results support

that this trend is also present in Portugal. MRSA-t2383 type is a rare relative of t011 and was never reported in raw milk or dairy cattle, but in human and other animals as pigs and seabream (Fanoy et al., 2009; Salgueiro et al., 2020). Thus, spa type/virulence/resistance factors can be a good way to assess the variation in *S. aureus* diversity over time. In most instances, a *spa* types are highly associated with a specific multilocus sequence type (MLST), which is usually related with a specific clonal lineage (Tegegne et al., 2019). As an example, identified spa-types t002, t108, t117, t529, t1334, and t1207 are usually assigned to either sequence type (ST) ST151 or ST504, both belonging to clonal complex (CC) CC70, while t1403 is usually associated with ST97-CC97 or ST133-CC133. In the case of MRSA isolates, the spa-types here identified are usually assigned to ST398-CC398, which is the prevalent LA-MRSA in Europe, occasionally involved in human disease (Bortolami et al., 2017). Overall, most of the ST/CC associated with the spa-types identified in this study are commonly associated with livestock infection or carriage, especially of bovine origin (Smith et al., 2005; Hasman et al., 2010; Boss et al., 2016).

CONCLUSION

In conclusion, the high prevalence of enterotoxigenic *S. aureus* and the detection of MRSA strains in raw cow's milk collected from bulk cooling tanks on dairy farms from the main dairy region of mainland Portugal is of particular concern. Furthermore, a high diversity of *S. aureus* was found in a relatively small geographical area, however, most with genomic lineages associated with livestock infection or carriage. This study also points out the predominance of SE-encoding genes that are not currently covered by the gold standard methodology (ISO 19020:2017) applied in the control of food samples. The higher prevalence of non-classical SEs, mainly SEG, SEH and SEI, should not be ignored, as these have been implicated in food poisoning outbreaks (Jørgensen et al., 2005a; Tang et al., 2011; Ciupescu et al., 2018; Hait et al., 2018). Commercial tests with proven effectiveness for the non-classic SEs are available on the market, which may facilitate their integration in food safety control standard for SEs analysis of foods (Hait et al., 2018). As SFP outbreaks linked to raw cow's milk consumption and raw milk products have been increasing, the presented data on the characterization of *S. aureus* and its virulence determinants are important to improve risk assessment and develop solutions to limit the dissemination of this pathogen in Portugal.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RO, EP, GA, NA, and CA designed the experiments. RO performed the experiments. RO, EP, and CA analyzed the data. All authors contributed to the writing of the manuscript.

FUNDING

This work was financially supported by LA/P/0045/2020 (ALiCE), UIDB/00511/2020 and UIDP/00511/2020 (LEPABE), funded by national funds through FCT/MCTES (PIDDAC); Project POCI-01-0145-FEDER-028659, funded by FEDER funds through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI); and by national funds (PIDDAC) through FCT/MCTES. The authors also thank FCT for the Ph.D. Fellowship SFRH/BD/138883/2018.

ACKNOWLEDGMENTS

We sincerely thank all the dairy farmers who voluntarily participated in this study and the professionals in the field who assisted in collecting the samples. We would also like to thank the Reference Laboratory for Coagulase Positive Staphylococci (EURL CPS) for providing the control strains.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.846653/full#supplementary-material

REFERENCES

Argudín, M. Á, Mendoza, M. C., and Rodicio, M. R. (2010). Food Poisoning and Staphylococcus aureus Enterotoxins. *Toxins* 2, 1751–1773. doi: 10.3390/toxins2071751

Asiimwe, B. B., Baldan, R., Trovato, A., and Cirillo, D. M. (2017). Prevalence and molecular characteristics of Staphylococcus aureus, including methicillin resistant strains, isolated from bulk can milk and raw milk products in pastoral communities of South-West Uganda. *BMC Infect. Dis.* 17:422. doi: 10.1186/s12879-017-2524-4

Benford, D., Ceccatelli, S., Cottrill, B., DiNovi, M., Dogliotti, E., Edler, L., et al. (2014). Scientific Opinion on Chloramphenicol in food and feed. *EFSA J.* 12:3907. doi: 10.2903/j.efsa.2014.3907

Bergonier, D., de Crémoux, R., Rupp, R., Lagriffoul, G., and Berthelot, X. (2003). Mastitis of dairy small ruminants. *Vet. Res.* 34, 689–716. doi: 10.1051/vetres:2003030

Bianchi, D. M., Gallina, S., Bellio, A., Chiesa, F., Civera, T., and Decastelli, L. (2014). Enterotoxin gene profiles of Staphylococcus aureus isolated from milk and dairy products in Italy. *Lett. Appl. Microbiol.* 58, 190–196. doi: 10.1111/lam.12182

Blaiotta, G., Fusco, V., Von Eiff, C., Villani, F., and Becker, K. (2006). Biotyping of enterotoxigenic Staphylococcus aureus by enterotoxin gene cluster (egc) polymorphism and spa typing analyses. *Appl. Environ. Microbiol.* 72, 6117–6123. doi: 10.1128/AEM.00773-06

Bortolami, A., Verin, R., Chantrey, J., Corrò, M., Ashpole, I., Lopez, J., et al. (2017). Characterization of Livestock-Associated Methicillin-Resistant Staphylococcus aureus CC398 and mecC-positive CC130 from Zoo Animals in

the United Kingdom. *Microb. Drug Resist.* 23, 908–914. doi: 10.1089/mdr.2017.0161

Boss, R., Cosandey, A., Luini, M., Artursson, K., Bardiau, M., Breitenwieser, F., et al. (2016). Bovine Staphylococcus aureus: subtyping, evolution, and zoonotic transfer. *J. Dairy Sci.* 99, 515–528. doi: 10.3168/jds.2015-9589

Brakstad, O. G., Aasbakk, K., and Maeland, J. A. (1992). Detection of Staphylococcus aureus by polymerase chain reaction amplification of the nuc gene. *J. Clin. Microbiol.* 30, 1654–1660. doi: 10.1128/jcm.30.7.1654-1660.1992

Chambers, H. F., and DeLeo, F. R. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat. Rev. Microbiol.* 7, 629–641. doi: 10.1038/nrmicro2200

Chao, G., Bao, G., Cao, Y., Yan, W., Wang, Y., Zhang, X., et al. (2015). Prevalence and diversity of enterotoxin genes with genetic background of Staphylococcus aureus isolates from different origins in China. *Int. J. Food Microbiol.* 211, 142–147. doi: 10.1016/j.ijfoodmicro.2015.07.018

Ciupescu, L. M., Auvray, F., Nicorescu, I. M., Meheut, T., Ciupescu, V., Lardeux, A. L., et al. (2018). Characterization of Staphylococcus aureus strains and evidence for the involvement of non-classical enterotoxin genes in food poisoning outbreaks. *FEMS Microbiol. Lett.* 365:139. doi: 10.1093/femsle/fny139

Clinical and Laboratory Standards Institute [CLSI] (2020). *VET01 | Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals*, 5th Edn. Available online at: https://clsi.org/standards/products/veterinary-medicine/documents/vet01/ (Accessed February 4, 2022).

Clinical and Laboratory Standards Institute [CLSI] (2021). *M100 | Performance Standards for Antimicrobial Susceptibility Testing*, 31st Edn. Pennsylvania: Clinical & Laboratory Standards Institute.

EU Commission (2007). Commission regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. *Off. J. Eur. Union* 322, 2–29.

Cortimiglia, C., Luini, M., Bianchini, V., Marzagalli, L., Vezzoli, F., Avisani, D., et al. (2016). Prevalence of Staphylococcus aureus and of methicillin-resistant S. aureus clonal complexes in bulk tank milk from dairy cattle herds in Lombardy Region (Northern Italy). *Epidemiol. Infect.* 144, 3046–3051. doi: 10.1017/S0950268816001576

Dai, J., Wu, S., Huang, J., Wu, Q., Zhang, F., Zhang, J., et al. (2019). Prevalence and Characterization of Staphylococcus aureus Isolated from Pasteurized Milk in China. *Front. Microbiol.* 10:641. doi: 10.3389/FMICB.2019.00641/BIBTEX

Dhanashekar, R., Akkinepalli, S., and Nellutla, A. (2012). Milk-borne infections. an analysis of their potential effect on the milk industry. *Germs* 2, 101–109. doi: 10.11599/germs.2012.1020

European Food Safety Authority [EFSA] (2019). *Trends and Sources of Zoonoses and in Humans, Foodstuffs, Animals and Feedingstuffs - Portugal*. Parma: European Food Safety Authority. Available online at: http://www.efsa.europa.eu/sites/default/files/zoocountryreport18it.pdf

European Food Safety Authority, European Centre for Disease Prevention and Control (2021b). The European Union One Health 2020 Zoonoses report. *EFSA J.* 19:e06971. doi: 10.2903/j.efsa.2021.6971

European Food Safety Authority, and European Centre for Disease Prevention and Control (2021a). The European Union One Health 2019 Zoonoses Report. *EFSA J* 19:e06406. doi: 10.2903/j.efsa.2021.6406

Fanoy, E., Helmhout, L. C., van der Vaart, W. L., Weijdema, K., van Santen-Verheuvel, M. G., Thijsen, S. F., et al. (2009). An outbreak of non-typeable MRSA within a residential care facility. *Euro Surveill.* 14:19080. doi: 10.2807/ese.14.01.19080-en

Fisher, E. L., Otto, M., and Cheung, G. Y. C. (2018). Basis of virulence in enterotoxin-mediated staphylococcal food poisoning. *Front. Microbiol.* 9:436. doi: 10.3389/fmicb.2018.00436

Gallo, M., Ferrara, L., Calogero, A., Montesano, D., and Naviglio, D. (2020). Relationships between food and diseases: what to know to ensure food safety. *Food Res. Int.* 137:109414. doi: 10.1016/j.foodres.2020.109414

Garcia, S. N., Osburn, B. I., and Jay-Russell, M. T. (2020). One Health for Food Safety, Food Security, and Sustainable Food Production. *Front. Sustain. Food Syst.* 4:1. doi: 10.3389/fsufs.2020.00001

Giacinti, G., Carfora, V., Caprioli, A., Sagrafoli, D., Marri, N., Giangolini, G., et al. (2017). Prevalence and characterization of methicillin-resistant Staphylococcus aureus carrying mecA or mecC and methicillin-susceptible Staphylococcus aureus in dairy sheep farms in central Italy. *J. Dairy Sci.* 100, 7857–7863. doi: 10.3168/JDS.2017-12940

Grundmann, H., Aanensen, D. M., Van Den Wijngaard, C. C., Spratt, B. G., Harmsen, D., Friedrich, A. W., et al. (2010). Geographic distribution of Staphylococcus aureus causing invasive infections in Europe: a molecular-epidemiological analysis. *PLoS Med.* 7:e1000215. doi: 10.1371/journal.pmed.1000215

Guimarães, F. F., Manzi, M. P., Joaquim, S. F., Richini-Pereira, V. B., and Langoni, H. (2017). Short communication: outbreak of methicillin-resistant Staphylococcus aureus (MRSA)-associated mastitis in a closed dairy herd. *J. Dairy Sci.* 100, 726–730. doi: 10.3168/JDS.2016-11700

Hait, J. M., Nguyen, A. T., and Tallent, S. M. (2018). Analysis of the VIDAS® Staph Enterotoxin III (SET3) for detection of staphylococcal enterotoxins G. H, and I in foods. *J. AOAC Int.* 101, 1482–1489. doi: 10.5740/jaoacint.17-0501

Hansen, J. E., Ronco, T., Stegger, M., Sieber, R. N., Fertner, M. E., Martin, H. L., et al. (2019). LA-MRSA CC398 in Dairy Cattle and Veal Calf Farms Indicates Spillover From Pig Production. *Front. Microbiol.* 10:2733. doi: 10.3389/FMICB.2019.02733/BIBTEX

Hasman, H., Moodley, A., Guardabassi, L., Stegger, M., Skov, R. L., and Aarestrup, F. M. (2010). spa type distribution in Staphylococcus aureus originating from pigs, cattle and poultry. *Vet. Microbiol.* 141, 326–331. doi: 10.1016/j.vetmic.2009.09.025

Hill, B., Smythe, B., Lindsay, D., and Shepherd, J. (2012). Microbiology of raw milk in New Zealand. *Int. J. Food Microbiol.* 157, 305–308. doi: 10.1016/j.ijfoodmicro.2012.03.031

International Organization for Standardization [ISO] (2017). *ISO 19020:2017 - Microbiology of the food chain — Horizontal method for the immunoenzymatic detection of enterotoxins in foodstuffs. 2017–06, undefined-undefined*. Available online at: https://www.mendeley.com/catalogue/6735a125-303d-3f3c-aabf-e8d701b029bb/?utm_source=desktop&utm_medium=1.19.8&utm_campaign=open_catalog&userDocumentId=%7B823ad3bb-0c0f-3d1f-a3d4-30ba3a6d0fad%7D (Accessed December 23, 2021)

International Organization for Standardization [ISO] (2021). *ISO 6888-2:2021 - Microbiology of the food chain — Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) — Part 2: Method using rabbit plasma fibrinogen agar medium*. Available online at: https://www.iso.org/standard/76673.html (Accessed December 23, 2021)

Jakobsen, R. A., Heggebø, R., Sunde, E. B., and Skjervheim, M. (2011). Staphylococcus aureus and Listeria monocytogenes in Norwegian raw milk cheese production. *Food Microbiol.* 28, 492–496. doi: 10.1016/j.fm.2010.10.017

Johler, S., Layer, F., and Stephan, R. (2011). Comparison of virulence and antibiotic resistance genes of food poisoning outbreak isolates of Staphylococcus aureus with isolates obtained from bovine mastitis milk and pig carcasses. *J. Food Prot.* 74, 1852–1859. doi: 10.4315/0362-028X.JFP-11-192

Johler, S., Macori, G., Bellio, A., Acutis, P. L., Gallina, S., and Decastelli, L. (2018). Short communication: characterization of Staphylococcus aureus isolated along the raw milk cheese production process in artisan dairies in Italy. *J. Dairy Sci.* 101, 2915–2920. doi: 10.3168/jds.2017-13815

Johnson, W. M., Tyler, S. D., Ewan, E. P., Ashton, F. E., Pollard, D. R., and Rozee, K. R. (1991). Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in Staphylococcus aureus by the polymerase chain reaction. *J. Clin. Microbiol.* 29, 426–430. doi: 10.1128/jcm.29.3.426-430.1991

Jørgensen, H. J., Mathisen, T., Løvseth, A., Omoe, K., Qvale, K. S., and Loncarevic, S. (2005a). An outbreak of staphylococcal food poisoning caused by enterotoxin H in mashed potato made with raw milk. *FEMS Microbiol. Lett.* 252, 267–272. doi: 10.1016/j.femsle.2005.09.005

Jørgensen, H. J., Mork, T., Høgåsen, H. R., and Rørvik, L. M. (2005b). Enterotoxigenic Staphylococcus aureus in bulk milk in Norway. *J. Appl. Microbiol.* 99, 158–166. doi: 10.1111/j.1365-2672.2005.02569.x

Kadariya, J., Smith, T. C., and Thapaliya, D. (2014). Staphylococcus aureus and Staphylococcal Food-Borne Disease: an Ongoing Challenge in Public Health. *Biomed Res. Int.* 2014:827965. doi: 10.1155/2014/827965

Kou, X., Cai, H., Huang, S., Ni, Y., Luo, B., Qian, H., et al. (2021). Prevalence and Characteristics of Staphylococcus aureus Isolated From Retail Raw Milk in Northern Xinjiang, China. *Front. Microbiol.* 12:2187. doi: 10.3389/fmicb.2021.705947

Lienen, T., Schnitt, A., Hammerl, J. A., Maurischat, S., and Tenhagen, B. A. (2021). Genomic Distinctions of LA-MRSA ST398 on Dairy Farms From Different German Federal States With a Low Risk of Severe Human Infections. *Front. Microbiol.* 11:3390. doi: 10.3389/FMICB.2020.575321/BIBTEX

Liu, H., Li, S., Meng, L., Dong, L., Zhao, S., Lan, X., et al. (2017). Prevalence, antimicrobial susceptibility, and molecular characterization of Staphylococcus aureus isolated from dairy herds in northern China. *J. Dairy Sci.* 100, 8796–8803. doi: 10.3168/jds.2017-13370

Malachowa, N., and Deleo, F. R. (2010). Mobile genetic elements of Staphylococcus aureus. *Cell. Mol. Life Sci.* 67, 3057–3071. doi: 10.1007/s00018-010-0389-4

Naik, S., Smith, F., Ho, J., Croft, N. M., Domizio, P., Price, E., et al. (2008). Staphylococcal Enterotoxins G and I, a Cause of Severe but Reversible Neonatal Enteropathy. *Clin. Gastroenterol. Hepatol.* 6, 251–254. doi: 10.1016/j.cgh.2007.09.004

Owusu-Kwarteng, J., Akabanda, F., Agyei, D., and Jespersen, L. (2020). Microbial safety of milk production and fermented dairy products in africa. *Microorganisms* 8:752. doi: 10.3390/microorganisms8050752

Park, J. Y., Fox, L. K., Seo, K. S., McGuire, M. A., Park, Y. H., Rurangirwa, F. R., et al. (2011). Detection of classical and newly described staphylococcal superantigen genes in coagulase-negative staphylococci isolated from bovine intramammary infections. *Vet. Microbiol.* 147, 149–154. doi: 10.1016/j.vetmic.2010.06.021

Pereira, V., Lopes, C., Castro, A., Silva, J., Gibbs, P., and Teixeira, P. (2009). Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of Staphylococcus aureus isolates from various foods in Portugal. *Food Microbiol.* 26, 278–282. doi: 10.1016/j.fm.2008.12.008

Pérez-Rodríguez, F., and Mercanoglu Taban, B. (2019). A State-of-Art Review on Multi-Drug Resistant Pathogens in Foods of Animal Origin: risk Factors and Mitigation Strategies. *Front. Microbiol.* 10:2091. doi: 10.3389/fmicb.2019.02091

Podkowik, M., Park, J. Y., Seo, K. S., Bystroń, J., and Bania, J. (2013). Enterotoxigenic potential of coagulase-negative staphylococci. *Int. J. Food Microbiol.* 163, 34–40. doi: 10.1016/j.ijfoodmicro.2013.02.005

Rola, J. G., Czubkowska, A., Korpysa-Dzirba, W., and Osek, J. (2016). Occurrence of Staphylococcus aureus on farms with small scale production of raw milk cheeses in poland. *Toxins* 8:62. doi: 10.3390/toxins8030062

Rola, J. G., Korpysa-Dzirba, W., Czubkowska, A., and Osek, J. (2015). Prevalence of enterotoxin genes and antimicrobial resistance of coagulase-positive staphylococci recovered from raw cow milk. *J. Dairy Sci.* 98, 4273–4278. doi: 10.3168/jds.2014-9064

Ronco, T., Klaas, I. C., Stegger, M., Svennesen, L., Astrup, L. B., Farre, M., et al. (2018). Genomic investigation of Staphylococcus aureus isolates from bulk tank milk and dairy cows with clinical mastitis. *Vet. Microbiol.* 215, 35–42. doi: 10.1016/j.vetmic.2018.01.003

Roussel, S., Felix, B., Vingadassalon, N., Grout, J., Hennekinne, J. A., Guillier, L., et al. (2015). Staphylococcus aureus strains associated with food poisoning outbreaks in France: comparison of different molecular typing methods, including MLVA. *Front. Microbiol.* 6:882. doi: 10.3389/fmicb.2015.00882

Sabat, A. J., Budimir, A., Nashev, D., Sá-Leão, R., van Dijl, J. M., Laurent, F., et al. (2013). Overview of molecular typing methods for outbreak detection and epidemiological surveillance. *Euro Surveill.* 18:20380. doi: 10.2807/ese.18.04.20380-en

Salgueiro, V., Manageiro, V., Bandarra, N. M., Ferreira, E., Clemente, L., and Caniça, M. (2020). Genetic relatedness and diversity of staphylococcus aureus from different reservoirs: humans and animals of livestock, poultry, zoo, and aquaculture. *Microorganisms* 8:1345. doi: 10.3390/microorganisms8091345

Schnitt, A., Lienen, T., Wichmann-Schauer, H., Cuny, C., and Tenhagen, B. A. (2020). The occurrence and distribution of livestock-associated methicillin-resistant Staphylococcus aureus ST398 on German dairy farms. *J. Dairy Sci.* 103, 11806–11819. doi: 10.3168/jds.2020-18958

Schultz, A. M., Mcmahon, W. A., Aleo, V. A., and Johnson, R. L. (2004). *VIDAS Staphylococcal Enterotoxin II (SET2) Pre-collaborative Study Report: AOAC Performance Tested Method. AOAC interational Anu. Meet., 2004.* Available online at: http://www.biomerieux-usa.com/sites/subsidiary_us/files/doc/staph_poster-1.pdf (Accessed February 15, 2022)

Sharma, C., Rokana, N., Chandra, M., Singh, B. P., Gulhane, R. D., Gill, J. P. S., et al. (2018). Antimicrobial resistance: its surveillance, impact, and alternative management strategies in dairy animals. *Front. Vet. Sci.* 4:237. doi: 10.3389/fvets.2017.00237

Shopsin, B., Gomez, M., Montgomery, S. O., Smith, D. H., Waddington, M., Dodge, D. E., et al. (1999). Evaluation of protein A gene polymorphic region DNA sequencing for typing of Staphylococcus aureus strains. *J. Clin. Microbiol.* 37, 3556–3563. doi: 10.1128/jcm.37.11.3556-3563.1999

Smith, E. M., Green, L. E., Medley, G. F., Bird, H. E., Fox, L. K., Schukken, Y. H., et al. (2005). Multilocus sequence typing of intercontinental bovine Staphylococcus aureus isolates. *J. Clin. Microbiol.* 43, 4737–4743. doi: 10.1128/JCM.43.9.4737-4743.2005

Stegger, M., Andersen, P. S., Kearns, A., Pichon, B., Holmes, M. A., Edwards, G., et al. (2012). Rapid detection, differentiation and typing of methicillin-resistant Staphylococcus aureus harbouring either mecA or the new mecA homologue mecALGA251. *Clin. Microbiol. Infect.* 18, 395–400. doi: 10.1111/j.1469-0691.2011.03715.x

Sudhanthiramani, S., Swetha, C. S., and Bharathy, S. (2015). Prevalence of antibiotic resistant Staphylococcus aureus from raw milk samples collected from the local vendors in the region of Tirupathi, India. *Vet. World* 8, 478–481. doi: 10.14202/vetworld.2015.478-481

Tang, J., Tang, C., Chen, J., Du, Y., Yang, X. N., Wang, C., et al. (2011). Phenotypic characterization and prevalence of enterotoxin genes in Staphylococcus aureus isolates from outbreaks of illness in Chengdu city. *Foodborne Pathog. Dis.* 8, 1317–1320. doi: 10.1089/fpd.2011.0924

Taponen, S., Simojoki, H., Haveri, M., Larsen, H. D., and Pyörälä, S. (2006). Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. *Vet. Microbiol.* 115, 199–207. doi: 10.1016/j.vetmic.2006.02.001

Tegegne, H. A., Koláčková, I., Florianová, M., Gelbíčová, T., Wattiau, P., Boland, C., et al. (2019). Genome Sequences of Livestock-Associated Methicillin-Resistant Staphylococcus aureus spa Type t899 Strains Belonging to Three Different Sequence Types (ST398, ST9, and ST4034). *Microbiol. Resour. Announc.* 8, e01351–18. doi: 10.1128/mra.01351-18

Tenhagen, B. A., Alt, K., Pfefferkorn, B., Wiehle, L., Käsbohrer, A., and Fetsch, A. (2018). Short communication: methicillin-resistant Staphylococcus aureus in conventional and organic dairy herds in Germany. *J. Dairy Sci.* 101, 3380–3386. doi: 10.3168/jds.2017-12939

Tenhagen, B. A., Vossenkuhl, B., Käsbohrer, A., Alt, K., Kraushaar, B., Guerra, B., et al. (2014). Methicillin-resistant Staphylococcus aureus in cattle food chains - Prevalence, diversity, and antimicrobial resistance in Germany. *J. Anim. Sci.* 92, 2741–2751. doi: 10.2527/jas.2014-7665

Titouche, Y., Hakem, A., Houali, K., Meheut, T., Vingadassalon, N., Ruiz-Ripa, L., et al. (2019). Emergence of methicillin-resistant Staphylococcus aureus (MRSA) ST8 in raw milk and traditional dairy products in the Tizi Ouzou area of Algeria. *J. Dairy Sci.* 102, 6876–6884. doi: 10.3168/jds.2018-16208

Ünal, N., and Çinar, O. D. (2012). Detection of stapylococcal enterotoxin, methicillin-resistant and Panton-Valentine leukocidin genes in coagulase-negative staphylococci isolated from cows and ewes with subclinical mastitis. *Trop. Anim. Health Prod.* 44, 369–375. doi: 10.1007/s11250-011-0032-x

van Duijkeren, E., Hengeveld, P. D., Albers, M., Pluister, G., Jacobs, P., Heres, L., et al. (2014). Prevalence of methicillin-resistant Staphylococcus aureus carrying mecA or mecC in dairy cattle. *Vet. Microbiol.* 171, 364–367. doi: 10.1016/j.vetmic.2013.12.024

Wang, W., Lin, X., Jiang, T., Peng, Z., Xu, J., Yi, L., et al. (2018). Prevalence and characterization of Staphylococcus aureus cultured from raw milk taken from dairy cows with mastitis in Beijing, China. *Front. Microbiol.* 9:1123. doi: 10.3389/fmicb.2018.01123

Wu, S., Huang, J., Wu, Q., Zhang, F., Zhang, J., Lei, T., et al. (2018). Prevalence and characterization of Staphylococcus aureus isolated from retail vegetables in China. *Front. Microbiol.* 9:1263. doi: 10.3389/FMICB.2018.01263/BIBTEX

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 01 April 2022
doi: 10.3389/fmicb.2022.816880

Phylogenetic and Evolutionary Genomic Analysis of *Listeria monocytogenes* Clinical Strains in the Framework of Foodborne Listeriosis Risk Assessment

*Antonietta Gattuso[1], Eleonora Cella[2], Silvia Fillo[3], Marco Francesco Ortoffi[1], Silvia Angeletti[2], Massimo Ciccozzi[2], Dario De Medici[1], Florigio Lista[3] and Alfonsina Fiore[1]**

[1] Dipartimento di Sicurezza Alimentare, Nutrizione e Sanità Pubblica Veterinaria, Istituto Superiore di Sanità, Rome, Italy, [2] Unità di Statistica Medica ed Epidemiologia Molecolare, Università Campus Bio-Medico di Roma, Rome, Italy, [3] Scientific Department, Army Medical Center, Rome, Italy

OPEN ACCESS

Edited by:
Paula Teixeira,
Catholic University of Portugal, Portugal

Reviewed by:
Dara Leong,
Teagasc Food Research Centre, Teagasc, Ireland
Sarah Hwa In Lee,
University of São Paulo, Brazil
David A. Montero,
University of Chile, Chile

***Correspondence:**
Alfonsina Fiore
alfonsina.fiore@iss.it

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 17 November 2021
Accepted: 01 February 2022
Published: 01 April 2022

Citation:
Gattuso A, Cella E, Fillo S, Ortoffi MF, Angeletti S, Ciccozzi M, De Medici D, Lista F and Fiore A (2022) Phylogenetic and Evolutionary Genomic Analysis of Listeria monocytogenes Clinical Strains in the Framework of Foodborne Listeriosis Risk Assessment. Front. Microbiol. 13:816880. doi: 10.3389/fmicb.2022.816880

Listeria monocytogenes is one of the most important foodborne pathogens responsible for listeriosis, a severe disease with symptoms ranging from septicemia, meningoencephalitis, and abortion. Given the strong impact of listeriosis on human health and the difficulty of controlling *L. monocytogenes* along the food production chain, listeriosis has become a priority subjected to molecular surveillance in European Union/European Economic Area since 2007. From 2018, surveillance is based on whole-genome sequence using the core genome multilocus sequence type. The complete sequences of 132 clinical strains were used to define the evolutionary relatedness among subtypes of *L. monocytogenes* isolated in Italy from 2010 to 2016, allowing the identification of clades and/or clusters associated with outbreaks or sporadic cases of listeriosis. All the strains analyzed are clustered in lineages I and II, and the majority of the strains were classified as lineage II. A probable epidemic entrance in different years for every clade and cluster from each different region was defined. The persistence of the same specific clonal complexes of *L. monocytogenes* has been found over long periods; this may be related to the fact that some strains are able to survive better than others in a food production environment. Phylogenic studies, using whole-genome sequence data, are able to identify the emergence of highly persistent pathogenic variants, contributing to improving the hazard characterization of *L. monocytogenes*.

Keywords: *Listeria monocytogenes*, listeriosis, whole-genome sequencing (WGS), clonal complexes (CCs), sequence types (STs), phylogenetic analysis, lineage, hazard characterization

INTRODUCTION

Listeria monocytogenes, one of the most important foodborne pathogens, is the causative agent of invasive listeriosis that typically presents as sepsis or meningoencephalitis in the elderly (>65 years) and in people with chronic illnesses and undergoing immunosuppression (de Noordhout et al., 2014). Infections during pregnancy can cause fever and other non-specific symptoms in the mother with severe outcomes such as fetal loss, premature labor, neonatal illness, and death

(Lamont et al., 2011). Listeriosis can have a relatively long incubation period, many cases are considered sporadic, and detected outbreaks usually involve a small number of patients, so most reported cases of listeriosis are difficult to link to a specific food product or food business operator (Van Walle et al., 2018). *L. monocytogenes* is able to form biofilms to grow at refrigeration temperature, tolerate high salt and nitrite concentrations, and resist disinfectants (Lundén et al., 2003). These properties contribute to its ability to persist and multiply in the food-processing environment and make it difficult to control. *L. monocytogenes* demonstrated a high genome-level of diversity, forming four evolutionary lineages (I–IV; Ragon et al., 2008); the majority of human illnesses were caused by strains belonging to lineages II and I. Given the strong impact of listeriosis on human health and the difficulty of controlling the pathogen along the food production chain, in 2007, the European Center for Diseases Prevention and Control (ECDC) identified within the food- and waterborne diseases listeriosis as a priority to be subjected to enhanced surveillance. In 2008, both sporadic and outbreak-associated cases of infection started to be collected and disseminated through The European Surveillance System. In 2012, ECDC implemented The European Surveillance System with Molecular Surveillance System to routinely collect pulsed-field gel electrophoresis (PFGE) molecular-typing data of *L. monocytogenes* and other foodborne pathogen strains isolated from humans (Acciari et al., 2016). Since 2018 within the European Union/European Economic Area and in Italy in 2019, whole-genome sequencing (WGS) has replaced PFGE for typing and cluster analyses of *L. monocytogenes* cases of infection, using the core genome multilocus sequence type scheme established by Moura et al. (2016). ECDC aimed to evaluate the effectiveness of WGS in a routine epidemiological surveillance system that promoted a large-scale, retrospective, multicenter study on *L. monocytogenes* strains isolated in human case sequences from European Union/European Economic Area countries from 2010 to 2016. Italy participated in the study, with sequences of 132 *L. monocytogenes* clinical strains selected by geographic distribution and PFGE profiles (Van Walle et al., 2018). In the present study, the complete sequences of 132 clinical strains were used to define the evolutionary relatedness among subtypes of *L. monocytogenes* with the aim of (a) identifying clades or clusters more often associated with outbreaks or sporadic cases (Jackson et al., 2016; Moura et al., 2016) and (b) investigating on the persistence of particular strains isolated in different Italian regions from 2010 to 2016.

MATERIALS AND METHODS

Bacterial Strains

During the period 2010–2016, the Istituto Superiore di Sanità collected 826 *L. monocytogenes* clinical strains from patients with invasive listeriosis from different Italian regions, including from the north (82.2%), center (16%), and south (1.8%) of the country. For this study, 132 *L. monocytogenes* strains that were selected both on the geographical distribution and serological and PFGE profiles (Van Walle et al., 2018) were subjected to WGS. Sequencing data were deposited in European Nucleotide Archive (ENA) at http://www.ebi.ac.uk/ena/browser/view/PRJEB45702.

DNA Extraction and Whole-Genome Sequencing

DNA was extracted and purified from overnight bacterial cultures by MasterPure^{TM} Gram Positive DNA purification kit (Lucigen, Epicentre) according to the manufacturer's instructions. DNA concentration was evaluated by Quantus fluorimeter (Promega, United States), and 2 ng of total DNA was used for library preparation, using Nextera XT DNA kit and sequenced on NextSeq 500 (111 isolates) and MiSeq (20 isolates) (Illumina, Inc., San Diego, CA, United States). High Output Kit v2 with paired-end 150-nt reads (300 cycles) was used for NextSeq 500 and v3 Reagent kit (600 cycles) paired-end for MiSeq following manufacturer's instructions. Raw reads were mapped to a reference genome, *L. monocytogenes* FSL F2-208 (accession number CM001046), using the Bowtie2 v.2.3.5 followed by the samtools-bcftools-vcfutils pipeline[1] to extract the variants [single-nucleotide polymorphisms (SNPs)]. Missing data and ambiguous bases were not allowed at any position, and they were removed by filtering. A final alignment of 175,774 SNPs was created.

Multilocus Sequence Typing

To determine the level of genetic diversity between isolates, sequence types (STs) were determined by MLST using seven housekeeping genes, including ABC transporter (abcZ), beta-glucosidase (bglA), catalase (cat), succinyl-diaminopimelate desuccinylase (dapE), D-amino acid aminotransferase (dat), L-lactate dehydrogenase (ldh), and histidine kinase (IhkA). The contig files, *de novo* assembly, for each of the draft genomes were uploaded to the Center for Genomic Epidemiology MLST 1.8 with *L. monocytogenes* as the MLST scheme (Larsen et al., 2012). The clonal complexes (CCs) and the STs were defined based on the MLST profile of the isolate having matching profiles at six of seven genes (Ragon et al., 2008; Moura et al., 2016).

Phylogenetic and Evolutionary Analyses

To perform phylogenetic and evolutionary analysis, six datasets were created.

The first dataset included 33 *L. monocytogenes* strains, classified as lineage I with MLST analysis. The second dataset included 99 *L. monocytogenes* strains, classified as lineage II with MLST analysis. These two datasets were used to perform maximum likelihood (ML) trees.

The nucleotide substitution model was chosen according to the Bayesian information criterion for all the datasets. Statistical support for internal branches of the ML tree was evaluated by bootstrapping (1,000 replicates). ML analysis was performed with IQTREE v.1.6.11 (Trifinopoulos et al., 2016) on the first and second datasets.

[1]https://github.com/samtools

The other four datasets were built to perform evolutionary analysis, Bayesian dated-tree, and phylogeographic tree. These datasets included: *L. monocytogenes* strains classified as CC1 (third dataset), *L. monocytogenes* strains classified as CC7 (fourth dataset), *L. monocytogenes* strains classified as CC101 (fifth dataset), and *L. monocytogenes* strains classified as CC155 (sixth dataset). Phylogenetic signal was assessed by likelihood mapping using Tree Puzzle (Schmidt et al., 2002). Analysis of the temporal signal and "clock likeness" of molecular phylogenies on the datasets was performed using TempEst v.1.5.3 (Rambau et al., 2016). This analysis was performed to evaluate the robustness in the molecular clock of the third, fourth, fifth, and sixth datasets. Bayesian Markov chain Monte Carlo (MCMC) method, implemented in BEAST v. 1.10.4[2] (Suchard et al., 2018), was used to estimate the demographic history of *L. monocytogenes* by calibrating a molecular clock. To investigate the demographic history, independent MCMC runs were carried out enforcing both a strict and relaxed clock with an uncorrelated lognormal rate distribution and one of the following coalescent priors: constant population size, exponential growth, non-parametric smooth skyride plot Gaussian Markov random field, and non-parametric Bayesian skyline plot with ascertainment bias correction. Marginal likelihood estimates for each demographic model were obtained using path sampling and steppingstone analyses (Drummond et al., 2005). Uncertainty in the estimates was indicated by 95% highest posterior density (95% HPD) intervals, and the best-fitting model for each dataset was by calculating the Bayes factors (BF) (Suchard et al., 2018). In practice, any two models can be compared to evaluate the strength of evidence against the null hypothesis (H0), defined as the one with the lower marginal likelihood: 2lnBF < 2 indicates no evidence against H0; 2–6, weak evidence; 6–10: strong evidence; and >10 very strong evidence. Chains were conducted for at least 100×10^6 generations and sampled every 10,000 steps for each molecular clock model. The convergence of the MCMC was assessed by calculating the effective sample size for each parameter. Only parameter estimates with effective sample size's of >250 were accepted. The maximum clade credibility (MCC) tree was obtained from the tree's posterior distributions after a 10% burn-in, with the Tree-Annotator software v 1.10.4, included in the Beast package. Statistical support for specific monophyletic clades was assessed by calculating the posterior probability ($pp > 0.90$). The continuous-time Markov chain process over discrete sampling locations implemented in BEAST (Suchard et al., 2018) was used for the phylogeography inference using the Bayesian stochastic search variable selection model, which allows the diffusion rates to be zero with a positive prior probability. Locations considered were the different Italian regions. Comparison of the posterior and prior probabilities of the individual rates being zero provided a formal BF for testing the significance of the linkage between locations. The MCC tree with the phylogeographic reconstruction was selected from the posterior tree distribution after a 10% burn-in using the Tree Annotator.

[2]http://beast.bio.ed.ac.uk

TABLE 1 | Distribution of clonal complexes (CCs) and relative sequence types (STs) of *L. monocytogenes* sequences based on MLST.

CCs	*N*	%	STs	*N*	%
CC1	20	15	ST1	19	15
			ST595	1	1
CC2	2	2	ST2	2	2
CC3	1	1	ST3	1	1
CC4	5	4	ST4	4	3
			ST219	1	1
CC5	3	2	ST5	3	2
CC6	2	2	ST6	2	2
CC7	35	27	ST7	32	24
			ST24	1	1
			ST40	1	1
			ST511	1	1
CC8	8	6	ST8	7	5
			ST120	1	1
CC9	2	2	ST9	2	2
CC14	2	2	ST14	2	2
CC26	1	1	ST26	1	1
CC29	7	5	ST29	7	5
CC31	2	2	ST325	2	2
CC37	3	2	ST37	3	2
CC89	2	2	ST391	2	2
CC101	18	14	ST38	17	13
			ST101	1	1
CC121	2	2	ST121	2	2
CC155	14	11	ST155	14	11
CC398	2	2	ST398	2	2
CC451	1	1	ST451	1	1
Total	132	100	Total	132	100

RESULTS

Table 1 reports the distribution of CCs and the relative STs of the *L. monocytogenes* sequence strains based on MLST. The most frequent CCs were CC7 (35 isolates), CC1 (20 isolates), CC101 (18 isolates), and CC155 (14 isolates). Specifically, it was possible to show that ST7 was prevalent (32 isolates) in the CC7, ST1 (19 isolates) in CC1, ST38 (17 isolates) in CC 101, and all the sequence strains included in CC155 that belonged to ST 155 (14 isolates). Results proved that CC1, CC7, CC101, and CC155 persist over time in Italy, and some of these have spread to more than one region. Particularly, the CC1 seemed to have an epidemic entrance in 2004 in Emilia-Romagna, moving to Lombardia, Marche, and Piemonte in 2006. CC7 is the oldest CC in Italy, with a presumable entrance in 1999 in Lombardia, moving to Emilia-Romagna in 2010. The CC101 appears to have the entrance in Lombardia in 2008 and does not suggest having evident circulation outside the region, although strains that belonged to CC101 were also isolated in Piemonte in 2013 and in Trentino-Alto Adige in 2015.

Phylogenetic Analysis

Likelihood mapping analysis indicated star-like signal (phylogenetic noise) under 6.8% for all datasets; this signified that enough signal for phylogenetic inference was present. The

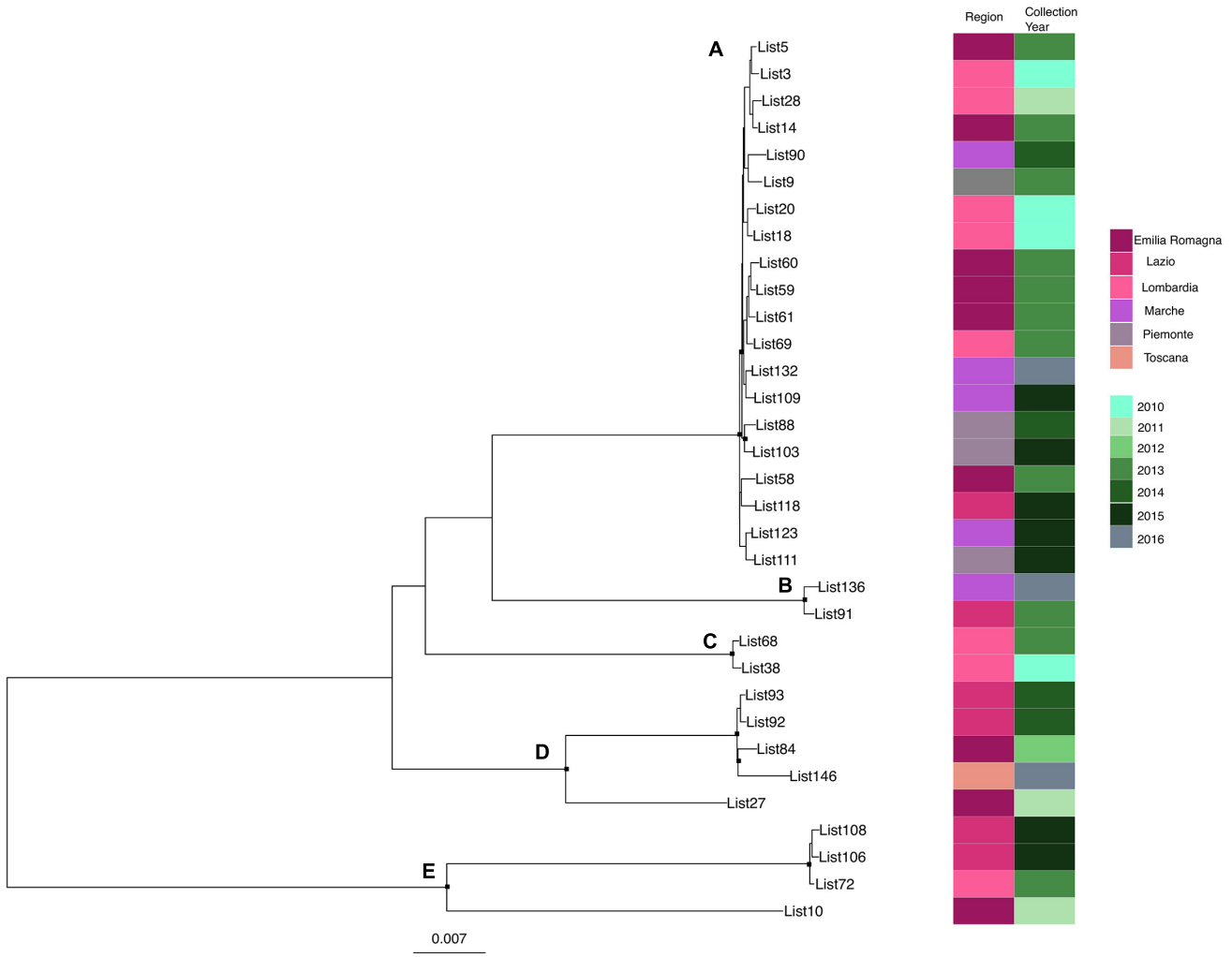


FIGURE 1 | Maximum likelihood of *L. monocytogenes* lineage I strain SNP alignment (first dataset). Significant statistical support (bootstrap values > 0.70%) as indicated by a black rectangle for clade subtending that branch. Clusters are highlighted. Scale bar at bottom of tree indicates 0.007 nucleotide substitutions per site. Heatmap on left showed Italian region membership and collection year for each strain. **(A,D,E)** Clades. **(B,C)** Mean clusters.

evolutionary model of substitution chosen according to the Bayesian information criterion was GTR + F + I for both the first and second datasets and HKY + G + I for the remaining datasets. **Figure 1** shows the ML of the *L. monocytogenes* lineage I strain SNP alignment (first dataset). There have been highlighted three clades (A–D–E) and two clusters (B–C) that were statistically supported (bootstrap values > 0.7). Clade A included the CC1 *L. monocytogenes* intermingled strains isolated from 2010 to 2015, forming different clusters (Lombardia, Emilia-Romagna, Marche, Piemonte, and Lazio); clade D included CC4 *L. monocytogenes* strains isolated from 2011 to 2016 forming different clusters (Emilia-Romagna, Toscana, and Lazio); clade E included different clusters: one *L. monocytogenes* strain belonged to ST3 isolated in 2011 in Emilia-Romagna, and three *L. monocytogenes* strains ST5 isolated in Lazio and Lombardia in 2013 and 2015. Cluster B included the two ST2 *L. monocytogenes* strains isolated in Marche and Lazio in 2013 and 2016; cluster C included two *L. monocytogenes* strains that belonged to ST6 isolated in Lombardia in 2010 and 2013. **Figure 2** shows the ML of the *L. monocytogenes* lineage II strain SNP alignment (second dataset). There were five clades (A–B–D–E–H) and three clusters (C–F–G), all statistically supported (bootstrap value > 0.7). With regard to clades, clade A included the *L. monocytogenes* intermingled strains that were classified as CC7 forming different clusters. The main clusters included strains that were mainly isolated in Marche (2015–2016) and in Umbria (2015), Emilia-Romagna, and Lombardia; clade B included *L. monocytogenes* intermingled strains forming different clusters that belonged to CC155 (Trentino, Lombardia, Campania, and Emilia-Romagna); clade D included eight *L. monocytogenes* intermingled strains that form different clusters that belonged to CC8 and two ST9 strains; clade E included three ST37 *L. monocytogenes* strains and seven *L. monocytogenes* strains, classified as ST29, and outside of this cluster, there was as an *L. monocytogenes* strain that belonged

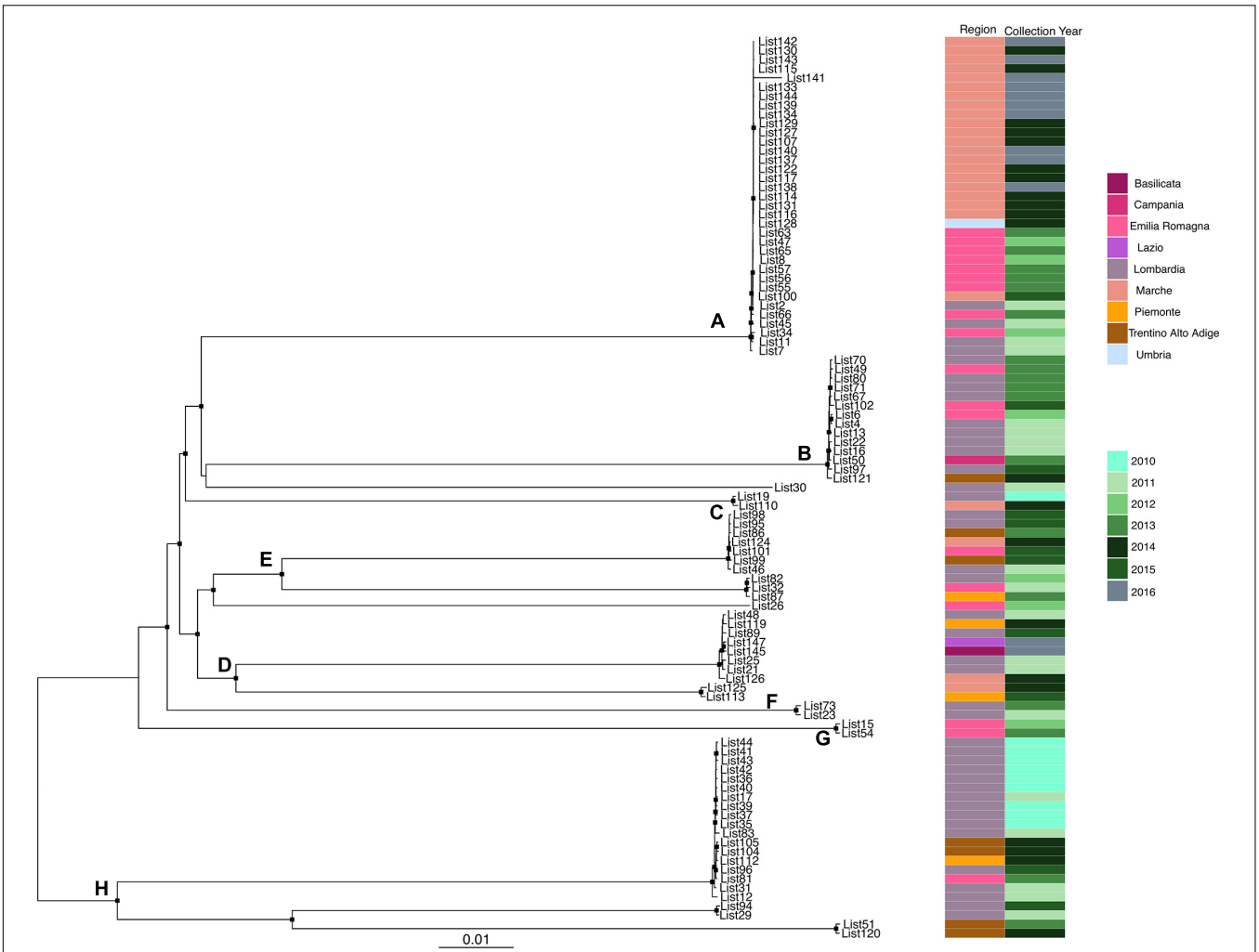


FIGURE 2 | Maximum likelihood of *L. monocytogenes* lineage II strain SNP alignment (second dataset). Significant statistical support (bootstrap values > 0.70%) as indicated by a black rectangle for clade subtending that branch. Clusters are highlighted. Scale bar at bottom of tree indicates 0.01 nucleotide substitutions per site. Heatmap on left showed Italian region membership and collection year for each strain. **(A,B,D,E,H)** Mean clades. **(C,F,G)** Mean clusters.

to ST26; clade H included two clusters, the ST121 and ST14 groups, with sequences from the same regions each (Lombardia and Trentino) and the CC101 *L. monocytogenes* intermingled strain group (Lombardia, Trentino, Piemonte, and Emilia-Romagna). With regard to clusters, cluster C included two ST398 *L. monocytogenes* strains that were isolated in Lombardia and Marche in 2010 and 2015, respectively; clusters F and G included two *L. monocytogenes* strains each, CC31 and CC89, respectively.

Bayesian Phylogenetic Analysis

Analysis of the temporal signal and "clock-likeness" of molecular phylogenies was performed on the third to the sixth dataset. A sufficient correlation between the genetic distance of each sequence to the root of *L. monocytogenes* strain SNP phylogeny and the date of sequence sampling for the datasets analyzed ($r > 0.54$) was found. The exponential growth model with a relaxed clock was selected as the most appropriate to describe the evolutionary history of *L. monocytogenes* strain SNPs' CC1 and CC155 alignment (lnBF > 6), whereas the Bayesian skyline plot as a demographic model with a relaxed molecular clock was the most appropriate to describe the evolutionary history of *L. monocytogenes* strain SNPs' CC7 and CC101 alignment (lnBF > 5). MCC tree with a phylogeographic reconstruction of *L. monocytogenes* strain CC1 SNP alignment is shown in **Figure 3**. The date of the time of the most common recent ancestor (tMRCA) of the root corresponded to 2004 (HPD 95% 1943–2006), probably originated in Emilia-Romagna. Four different statistically supported clusters have been identified (A–B–C–D): cluster A dated back to 2006, probably in Emilia-Romagna, including *L. monocytogenes* strains from Lombardia and Emilia-Romagna from 2010 to 2013; cluster B including two strains from Lombardia in 2010 and it originated in 2007 in Lombardia; cluster C was composed by two subclusters: one including Marche strains from 2015 and 2016 and the other subcluster including 2013 strains from Lombardia and Emilia-Romagna. This cluster dated back to 2006, probably originated

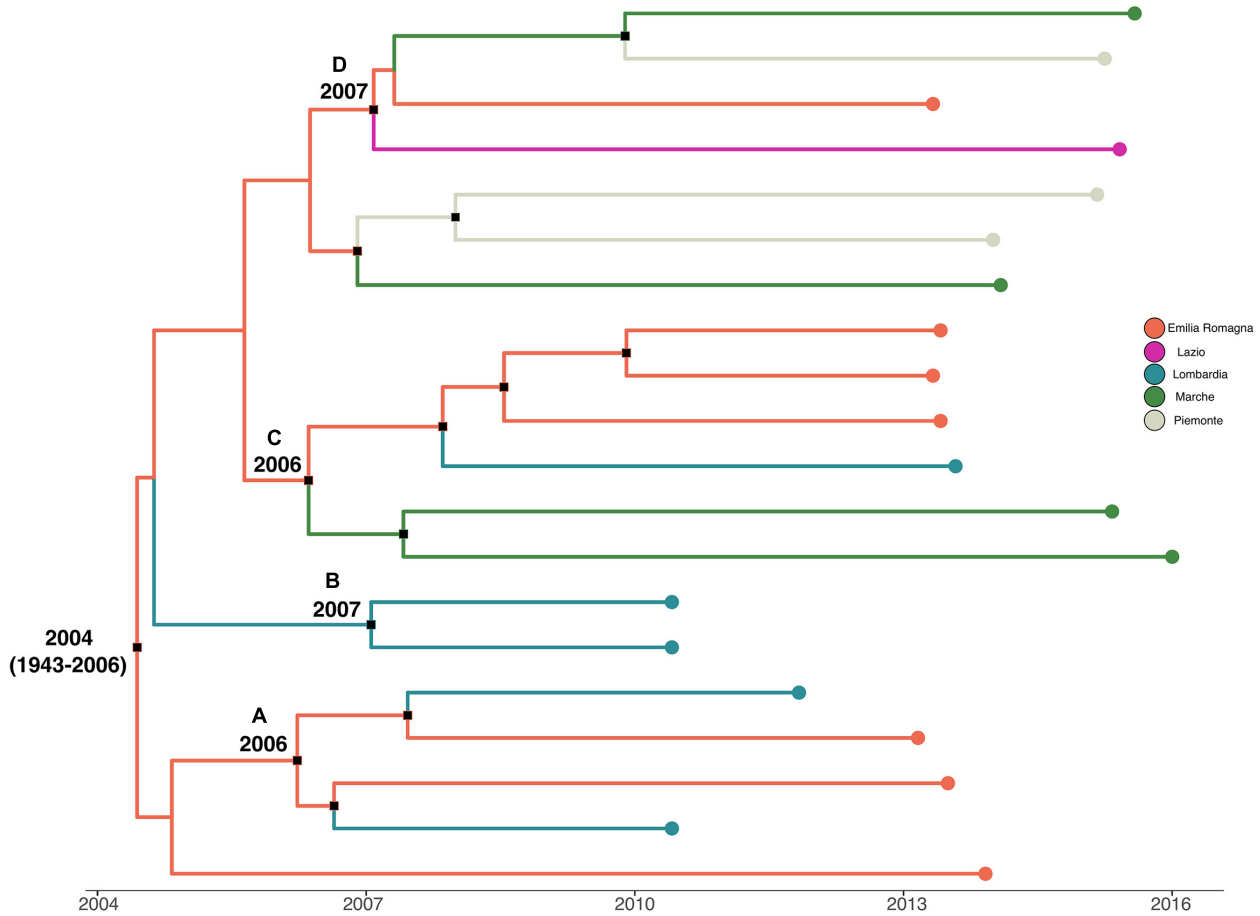


FIGURE 3 | Maximum clade credibility (MCC) tree with Bayesian phylogeographic reconstruction *L. monocytogenes* strain CC1 SNP alignment. Branches are scaled in time and colored according to legend to left, where each color represents geographic location of sampled sequence (tip branches), as well as of ancestral lineage (internal branches) inferred by Bayesian phylogeography. Significant posterior probability support ($pp \geq 0.9$) as indicated by a black rectangle. Clusters are highlighted.

in Emilia-Romagna; cluster D dated back to 2007 in Emilia-Romagna and included strains from Emilia-Romagna in 2013, Piemonte, Lazio, and Marche in 2015.

Maximum clade credibility tree with a phylogeographic reconstruction of *L. monocytogenes* strain CC7 SNP alignment is shown in **Figure 4**. The date of tMRCA of the root corresponded to 1999 (HPD 95% 1987–2007), probably originated in Lombardia. Two different statistically supported clades (A, B) have been identified: clade A dated back to May 2005, probably originated from Lombardia, which included two strains isolated in 2011 and 2012 from Lombardia and Emilia-Romagna, and clade B dated back to July 2005, probably in Lombardia. Clade B included three clusters statistically supported: cluster B1 included Emilia-Romagna outbreak in 2013, dated back to December 2012; cluster B2 included strains from Marche and Umbria in 2015–2016, dated back to February 2014; and lastly, cluster B3 included strains from Marche isolated in 2015 and 2016, probably originated in January 2015.

A phylodynamic reconstruction of *L. monocytogenes* strain CC101 SNP alignment is shown in **Figure 5**. The date of tMRCA of the root is December 2008 (HPD 95% 2007–2009), probably originated in Lombardia. Five statistically supported clusters have been found (A–B–C–D–E), all probably originated in Lombardia: cluster A included strains that originated in May 2009; clusters B, C, and D included strains that originated in December 2009 and October 2009 (for cluster D), and cluster E dated back to February 2010. The latter cluster included strains from Lombardia, Emilia-Romagna, Piemonte, and Trentino that were isolated in different years (2011, 2013, 2014, and 2015).

Figure 6 shows the MCC tree with a phylogeographic reconstruction of *L. monocytogenes* strain CC155 SNP alignment. Three different statistically supported clades have been highlighted (A–B–C). The date of tMRCA of the root is 2002 (HPD 95% 1987–2011), probably originated in Lombardia. Specifically, clade A included strains from Lombardia that were isolated in 2013, probably originated in Lombardia in 2007; clade B included two strains isolated in Lombardia and Emilia-Romagna in 2011 and 2012, respectively, and probably originated in Lombardia in 2006, and clade C included six strains that were isolated from Lombardia,

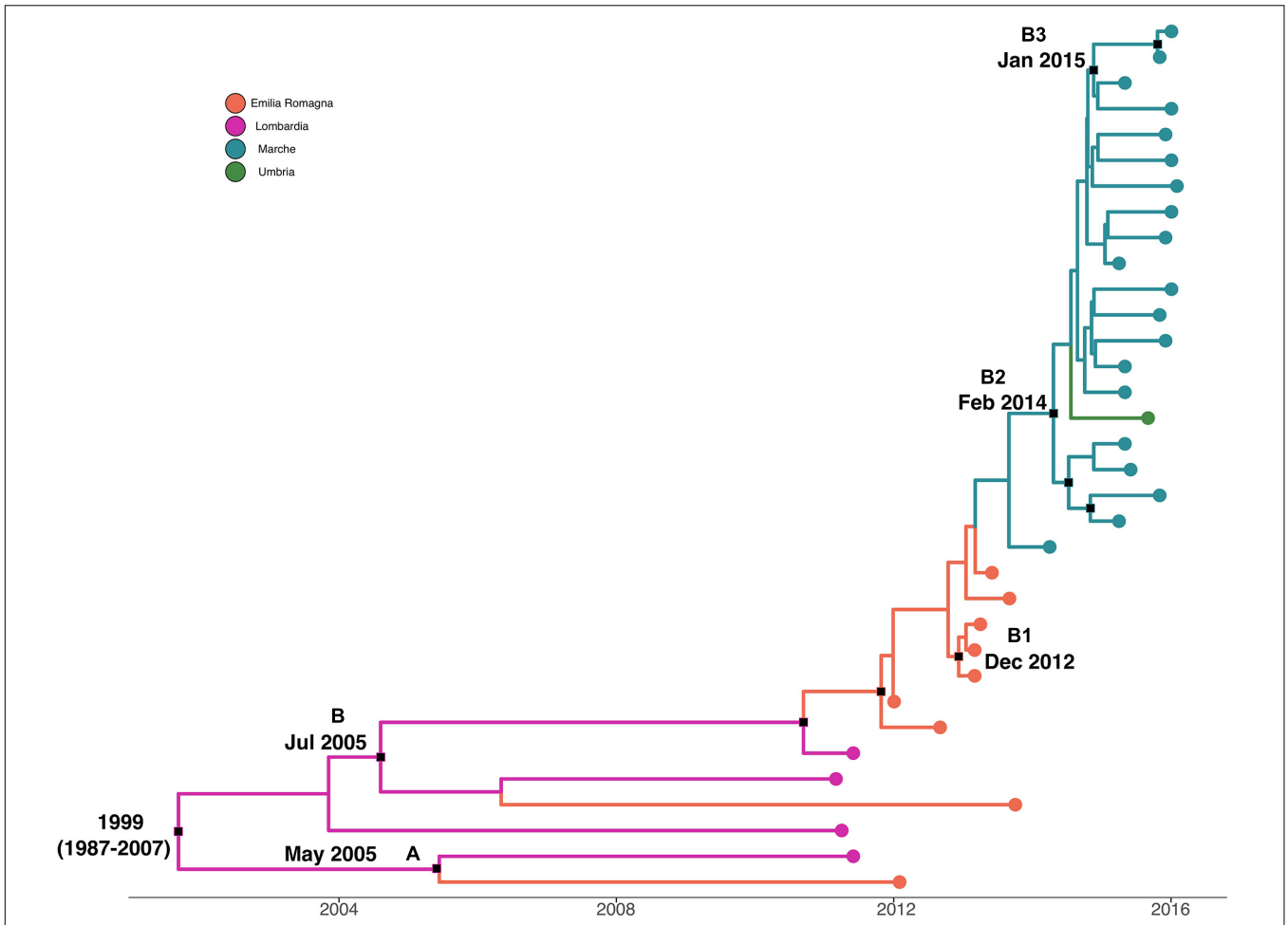


FIGURE 4 | Maximum clade credibility (MCC) tree with Bayesian phylogeography reconstruction *L. monocytogenes* strain CC7 SNP alignment. Branches are scaled in time and colored according to legend to right, where each color represents geographic location of sampled sequence (tip branches), as well as of ancestral lineage (internal branches) inferred by Bayesian phylogeography. Significant posterior probability support ($pp \geq 0.9$) as indicated by a black rectangle. Clusters are highlighted.

Trentino, and Campania, dated back to 2005, which probably originated in Lombardia.

DISCUSSION

Molecular characterization of the strains has been widely used for analyzing the genetic diversity of the *L. monocytogenes* isolates involved in temporally and geographically unrelated outbreaks to evaluate a communal ancestral strain. Recently, WGS has provided enhanced resolution over traditional subtyping methods and can accurately distinguish isolates that would otherwise be overlooked with traditional subtyping methods. Previous works have demonstrated the ability that WGS has to distinguish between *L. monocytogenes* strains and provide robust phylogenetic evidence linking clinical cases (Jackson et al., 2016; Moura et al., 2016). This ability is crucial for outbreaks of listeriosis that are often temporally extended and usually involve small numbers of (apparently) sporadic illnesses. In addition, epidemiological information is often difficult to collect because of the lengthy incubation period and the severity of the illness. On the other hand, WGS combined with epidemiological information has the potential to attribute relatedness among *L. monocytogenes* strains and thus establish stronger links between human listeriosis cases and causative foods (Bergholz et al., 2016). Therefore, timely typing of pathogens is essential to evaluate the real persistence of clusters in determinate geographic regions. In Italy, the majority of the listeriosis cases reported and the correlated strains collected come from Northern Italy, particularly from Lombardia and Emilia-Romagna. Even if this evidence could suggest that there is a strong underreporting/underdiagnosis in the listeriosis cases in the South of Italy (Pontello et al., 2021), it should also be possible to suspect a lower incidence due to different food practices. Recently, a study performed on food and clinical *L. monocytogenes* strains in northern Italy (Lombardia and Piemonte regions) demonstrated that the same clone was persistent for years (2004–2015) in the Gorgonzola processing

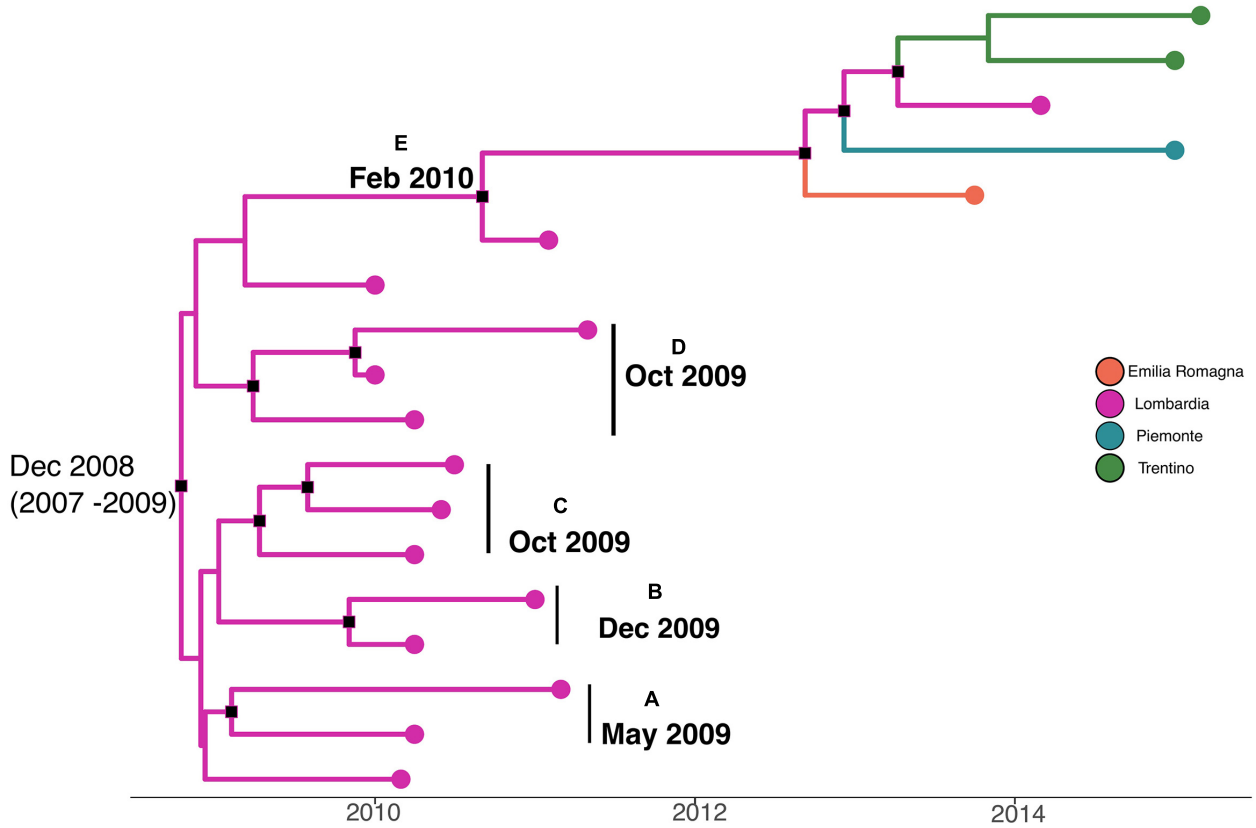


FIGURE 5 | Maximum clade credibility (MCC) tree with Bayesian phylogeography reconstruction *L. monocytogenes* strain CC101 SNP alignment. Branches are scaled in time and colored according to legend to left, where each color represents geographic location of sampled sequence (tip branches), as well as of ancestral lineage (internal branches) inferred by Bayesian phylogeography. Significant posterior probability support ($pp \geq 0.9$) as indicated by a black rectangle. Clusters are highlighted.

plants (Filipello et al., 2017). The strains included in this work have been chosen to represent the Italian situation concerning the clinical strains, isolated from patients with invasive listeriosis, in different Italian regions from 2010 to 2016. In this study, for every clade and cluster, probable epidemic entrances were defined for different years and in different regions. All the analyzed strains belonged to lineages I and II, and the majority of the strains were classified as lineage II (75%). Lineage II included also serotype 1/2a that resulted in the more frequently isolated in Italy in clinical cases (Gianfranceschi et al., 2009). Serotype 1/2a was found more frequently than serotype 4b in listeriosis cases and outbreaks occurring in Europe and the United States between 2010 and 2016 and underlining the hypothesis that serotype 1/2a may be better suited to survive and grow in food and food production (Lomonaco et al., 2015), probably due to its high resistance to disinfection procedures (Brauge et al., 2018). This study provides the first view of *L. monocytogenes* clonal diversity in Italy. The majority of the strains are included in four large CCs (CC1, CC7, CC101, and CC155) and appear to prove the presence of persistence of *L. monocytogenes* in Italy. Particularly in lineage I, approximately 60% of the strains (68.9% of the total *L. monocytogenes* serovars 4b) of *L. monocytogenes* belonged to CC1. The CC1 seemed to have an epidemic entrance in 2004 in Emilia-Romagna, moving to Lombardia, Marche, and Piemonte in 2006. CC1 was strongly associated with strains of clinical origin and reported as the most frequent clone isolated from dairy products (Maury et al., 2016, 2019). In lineage II, the majority of the strains of *L. monocytogenes* sequenced belonged to three main CCs (CC7, CC101, and CC155). In particular, the analysis of the temporal signal and "clock-likeness" of molecular phylogenies established different epidemic entrances of different CCs of lineage II. CC7 is the oldest CC in Italy, with a presumable entrance in 1999 in Lombardia, moving to Emilia-Romagna in 2010. The same CC caused a large outbreak in Marche in 2015 (Duranti et al., 2018). CC155 seems to have its entrance in Lombardia in 2002, where it is apparently limited. CC101 appears to have the entrance in Lombardia in 2008 and does not suggest having evident circulation outside the region, although strains that belonged to CC101 were also isolated in Piemonte in 2013 and in Trentino-Alto Adige in 2015. Among the most frequent CCs isolated in Italy during the period 2010–2016, CC1, CC101, and CC155 are widespread and linked to listeriosis cases in the world, as reported in several studies (Chenal-Francisque et al., 2011; Huang et al., 2015; Maury et al., 2016; Kuch et al., 2018; Zhang et al., 2020). Particularly, the CC1 is the most prevalent genotype in Europe

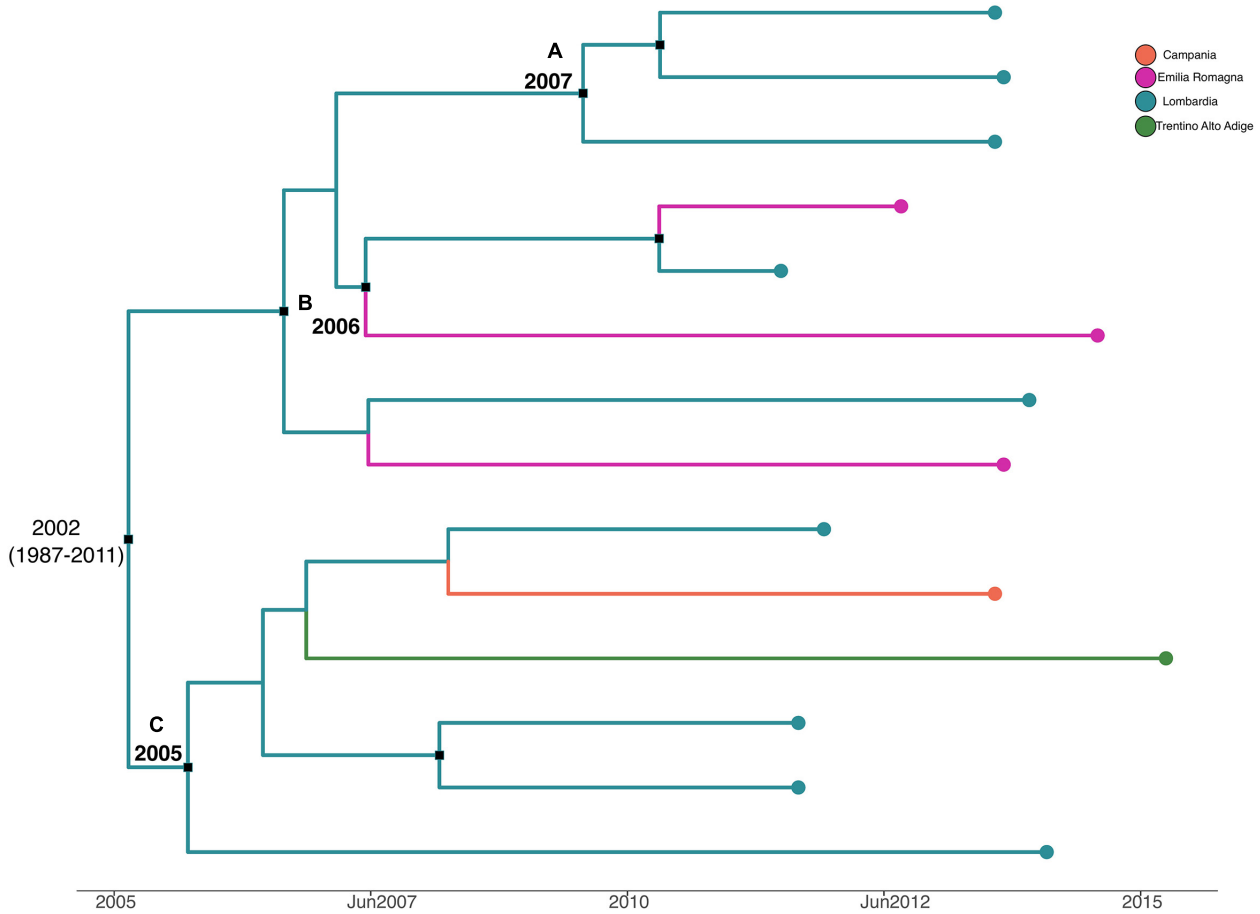


FIGURE 6 | Maximum clade credibility (MCC) tree with Bayesian phylogeography reconstruction *L. monocytogenes* strain CC155 SNP alignment. Branches are scaled in time and colored according to legend to left, where each color represents geographic location of sampled sequence (tip branches), as well as of ancestral lineage (internal branches) inferred by Bayesian phylogeography. Significant posterior probability support ($pp \geq 0.9$) as indicated by a black rectangle. Clusters are highlighted.

and America (Chenal-Francisque et al., 2011), and CC7 isolates were globally recovered in North and South Americas, Europe, Oceania, Africa, and Asia, from a variety of sources and human infections (Kim et al., 2018). Moreover, a recent study showed that CC1 and CC7 represented the most frequent and widespread clones in food-producing plants and retail stores in central Italy (Centorotola et al., 2021).

Our results highlight that WGS is valuable in epidemiological and microbiological surveillance of *L. monocytogenes* in Italy, allowing, also, the monitoring of the pathogen dissemination.

CONCLUSION

According to the European annual epidemiological report on sources of zoonoses, zoonotic agents, and foodborne outbreaks, *L. monocytogenes* is one of the main causes of hospitalization and death in Europe (EFSA and ECDC, 2021). Most human listeriosis cases appear to be related to the consumption of ready-to-eat foods contaminated with *L. monocytogenes* (Chlebicz and Ślizewska, 2018; Ricci et al., 2018). The persistence of specific CC strains in food-processing plants for many years, resulting in intermittent food contamination, has been suggested as the probable cause of many outbreaks (Lomonaco et al., 2015). In this study, the prolonged persistence of specific *L. monocytogenes* CCs was found, indicating that some strains are able to survive better than others in food production environments for extended periods. *L. monocytogenes* exploits different mechanisms of adapting to adverse conditions, such as the capacity to form biofilm or to resist the cleaning and disinfection procedures normally applied in food processing plans (Lundén et al., 2003; Manso et al., 2019). As the persistence of *L. monocytogenes* in food-processing environments is still considered the major source of ready-to-eat food contamination, the identification of these strains has to be considered as an integral part of the risk assessment for improving the hazard characterization of *L. monocytogenes* (Koutsoumanis et al., 2019). Phylogenetic and evolutionary genomic analysis using WGS data has demonstrated an ability to identify the persistence of specific strains in humans, the environment, and foods.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the European Nucleotide Archive (ENA) repository, accession number PRJEB45702. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AF, AG, SF, EC, MO, and FL contributed to the experimental work. EC, SA, and MC contributed to the data analysis. MC, AF, and DD contributed to the writing of the final manuscript. MO performed uploading sequencing data in ENA. All authors contributed significantly to the research, and read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

We gratefully acknowledge Fabrizio Anniballi for his technical support and valuable suggestions for this manuscript, Massimiliano Francia for his graphical support to perform the figures, and Gianni Ciccaglioni for collaborating in uploading sequencing data in ENA.

REFERENCES

Acciari, V. A., Iannetti, L., Gattuso, A., Sonnessa, M., Scavia, G., Montagna, C., et al. (2016). Tracing sources of listeria contamination in traditional italian cheese associated with a US outbreak: investigations in Italy. *Epidemiol. Infect.* 144, 2719–2727. doi: 10.1017/S095026881500254X

Bergholz, T. M., den Bakker, H. C., Katz, L. S., Silk, B. J., Jackson, K. A., Kucerova, Z., et al. (2016). Determination of evolutionary relationships of outbreak-associated listeria monocytogenes strains of serotypes 1/2a and 1/2b by whole-genome sequencing. *Appl. Environ. Microbiol.* 82, 928–938. doi: 10.1128/AEM.02440-15

Brauge, T., Faille, C., Sadovskaya, I., Charbit, A., Benezech, T., Shen, Y., et al. (2018). The absence of N-acetylglucosamine in wall teichoic acids of listeria monocytogenes modifies biofilm architecture and tolerance to rinsing and cleaning procedures. *PLoS One* 13:e0190879. doi: 10.1371/journal.pone.0190879

Centorotola, G., Guidi, F., D'Aurizio, G., Salini, R., Di Domenico, M., Ottaviani, D., et al. (2021). Intensive environmental surveillance plan for listeria monocytogenes in food producing plants and retail stores of central italy: prevalence and genetic diversity. *Foods* 10:1944. doi: 10.3390/foods10081944

Chenal-Francisque, V., Lopez, J., Cantinelli, T., Caro, V., and Tran, C. (2011). Worldwide distribution of major clones of Listeria monocytogenes. *Emerg. Infect. Dis.* 17, 1110–1112. doi: 10.3201/eid/1706.101778

Chlebicz, A., and Ślizewska, K. (2018). Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. *Int. J. Environ. Res. Public Health.* 15:863. doi: 10.3390/ijerph15050863

de Noordhout, C. M., Devleesschauwer, B., Angulo, F. J., Verbeke, G., Haagsma, J., Kirk, M., et al. (2014). The global burden of listeriosis: a systematic review and meta-analysis. *Lancet Infect. Dis.* 14, 1073–1082. doi: 10.1016/S1473-3099(14)70870-9

Drummond, A. J., Rambaut, A., Shapiro, B., and Pybus, O. G. (2005). Bayesian coalescent inference of past population dynamics from molecular sequences. *Mol. Biol. Evol.* 22, 1185–1192. doi: 10.1093/molbev/msi103

Duranti, A., Sabbatucci, M., Blasi, G., Acciari, V. A., Ancora, M., Bella, A., et al. (2018). A severe outbreak of listeriosis in central italy with a rare pulsotype associated with processed pork products. *J. Med. Microbiol.* 67, 1351–1360. doi: 10.1099/jmm.0.000785

EFSA, and ECDC. (2021). The european union one health 2019 zoonoses report. *EFSA J.* 19:6406. doi: 10.2903/j.efsa.2021.6406

Filipello, V., Amato, E., Gori, M., Huedo, P., Ciceri, G., Lomonaco, S., et al. (2017). Epidemiology and molecular typing of pregnancy-associated listeriosis cases in lombardy, italy, over a 10-year period (2005-2014). *Infect. Dis. Obstet. Gynecol.* 2017:6479121. doi: 10.1155/2017/6479121

Gianfranceschi, M. V., D'Ottavio, M. C., Gattuso, A., Bella, A., and Aureli, P. (2009). Distribution of serotypes and pulsotypes of listeria monocytogenes from humans, food and environmental isolates (italy 2002-2005). *Food Microbiol.* 26, 520–526. doi: 10.1016/j.fm.2009.03.003

Huang, Y. T., Ko, W. C., Chan, Y. J., Lu, J. J., Tsai, H. Y., Liao, C. H., et al. (2015). Disease burden of invasive listeriosis and molecular characterization of clinical isolates in taiwan, 2000–2013. *PLos One* 10:e0141241. doi: 10.1371/journal.pone.0141241

Jackson, B. R., Tarr, C., Strain, E., Jackson, K. A., Conrad, A., Carleton, H., et al. (2016). Implementation of nationwide real-time whole-genome sequencing to enhance listeriosis outbreak detection and investigation. *Clin. Infect. Dis.* 63, 380–386. doi: 10.1093/cid/ciw242

Kim, S. W., Haendiges, J., Keller, E. N., Myers, R., Kim, A., Lombard, J. E., et al. (2018). Genetic diversity and virulence profiles of *Listeria monocytogenes* recovered from bulk tank milk, milk filters, and milking equipment from dairies in the United States (2002 to 2014). *PLos One* 13:e0197053. doi: 10.1371/journal.pone.0197053

Koutsoumanis, K., Allende, A., Alvarez-Ordónez, A., Bolton, D., Bover-Cid, S., Chemaly, M., et al. (2019). Scientific opinion on the whole genome sequencing and metagenomics for outbreak investigation, source attribution and risk assessment of food-borne microorganisms. *EFSA J.* 17:5898. doi: 10.2903/j.efsa.2019.5898

Kuch, A., Goc, A., Belkiewicz, K., Filipello, V., Ronkiewicz, P., Golebiewska, A., et al. (2018). Molecular diversity and antimicrobial susceptibility of listeria monocytogenes isolates from invasive infections in poland (1997–2013). *Sci. Rep.* 8:14562. doi: 10.1038/s41598-018-32574-0

Lamont, R. F., Sobel, J., Mazaki-Tovi, S., Kusanovic, J. P., Vaisbuch, E., Kim, S. K., et al. (2011). Listeriosis in human pregnancy: a systematic review. *J. Perinat. Med.* 39, 227–236. doi: 10.1515/jpm.2011.035

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. L., et al. (2012). Multilocus sequence typing of total-genome-sequenced bacteria. *J. Clin. Microbiol.* 50, 1355–1361. doi: 10.1128/JCM.06094-11

Lomonaco, S., Nucera, D., and Filipello, V. (2015). The evolution and epidemiology of listeria monocytogenes in europe and the united states. *Infect. Genet. Evol.* 35, 172–183. doi: 10.1016/j.meegid.2015.08.008

Lundén, J., Autio, T., Markkula, A., Hellström, S., and Korkeala, H. (2003). Adaptive and cross-adaptive responses of persistent and non-persistent listeria monocytogenes strains to disinfectants. *Int. J. Food Microbiol.* 82, 265–272. doi: 10.1016/S0168-1605(02)00312-4

Manso, B., Melero, B., Stessl, B., Fernández-Natal, I., Jaime, I., Hernández, M., et al. (2019). Characterization of virulence and persistence abilities of listeria monocytogenes strains isolated from food processing premises. *J. Food Prot.* 82, 1922–1930. doi: 10.4315/0362-028X.JFP-19-109

Maury, M. M., Bracq-Dieye, H., Huang, L., Vales, G., Lavina, M., Thouvenot, P., et al. (2019). Hypervirulent listeria monocytogenes clones' adaption to mammalian gut accounts for their association with dairy products. *Nat. Commun.* 10:2488. doi: 10.1038/s41467-019-10380-0

Maury, M. M., Tsai, Y. H., Charlier, C., Touchon, M., Chenal-Francisque, V., Leclercq, A., et al. (2016). Uncovering listeria monocytogenes hypervirulence by harnessing its biodiversity. *Nat. Genet.* 48, 308–313. doi: 10.1038/ng.3501

Moura, A., Criscuolo, A., Pouseele, H., Maury, M. M., Leclercq, A., Tarr, C., et al. (2016). Whole genome-based population biology and epidemiological surveillance of Listeria monocytogenes. *Nat. Microbiol.* 2:16185. doi: 10.1038/nmicrobiol.2016.185

Pontello, M., Guaita, A., Sala, G., Cipolla, M., Gattuso, A., Sonnessa, M., et al. (2021). Listeria monocytogenes serotypes in human infections (italy, 2000-2010). *Ann. Ist. Super. Sanità.* 48, 146–150. doi: 10.4415/ANN_12_02_07

Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecui, M., Le Monnier, A., et al. (2008). A new perspective on listeria monocytogenes evolution. *PLoS Pathog.* 4:146. doi: 10.1371/journal.ppat.1000146

Rambau, A., Lam, T. T., Max Carvalho, L., and Pybus, O. G. (2016). Exploring the temporal structure of heterochronous sequences using TempEst (formerly path-o-gen). *Virus Evol.* 2:e07. doi: 10.1093/ve/vew007

Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Fernàndez Escàmez, P. S., et al. (2018). Scientific opinion on the listeria monocytogenes contamination of ready-to-eat foods and the risk for human health in the EU. *EFSA J.* 16:5134. doi: 10.2903/j.efsa.2018.5134

Schmidt, H. A., Strimmer, K., Vingron, M., and von Haeseler, A. (2002). TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* 18, 502–504. doi: 10.1093/bioinformatics/18.3.50.2

Suchard, M. A., Lemey, P., Baele, G., Ayres, D. L., Drummond, A. J., and Rambaut, A. (2018). Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol.* 4:e016. doi: 10.1093/ve/vey016

Trifinopoulos, J., Nguyen, L. T., von Haeseler, A., and Minh, B. Q. (2016). W-IQ-TREE: a fast-online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res.* 44, W232–W235. doi: 10.1093/nar/gkw256

Van Walle, I., Björkman, J. T., Cormican, M., Dallman, T., Mossong, J., Moura, A., et al. (2018). Retrospective validation of whole genome sequencing-enhanced surveillance of listeriosis in Europe, 2010 to 2015. *Euro Surveill.* 23:798. doi: 10.2807/1560-7917.es.2018.23.33.1700798

Zhang, H., Chen, W., Wang, J., Xu, B., Liu, H., Dong, Q., et al. (2020). 10-year molecular surveillance of listeria monocytogenes using whole-genome sequencing in shanghai, china, 2009–2019. *Front. Microbiol.* 11:551020. doi: 10.3389/fmicb.2020.551020

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 19 May 2022
doi: 10.3389/fmicb.2022.883931

Bacterial Resistance Toward Antimicrobial Ionic Liquids Mediated by Multidrug Efflux Pumps

*Tobias Gundolf[1], Roland Kalb[2,3], Peter Rossmanith[3,4] and Patrick Mester[4]**

[1]Christian Doppler Laboratory for Monitoring of Microbial Contaminants, Unit for Food Microbiology, Department of Veterinary Public Health and Food Science, University of Veterinary Medicine, Vienna, Austria, [2]Proionic Production of Ionic Substances GmbH, Grambach, Austria, [3]Joint BioEnergy Institute, Lawrence Berkeley National Laboratory, Berkeley, CA, United States, [4]Unit for Food Microbiology, Department of Veterinary Public Health and Food Science, University of Veterinary Medicine, Vienna, Austria

The effective elimination of foodborne pathogens through cleaning and disinfection measures is of great importance to the food processing industry. As food producers rely heavily on disinfectants to control pathogenic bacteria in their facilities, the increasing spread of tolerant, often even multidrug resistant, strains is of particular concern. In addition to efforts to prevent or at least reduce development and spread of strains resistant to disinfectants and sanitizers, there is an urgent need for new and effective antimicrobials. One new class of promising antimicrobials is ionic liquids (ILs), which have been reported to be effective against resistant strains as they interact with bacterial cells in multiple ways, but investigations of their effectivity against MDR bacteria or specific defense mechanisms are still limited. This study investigates the role of multidrug efflux pumps of the Resistance Nodulation-Division family (RND) on the resistance of bacterial pathogens *Escherichia coli* and *Salmonella enterica* serovar Typhimurium toward 10 antimicrobial active ILs. Results reveal that, while known structure–activity relationships (SARs), such as the side-chain effect, were found for all strains, antimicrobial ILs with one elongated alkyl side chain were significantly affected by the RND efflux pump, highlighting the importance of efflux pumps for future IL toxicity studies. In case of antimicrobial ILs with multiple side chains and different cationic head groups, two ILs were identified that were highly active against all investigated strains with little to no effect of the efflux pump. The results obtained in this study for RND efflux pumps can serve as a starting point for identifying and designing antimicrobial ILs as effective biocides against MDR bacteria.

Keywords: ionic liquids, antimicrobial, multidrug resistance, efflux pump, Resistance Nodulation Division, structure–activity relationship, *Escherichia coli*, *Salmonella enterica*

OPEN ACCESS

Edited by:
Laurent Dufossé,
Université de la Réunion, France

Reviewed by:
Ahmed Ghamry Abdelhamid,
The Ohio State University, United States
Kunihiko Nishino,
Osaka University, Japan

****Correspondence:***
Patrick Mester
patrick-julian.mester@vetmeduni.ac.at

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *25 February 2022*
Accepted: *27 April 2022*
Published: *19 May 2022*

Citation:
Gundolf T, Kalb R, Rossmanith P and Mester P (2022) Bacterial Resistance Toward Antimicrobial Ionic Liquids Mediated by Multidrug Efflux Pumps. Front. Microbiol. 13:883931. doi: 10.3389/fmicb.2022.883931

INTRODUCTION

Humanity's history is a continuous battle between us and microbial pathogens and for the most part, we were on the losing side with bacterial and viral infections being among the major causes of morbidity and mortality worldwide. Thanks to the development and improvement of sanitation, hygiene practices and especially discovery of disinfectants, antibiotics, and vaccinations since the

early 20th century, deaths from infectious diseases have declined markedly. This decline can be considered one of the biggest success stories in human history (CDC on infectious diseases in the United States: 1900-99, 1999) but is under pressure due to a dramatic increase of multidrug-resistant species (Andersson and Hughes, 2014; Qiao et al., 2018). Disinfectants play an important role in maintaining acceptable health standards by significantly reducing microbial loads as well as reducing, if not eliminating, pathogens (Tezel and Pavlostathis, 2015). Quaternary ammonium compounds (QAC) are among the most commonly used disinfectants in a variety of different industries including hospital, water, cosmetic, and the food industry, where bacteria are often exposed to disinfectants through the entire food chain (Buffet-Bataillon et al., 2016; Martínez-Suárez et al., 2016; Duze et al., 2021). Upon continued exposure, bacteria can adapt to biocides, a phenomenon known as biocide resistance (Poole, 2002; Andersson and Hughes, 2014; Grande Burgos et al., 2016; Gadea et al., 2017), which can increase the ability of pathogens to persist in food environments (Martínez-Suárez et al., 2016; Chmielowska et al., 2021; Guidi et al., 2021) and can be transferred from one species to another in the food environment (Hansen et al., 2007; Szmolka and Nagy, 2013). Bacteria can elicit non-specific mechanisms of resistance mediated by efflux pumps, which can accommodate a diversity of chemical structures as substrates including biocides and antibiotics (Poole, 2002, 2004, 2005, 2007; Grande Burgos et al., 2016; Shafaati et al., 2016).

Bacterial efflux pumps actively transport many antimicrobials and/or antibiotics out of the cell and are major contributors to the intrinsic resistance of bacteria (Putman et al., 2000; Tezel and Pavlostathis, 2015; Dam et al., 2018). While some efflux pumps have narrow substrate specificity, many transport a wide range of structurally dissimilar substrates and are known as multidrug resistance (MDR) efflux pumps. There are well-studied examples of MDR efflux pumps that are present in all bacteria, and new pumps that export antibiotics continue to be described. There are five classes of MDR efflux pumps: the ATP-binding cassette family (ABC), the Major Facilitator Superfamily (MFS), the Multidrug and Toxic Compound Extrusion family (MATE), the Resistance Nodulation-Division family (RND), and the Small-Multidrug Resistance family (SMR). Family division is based on the number of structural components that compromise each pump, the number of membranes they span, their substrate specificity, and the energy source used (Putman et al., 2000; Tezel and Pavlostathis, 2015). Whereas ABC, MATE, MFS, and SMR efflux pumps are widely distributed in both Gram-negative and Gram-positive species, the RND transporter type is exclusively found in Gram-negative bacteria, as it forms a tripartite complex that span from the inner to the outer membranes and is one of the best-characterized clinically relevant MDR efflux transporter type. Well-studied examples include the multidrug efflux pump AcrB in *Escherichia coli* and MexB in *Pseudomonas aeruginosa*. RND pumps, such as AcrB, are homo-trimers that reside in the inner membrane and form a tripartite complex with a periplasmic adaptor protein, such as AcrA and an outer-membrane channel, such as TolC (Chetri et al., 2019). Collectively, it is clear that there is an urgent need to identify novel antibacterial agents and biocides to combat the plethora of resistant bacterial genotypes (Andersson and Hughes, 2014; Bodro et al., 2014; Qiao et al., 2018; Prudêncio et al., 2020).

One promising new chemical class in this regard has been ionic liquids (ILs). Ionic liquids, defined as organic salts with a melting points below 100°C (Wasserscheid and Welton, 2008), have attracted substantial attention from both academia and industry due to their unique physiochemical properties and high tuneability (Rogers and Seddon, 2003; Aschenbrenner et al., 2009; Cevasco and Chiappe, 2014) including applications in medicine and as pharmaceuticals. In this context, ILs are applied in mostly one of two ways. Either as an antimicrobial active agent itself, as components of drug or drug delivery systems and as solvents in drug synthesis (Ferraz et al., 2011; Prudêncio et al., 2020). So-called API-ILs facilitate the incorporation of active pharmaceutical ingredients (API) into an IL form (Hough et al., 2007; Ferraz et al., 2011; Mester et al., 2016; Bromberger et al., 2020). One advantage of ILs, in comparison to other antimicrobials, is that they act on bacterial cells in multiple ways. Previous studies have demonstrated that ILs are (i) interacting with bacterial membrane and wall (Mester et al., 2016; Borkowski et al., 2017); (ii) disrupting cell integrity (Venkata Nancharaiah et al., 2012; Cook et al., 2019); (iii) destabilizing proteins and hindering their enzymatic activity (Mester et al., 2019; Tarannum et al., 2019; Bromberger et al., 2020); (iv) dysregulating bacterial metabolism (Yu et al., 2016; Cłapa et al., 2021); (v) triggering oxidative stress response (Yu et al., 2016); and (vi) leading to DNA damage (Kowalczyk et al., 2018). Consequently, ILs can be considered versatile antimicrobials of great potential with a wide spectrum of antibacterial mechanisms, thus potentially having an advantage against MDR resistant bacteria.

Nevertheless, there is still only a very limited amount of studies investigating the effectivity against MDR bacteria and to understand the impact of different bacterial defense strategies and mechanisms. What could be demonstrated in previous studies is a significant difference of antimicrobial IL efficacy between Gram-positive and Gram-negative bacteria. This difference is mainly attributed to the presence of outer membrane and lipopolysaccharide (LPS) layer in Gram-negative microorganisms (Cole et al., 2011; Weyhing-Zerrer et al., 2017). Due to its hydrophilic nature, LPS can prevent large, hydrophobic compounds from passing through the membrane and the impact of the LPS structure on overall IL susceptibility has been previously demonstrated (Gundolf et al., 2018; Kowalczyk et al., 2018). Interestingly, the influence of bacterial efflux pumps, one of the most important resistance mechanism toward antimicrobials as well as antibiotics, on antimicrobial IL efficacy has been scarcely investigated up to this point. Concerning antimicrobial ILs, only few studies investigated the impact of efflux pumps on bacterial resistance. For the Gram-positive pathogen *Listeria monocytogenes*, the SMR transporter QacH was shown to significantly increase the resistance of bacterial cells against classic QAC-based biocides as well as ILs with long alkyl side chains (Mester et al., 2015). For the Gram-negative bacterium *Enterobacter lignolyticus*, an efficient transport of $[C_2mim]^+$ cations outside the cell was found for efflux pump from a MFS encoded by the *eilA* gene (Ruegg et al., 2014).

Taken together, it is clear that the role of bacterial efflux pumps in regard to antimicrobial active ILs is understudied.

Consequently, the aim of our study was to determine the impact of some of the most important efflux pump types on susceptibility of bacterial pathogens to antimicrobial ILs, which could subsequently influence the future design of these substances. To accomplish this objective, we investigated the impact of the multidrug efflux pump belonging the RND on the resistance of the bacterial pathogens *E. coli* and *Salmonella enterica* serovar Typhimurium toward a set of 10 antimicrobial active ILs.

MATERIALS AND METHODS

Ionic Liquids and Other Chemical Substances

QACs: benzalkonium chloride (BC), benzethonium chloride (BZ), cetylpyridinium chloride (CP), cetyltrimethylpyridinium chloride (CTAB), and domiphen bromide (DB) were purchased from Sigma-Aldrich (Steinheim, Germany).

The ILs used in this study were either (**a**) provided by Proionic GmbH (Grambach, Austria) with a nominal purity of >98%, (**b**) purchased from Iolitec (Ionic Liquid Technologies GmbH, Heilbronn, Germany) with a nominal purity of >98%, or (**c**) synthesized in our laboratory, according to the CBILS© route (CBILS is a registered trademark of Proionic GmbH; Kalb et al., 2005, 2016). The following ILs were investigated as: ILs with one elongated alkyl side chain 1-decyl-3-methylimidazolium chloride ([C_{10}mim][Cl];**a**), 1-dodecyl-3-methylimidazolium chloride ([C_{12}mim][Cl];**b**), 1-methyl-3-tetradecylimidazolium chloride ([C_{14}mim][Cl];**b**), 1-hexadecyl-3-methylimidazolium chloride ([C_{16}mim][Cl];**b**) trimethyldecylammonium chloride ([TMC_{10}A][Cl];**c**), trimethylhexadecylammonium chloride ([TMC_{16}A][Cl];**c**). ILs with two elongated alkyl side chains dioctyldimethylammonium chloride ([DC_8DMA][Cl];**c**) and 1-3-didecyl-2-imidazolium chloride ([$C_{10}C_{10}$im][Cl];**b**). ILs with three elongated alkyl side chains trioctylmethylammonium chloride ([TC_8MA][Cl];**a**) and trioctylmethylphosphonium chloride ([TMC_{10}P][Cl];**c**).

Bacterial Strains and Culture Conditions

Escherichia coli BW25113 (wild-type), *E. coli* JW0451-2 (Δ*acrB*), *E. coli* JW0452-3 (Δ*acrA*), and *E. coli* JW5503-1 (Δ*tolC*) were obtained from the Coli Genetic Stock Centre (CGSC, Yale University) and are part of the Keio collection of *E. coli K-12* single-gene knockout mutants (Baba et al., 2006). The Keio collection contains a set of precisely defined, single-gene deletions of all nonessential genes in *E. coli* K-12, which enables systematic analyses of unknown gene functions and gene regulatory networks but also for genome-wide testing of mutational effects in a common strain background, *E. coli* K-12 BW25113. Open-reading frame coding regions were replaced with a kanamycin cassette flanked by FLP recognition target sites by using a one-step method for inactivation of chromosomal genes and primers designed to create in-frame deletions upon excision of the resistance cassette (Baba et al., 2006). *S. enterica* serovar Typhimurium ATCC 14028s (wild-type) and *S. enterica* serovar Typhimurium NKS148 (Δ*acrB*) were kindly provided by Kunihiko Nishino (Osaka University; Horiyama et al., 2010; Yamasaki et al., 2013). A detailed description of the respective strains is provided in the supplement section (**Supplementary Table S1**). Bacterial strains were grown overnight in tryptone soy broth supplemented with 0.6% (w/v) yeast extract (Oxoid™, Hamsphire, United Kingdom) and 30 μg/ml kanamycin at 37°C, with the exception of the wild-type strain *E. coli* BW25113 and the *S. enterica* strains that lack the kanamycin resistance cassette. Twenty-four hours growth curves were performed in 96-well microtiter plates (Corning B.V Life Sciences, Amsterdam, Netherlands) on measuring their optical densities every hour at a wavelength of 610 nm in a TECAN F100 microplate reader (Tecan Austria GmbH., Groeding, Austria). Bacteria were maintained at −80°C using Microbank™ technology (Pro-Lab Diagnostics, Richmond Hill, Canada).

Minimal Inhibitory Concentration Assessment

MICs of the test chemicals (ILs, QACs, NaCl, KH_2PO_4, ethanol, and urea as well as pH) were assessed by applying the serial 2-fold dilution microtiter plate method in TSB-Y medium (Morrissey et al., 2009). In order to create a constant cell status for each experiment, 1 ml aliquots of the respective overnight cultures were transferred into 9 ml of fresh TSB-Y medium (1:10 dilution) and incubated for 3 h at 37°C to ensure that cells were in a logarithmic growth phase. Subsequently, each well, which contained a serial diluted antimicrobial substance (dilution 1:2), was inoculated with 5×10^5 CFU of the respective bacterial cells. After inoculation with the respective bacteria, absorbance of the 96-well microtiter plates (Corning B.V Life Sciences, Amsterdam, Netherlands) was measured at a wavelength of 610 nm in a TECAN F100 microplate reader (Tecan Austria GmbH, Groeding, Austria) to monitor for any possible interference by the antimicrobial substances. The microtiter plates were then incubated for 24 h at the 37°C and bacterial growth assessed by measuring the absorbances at 610 nm. The MIC was defined as the lowest concentration of the tested antimicrobial substance where no bacterial growth could be measured after 24 h. Results are presented as mean MICs and upper and lower limits of 95% CIs of at least three experiments performed on different days. Each experiment included positive (bacterial growth control without ILs) and negative controls (medium without the addition of bacteria).

RESULTS AND DISCUSSION

Influence of Efflux Pumps on Bacterial Growth and Susceptibility to Chemical Substances

This study investigates the role of efflux pumps deletions on the susceptibility of four *E. coli* strains (wild type and three different single-gene deletion mutants), as well as Salmonella NKS (**Figure 1A**) toward antimicrobial ILs. For *E. coli*, three efflux pump deletion strains (Δ*acrA*, Δ*acrB*, and Δ*tolC*) are missing one part of the tripartite complex of the RND transporter family. The three different mutant strains were included in this study to investigate possible differences concerning IL susceptibility if only one part of the tripartite complex is

missing. For *S. enterica*, two strains (wild type and RND deletion mutant Δ*acrB*) were studied in order to investigate possible susceptibility differences between the two species.

To investigate if efflux pump deletion affects bacterial growth, the growth of wild type and deletions mutants was monitored for 24 h while measuring cell density hourly. No significantly impaired growth was observed for any of the four deletion strains compared to their respective wild type (data not shown). Further, strains were subjected to six hydrophilic chemical substances as an additional control to test strain viability/vitality. No significant differences between the wild-type strains and the deletion mutants were found indicating that the efflux strains were *per se* not less robust compared to the wild-type strains and the results for QACS and ILs can be interpreted accordingly (**Table 1**).

As mentioned in the introduction, one of the criteria to investigate the efflux pumps investigated in this study was their reported connection to QAC resistance. It therefore comes as no surprise that the wild-type *E. coli* and *S. enterica* had significantly higher MIC values for each of the five QACs than the respective deletion mutants (**Figure 1**).

Although there were slight differences regarding the susceptibilities for the different strains and QACs, on average, the wild-type strains had a four times higher MIC compared to the deletion mutants with the highest observed for BC (6x) and the lowest for CP (2x). In conclusion, the results confirmed the influence of the studied efflux pumps on the susceptibility of the studied pathogens toward QACS. Consequently, the chosen bacterial strains can serve as models for studying the antimicrobial effect of ILs.

Influence of Efflux Pumps on the Susceptibility to ILs

ILs With One Side Chain

Imidazolium ([$C_n$mim][Cl]) and ammonium-based ([TMC_nA][Cl]) ILs with varying alkyl side chain lengths were tested as representatives of ILs with one alkyl side chain (**Figure 2**).

In case of *S. enterica* wild type and the Δ*acrB* deletion mutant, for each of the six ILs with one elongated side chain, a significant higher resistance of the wild type compared to the deletion mutant was found. On average, the wild-type strain had a 4x higher MIC than the Δ*acrB* deletion mutant, with the highest observed for [C_{14}mim][Cl] (8x) and the lowest for [C_{16}mim][Cl] (2x).

In case, of *E. coli* very similar results for all three deletion mutants were obtained and will be discussed together. As was the case for *S. enterica*, the wild-type *E. coli* was significantly more resistant against ILs with one side chain than the efflux

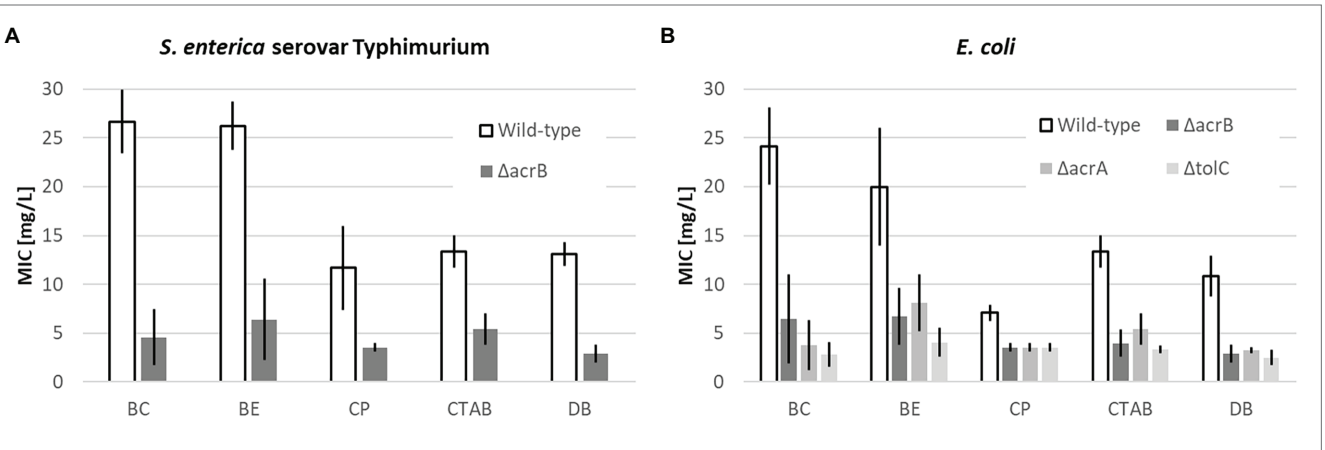


FIGURE 1 | Mean minimal inhibitory concentration (MIC) values for quaternary ammonium compounds (QACs; BC, BE, CP, CTAB, and DB) including upper and lower limits of 95% CIs of the respective *Salmonella enterica* **(A)** and *Escherichia coli* **(B)** strains after 24 h incubation at 37°C.

TABLE 1 | Mean MIC values (bold) for chemicals including upper and lower limits of 95% CIs (in brackets) of the respective Salmonella enterica and Escherichia coli strains after 24 h incubation at 37°C.

	S. enterica		*E. coli*			
	Wild-type	**Δ*acrB***	**Wild-type**	**Δ*acrB***	**Δ*acrA***	**Δ*tolC***
Methanol (%; v/v)	**4.7** (4.7)	**3.9** (2.4–5.4)	**9.4** (9.38)	**7.8** (4.8–10.9)	**6.3** (3.2–9.3)	**4.7** (4.7)
Ethanol (%; v/v)	**3.8** (2.3–5.2)	**4.5** (4.5)	**4.5** (4.5)	**4.5** (4.5)	**4.5** (4.5)	**3.8** (2.3–5.2)
KH_2PO_4 (%; w/v)	**13.5** (13.5)	**13.5** (13.5)	**13.5** (13.5)	**13.5** (13.5)	**13.5** (13.5)	**11.3** (6.8–15.7)
NaCl (%; w/v)	**11.3** (11.3)	**11.3** (11.3)	**11.3** (11.3)	**11.3** (11.3)	**11.3** (11.3)	**7.5** (3.8–11.2)
Urea (mol/L)	**0.8** (0.5–1.2)	**0.8** (0.5–1.2)	**1.0** (1.0)	**0.8** (0.5–1.2)	**0.8** (0.5–1.2)	**0.8** (0.5–1.2)

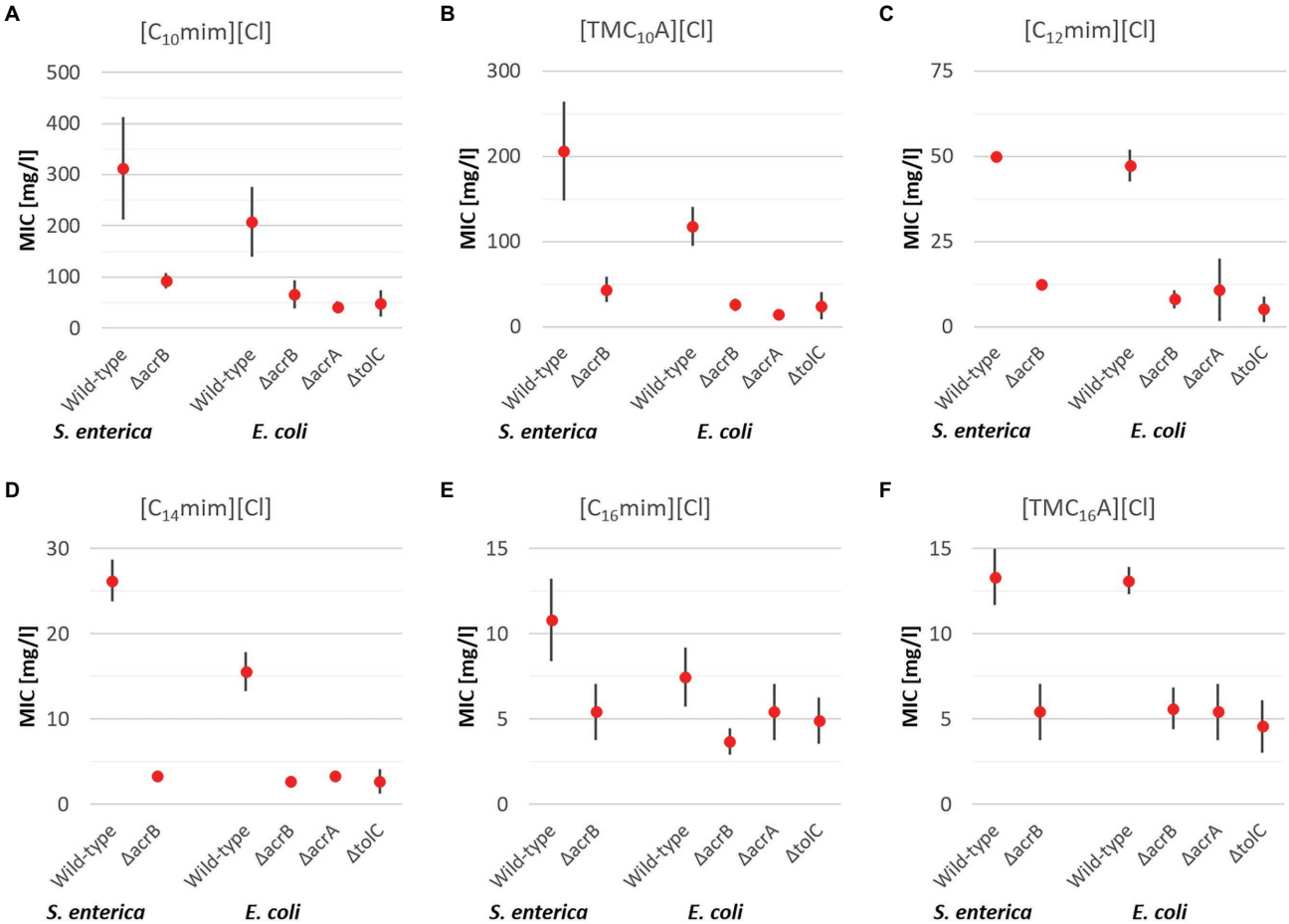


FIGURE 2 | Mean MIC values for antimicrobial ionic liquids (ILs) with one elongated alkyl side chain [C_{10}mim][Cl] **(A)**, [TMC_{10}A][Cl] **(B)**, [C_{12}mim][Cl] **(C)**, [C_{14}mim][Cl] **(D)**, [C_{16}mim][Cl] **(E)**, and [TMC_{16}A][Cl] **(F)** including upper and lower limits of 95% CIs for the respective *S. enterica* and *E. coli* strains after 24 h incubation at 37°C.

pump deletion mutants, all of which lack one gene necessary for a functional RND transporter. On average the wild type was able to withstand 4.3 times higher IL concentrations with the biggest differences observed for [C_{12}mim][Cl] (6.5x) and the smallest for [C_{16}mim][Cl] (1.7x).

Taken together for both bacterial species and all investigated efflux pump deletion mutants, the obtained results are quite similar to the results obtained for "classic" QACs.

ILs With Multiple Side Chains

In addition to antimicrobial ILs containing one elongated alkyl side chain, cations with multiple side chains have also been reported to have good antimicrobial activity and were thus included in the present study. The investigation included two ammonium-based ILs with two ([DC_8DMA][Cl]) and three octyl side chains ([TC_8MA][Cl]), one phosphonium based IL also with three octyl side chains ([TC_8MP][Cl]), and one imidazolium based IL with two decyl side chains ([$C_{10}C_{10}$im][Cl]) and the general trends regarding IL antimicrobial activity were confirmed. With an increasing number of alkyl side chains of identical length, the antimicrobial activity is also increasing (**Figures 2A, 3**) which was observed for both bacterial specials and all strains. However, in case of the efflux pump deletion mutants, different results were obtained.

In case of *S. enterica*, the results for ILs with multiple side chains were found to be similar to those for ILs with one side chain and the QACs. For all ILs with multiple side chains, an average 3.1x times higher MIC of the wild type compared to the Δ*acrB* deletion mutant was found, with the highest observed for [DC_8DMA][Cl] (5.9x) and the lowest for [$C_{10}C_{10}$im][Cl] (1.4x; **Figure 3**).

In case of *E. coli*, the results for ILs with multiple side chains were quite different for the different ILs and thus will discussed separately. In case of [DC_8DMA][Cl], a significantly 11x higher MIC of the wild type was observed, demonstrating the impact of all three efflux pump types on the resistance against the IL. In case of [TC_8MA][Cl], which has a similar cation core with one additional octyl side chain, a higher MIC (2x) for the wild type was observed in comparison with the three RND deletion mutants. Interestingly in case of [TC_8MP][Cl], the MIC for all *E. coli* strains was around 6 mg/l with no significant differences between the four strains. Thus, demonstrating for the first time no significant effect on bacterial resistance against this antimicrobial active IL

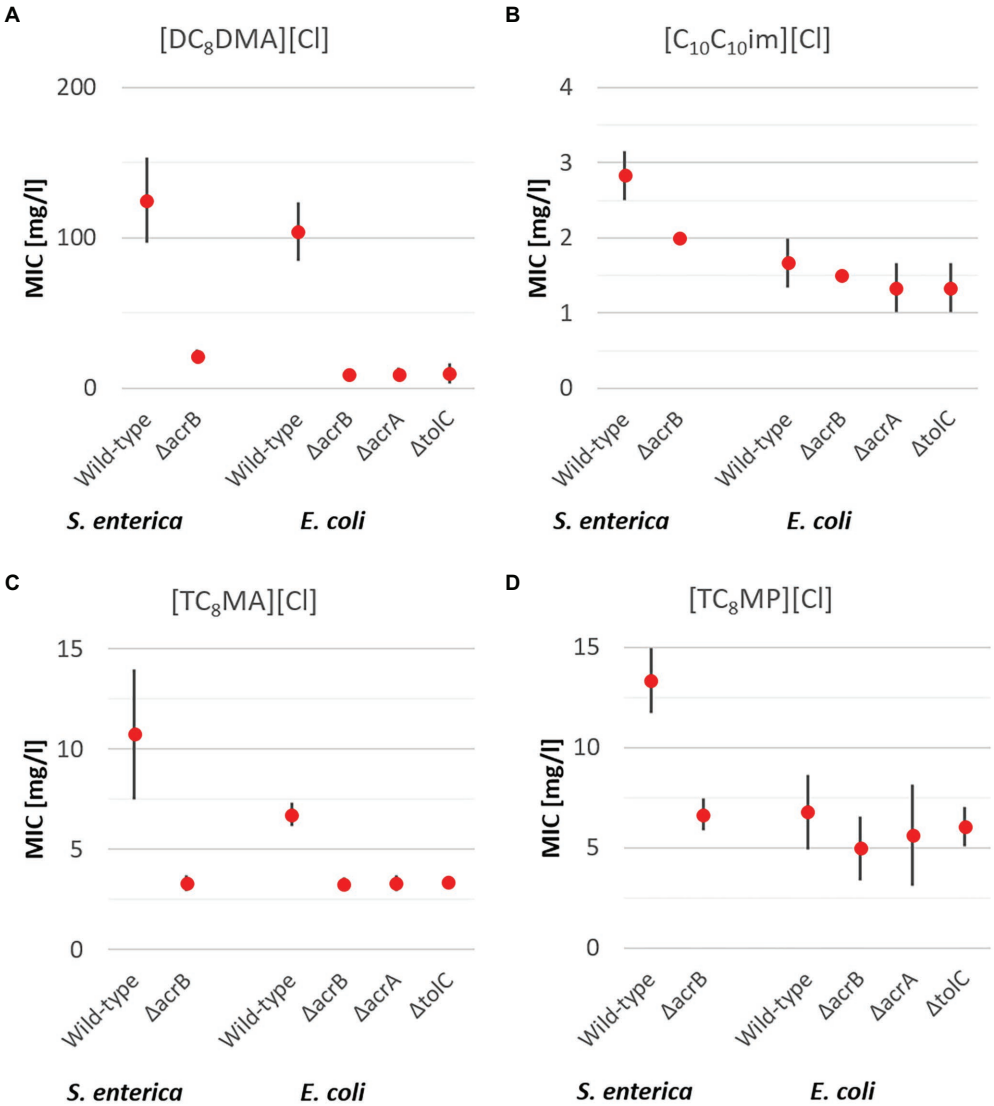


FIGURE 3 | Mean MIC values for antimicrobial ILs with two elongated alkyl side chains [DC_8DMA][Cl] **(A)** and [C_{10} C_{10}im][Cl] **(B)** and three elongated alkyl side chains [TC_8MA][Cl] **(C)** and [TC_8MP][Cl] **(D)** including upper and lower limits of 95% CIs for the respective *S. enterica* and *E. coli* strains after 24 h incubation at 37°C.

by the RND transporter. From these results, it seems as either the efflux pumps of *E. coli* are not able to remove the ILs from the cell inside, or the ILs have a different mode of action not acting inside the cell but for instance directly interact with the cell membrane as has been previously reported (Mester et al., 2016, 2019). Speaking against this hypothesis are the findings for *S. enterica*, as a clear and significant effect of the efflux pump was determined. As both bacterial species are Gram-negative bacteria with a similar cell membrane structure, it is more likely that the ILs act intracellularly and that the *E. coli* RND Transporter is less effective in transporting them out of the cell.

Similar results as for [TC_8MP][Cl] were also found in case of the IL [$C_{10}C_{10}$im][Cl] containing two decyl side chains. In general, [$C_{10}C_{10}$im][Cl] was found to be the IL with the highest antimicrobial activity showing an average MIC of 1.8 mg/L, being even lower than the MICs of all QACs that are established biocides. Additionally, while all QACs as well as structurally similar ILs ([C_{10}mim][Cl] and [DC_8DMA][Cl]) are affected by the RND transporter in both bacterial species, for [$C_{10}C_{10}$im][Cl] no differences between the four *E. coli* strains and only a marginal effect for *S. enterica* (1.4x higher MIC) were found. These results demonstrate the possibility to design and obtain ILs that are not affected by multidrug efflux pumps belonging to the RND transporter type.

CONCLUSION

This study aimed at investigating the effect of one of the most important multidrug efflux pump type belonging to the RND,

which is associated with biocide and antibiotic resistance, in regard to the antimicrobial activity of ILs. By comparing the activity of antimicrobial ILs against both the wild-type strains and the respective efflux pump deletion mutants in two different bacterial species, the impact of the efflux pump could be directly assessed.

Investigating 10 different antimicrobial ILs with different cation structures enabled the identification of structure–activity relationships (SARs) in regard to efflux pump impact. The results of this study confirmed known SARs, such as the side-chain effect, for all tested strains as well as the general effect of efflux pumps. For all antimicrobial ILs with one elongated alkyl side chain, a clear and significant effect of the efflux pump in both species could be determined, regardless of the length of the side chain, the cationic head group, or the MIC. These results demonstrate that such ILs affect bacterial cells mostly intracellularly, where ILs have been reported to act in multiple ways including destabilizing proteins and hindering their enzymatic activity (Mester et al., 2019), increasing oxidative stress (Yu et al., 2016), or leading to DNA damage (Kowalczyk et al., 2018). In contrast, for ILs with multiple side chains, this study could identify for the first antimicrobial ILs that were not affected by the multidrug efflux pump. While for ILs with two and three octyl side chains ([DC_8DMA][Cl] and [TC_8MA][Cl]) the efflux pump significantly increased the MIC for both strains, in case of the structurally similar [TC_8MP][Cl] no effect could be observed for *E. coli*. The antimicrobial IL that was found to be least affected by the efflux pump was [$C_{10}C_{10}$im][Cl], which was also the one with the highest antimicrobial activity of all investigated ILs, making it the most promising candidate as a novel biocide against multidrug-resistant bacterial species. At this point, it cannot be ultimately determined if the reduced effect of the efflux pump is either due to a different mode of action of these ILs, for example, a direct interaction with the cell membrane (Mester et al., 2016), or if the efflux pump is simply less effective to transport the ILs out of the cell. To further improve the antimicrobial activity, new ILs with the $[C_{10}C_{10}im]^+$ cation as a lead structural motif can be designed and should be investigated against a broader set of resistant bacterial strains from clinical and environmental sources.

Overall, this study demonstrated the effect of efflux pumps belonging to the RND as an intrinsic defense mechanism of Gram-negative bacteria against antimicrobial ILs. The results demonstrate that by studying the effect of individual efflux pumps, structural motifs of antimicrobial ILs can be identified that are not affected by these efflux pumps. As this study focused solely on RND efflux pumps, future studies should include a more diverse set of efflux pump types to investigate possible similarities or differences in regard to the antimicrobial activity of biocidal ILs. Starting from the identified structural motifs in this study, the unique tuneability of ILs can be utilized for the development of effective biocides against MDR bacteria.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TG, PR, and PM: conceptualization. TG and PM: methodology. TG: formal analysis and investigation. PR and RK: resources. TG, PR, RK, and PM: data curation and writing—review and editing. TG and PM: writing. PM: supervision and project administration. PR: funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

The financial support by the Austrian Federal Ministry for Digital and Economic Affairs and the National Foundation of Research, Technology and Development is gratefully acknowledged.

ACKNOWLEDGMENTS

We would like to thank Bernhard Rauch and Birgit Bromberger for their support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.883931/full#supplementary-material

REFERENCES

Andersson, D. I., and Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* 12, 465–478. doi: 10.1038/nrmicro3270

Aschenbrenner, O., Supasitmongkol, S., Taylor, M., and Styring, P. (2009). Measurement of vapour pressures of ionic liquids and other low vapour pressure solvents. *Green Chem.* 11, 1217–1221. doi: 10.1039/B904407H

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., et al. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* 2:2006.0008. doi: 10.1038/msb4100050

Bodro, M., Gudiol, C., Garcia-Vidal, C., Tubau, F., Contra, A., Boix, L., et al. (2014). Epidemiology, antibiotic therapy and outcomes of bacteremia caused by drug-resistant ESKAPE pathogens in cancer patients. *Support Care Cancer* 22, 603–610. doi: 10.1007/s00520-013-2012-3

Borkowski, A., Kowalczyk, P., Czerwonka, G., Cieśla, J., Cłapa, T., Misiewicz, A., et al. (2017). Interaction of quaternary ammonium ionic liquids with bacterial membranes – studies with *Escherichia coli* R1–R4-type lipopolysaccharides. *J. Mol. Liq.* 246, 282–289. doi: 10.1016/j.molliq.2017.09.074

Bromberger, B., Sommer, J., Robben, C., Trautner, C., Kalb, R., Rossmanith, P., et al. (2020). Evaluation of the antimicrobial activity of pyrithione-based ionic liquids. *Sep. Purif. Technol.* 251:117309. doi: 10.1016/j.seppur.2020.117309

Buffet-Bataillon, S., Tattevin, P., Maillard, J.-Y., Bonnaure-Mallet, M., and Jolivet-Gougeon, A. (2016). Efflux pump induction by quaternary ammonium compounds and fluoroquinolone resistance in bacteria. *Future Microbiol.* 11, 81–92. doi: 10.2217/fmb.15.131

CDC on infectious diseases in the United States: 1900-99 (1999). Popul. *Dev. Rev.* 25, 635–640. doi: 10.1111/j.1728-4457.1999.00635.x

Cevasco, G., and Chiappe, C. (2014). Are ionic liquids a proper solution to current environmental challenges? *Green Chem.* 16, 2375–2385. doi: 10.1039/C3GC42096E

Chetri, S., Bhowmik, D., Paul, D., Pandey, P., Chanda, D. D., Chakravarty, A., et al. (2019). AcrAB-TolC efflux pump system plays a role in carbapenem non-susceptibility in Escherichia coli. *BMC Microbiol.* 19:210. doi: 10.1186/s12866-019-1589-1

Chmielowska, C., Korsak, D., Szuplewska, M., Grzelecka, M., Maćkiw, E., Stasiak, M., et al. (2021). Benzalkonium chloride and heavy metal resistance profiles of listeria monocytogenes strains isolated from fish, fish products and food-producing factories in Poland. *Food Microbiol.* 98:103756. doi: 10.1016/j.fm.2021.103756

Cłapa, T., Michalski, J., Syguda, A., Narożna, D., van Oostrum, P., and Reimhult, E. (2021). Morpholinium-based ionic liquids show antimicrobial activity against clinical isolates of *Pseudomonas aeruginosa*. *Res. Microbiol.* 172:103817. doi: 10.1016/j.resmic.2021.103817

Cole, M. R., Li, M., El-Zahab, B., Janes, M. E., Hayes, D., and Warner, I. M. (2011). Design, synthesis, and biological evaluation of β-lactam antibiotic-based imidazolium- and pyridinium-type ionic liquids. *Chem. Biol. Drug Des.* 78, 33–41. doi: 10.1111/j.1747-0285.2011.01114.x

Cook, K., Tarnawsky, K., Swinton, A. J., Yang, D. D., Senetra, A. S., Caputo, G. A., et al. (2019). Correlating lipid membrane permeabilities of imidazolium ionic liquids with their cytotoxicities on yeast, bacterial, and mammalian cells. *Biomol. Ther.* 9:251. doi: 10.3390/biom9060251

Dam, S., Pagès, J.-M., and Masi, M. (2018). Stress responses, outer membrane permeability control and antimicrobial resistance in enterobacteriaceae. *Microbiology* 164, 260–267. doi: 10.1099/mic.0.000613

Duze, S. T., Marimani, M., and Patel, M. (2021). Tolerance of listeria monocytogenes to biocides used in food processing environments. *Food Microbiol.* 97:103758. doi: 10.1016/j.fm.2021.103758

Ferraz, R., Branco, L. C., Prudencio, C., Noronha, J. P., and Petrovski, Z. (2011). Ionic liquids as active pharmaceutical ingredients. *ChemMedChem* 6, 975–985. doi: 10.1002/cmdc.201100082

Gadea, R., Fernández Fuentes, M. Á., Pérez Pulido, R., Gálvez, A., and Ortega, E. (2017). Effects of exposure to quaternary-ammonium-based biocides on antimicrobial susceptibility and tolerance to physical stresses in bacteria from organic foods. *Food Microbiol.* 63, 58–71. doi: 10.1016/j.fm.2016.10.037

Grande Burgos, M. J., Fernández Márquez, M. L., Pérez Pulido, R., Gálvez, A., and Lucas López, R. (2016). Virulence factors and antimicrobial resistance in Escherichia coli strains isolated from hen egg shells. *Int. J. Food Microbiol.* 238, 89–95. doi: 10.1016/j.ijfoodmicro.2016.08.037

Guidi, F., Orsini, M., Chiaverini, A., Torresi, M., Centorame, P., Acciari, V. A., et al. (2021). Hypo-and hyper-virulent listeria monocytogenes clones persisting in two different food processing plants of Central Italy. *Microorganisms* 9, 1–21. doi: 10.3390/microorganisms9020376

Gundolf, T., Rauch, B., Kalb, R., Rossmanith, P., and Mester, P. (2018). Influence of bacterial lipopolysaccharide modifications on the efficacy of antimicrobial ionic liquids. *J. Mol. Liq.* 271, 220–227. doi: 10.1016/j.molliq.2018.08.134

Hansen, L. H., Jensen, L. B., Sørensen, H. I., and Sørensen, S. J. (2007). Substrate specificity of the OqxAB multidrug resistance pump in *Escherichia coli* and selected enteric bacteria. *J. Antimicrob. Chemother.* 60, 145–147. doi: 10.1093/jac/dkm167

Horiyama, T., Yamaguchi, A., and Nishino, K. (2010). TolC dependency of multidrug efflux systems in salmonella enterica serovar Typhimurium. *J. Antimicrob. Chemother.* 65, 1372–1376. doi: 10.1093/jac/dkq160

Hough, W. L., Smiglak, M., Rodriguez, H., Swatloski, R. P., Spear, S. K., Daly, D. T., et al. (2007). The third evolution of ionic liquids: active pharmaceutical ingredients. *New J. Chem.* 31, 1429–1436. doi: 10.1039/b706677p

Kalb, R. S., Stepurko, E. N., Emel'yanenko, V. N., and Verevkin, S. P. (2016). Carbonate based ionic liquid synthesis (CBILS[registered sign]): thermodynamic analysis. *Phys. Chem. Chem. Phys.* 18, 31904–31913. doi: 10.1039/c6cp06594e

Kalb, R., Wesner, W., Hermann, R., Kotschan, M., Schelch, M., and Staber, W. (2005). WO2005021484. Verfahren zur Herstllung ionischer Flüssigkeiten, ionischer Feststoffe oder Gemische derselben.

Kowalczyk, P., Borkowski, A., Czerwonka, G., Cłapa, T., Cieśla, J., Misiewicz, A., et al. (2018). The microbial toxicity of quaternary ammonium ionic liquids is dependent on the type of lipopolysaccharide. *J. Mol. Liq.* 266, 540–547. doi: 10.1016/j.molliq.2018.06.102

Martínez-Suárez, J. V., Ortiz, S., and López-Alonso, V. (2016). Potential impact of the resistance to quaternary ammonium disinfectants on the persistence of listeria monocytogenes in food processing environments. *Front. Microbiol.* 7:638. doi: 10.3389/fmicb.2016.00638

Mester, P., Gundolf, T., Kalb, R., Wagner, M., and Rossmanith, P. (2015). Molecular mechanisms mediating tolerance to ionic liquids in *Listeria monocytogenes*. *Sep. Purif. Technol.* 155, 32–37. doi: 10.1016/j.seppur.2015.01.017

Mester, P., Jehle, A. K., Leeb, C., Kalb, R., Grunert, T., and Rossmanith, P. (2016). FTIR metabolomic fingerprint reveals different modes of action exerted by active pharmaceutical ingredient based ionic liquids (API-ILs) on: salmonella typhimurium. *RSC Adv.* 6, 32220–32227. doi: 10.1039/c5ra24970h

Mester, P., Robben, C., Witte, A. K., Kalb, R., Ehling-Schulz, M., Rossmanith, P., et al. (2019). FTIR spectroscopy suggests a revised mode of action for the cationic side-chain effect of ionic liquids. *ACS Comb. Sci.* 21, 90–97. doi: 10.1021/acscombsci.8b00141

Morrissey, S., Pegot, B., Coleman, D., Garcia, M. T., Ferguson, D., Quilty, B., et al. (2009). Biodegradable, non-bactericidal oxygen-functionalised imidazolium esters: a step towards 'greener' ionic liquids. *Green Chem.* 11:475. doi: 10.1039/b812809j

Poole, K. (2002). Mechanisms of bacterial biocide and antibiotic resistance. *J. Appl. Microbiol.* 92, 55S–64S. doi: 10.1046/j.1365-2672.92.5s1.8.x

Poole, K. (2004). Efflux-mediated multiresistance in gram-negative bacteria. *Clin. Microbiol. Infect.* 10, 12–26. doi: 10.1111/j.1469-0691.2004.00763.x

Poole, K. (2005). Efflux-mediated antimicrobial resistance. *J. Antimicrob. Chemother.* 56, 20–51. doi: 10.1093/jac/dki171

Poole, K. (2007). Efflux pumps as antimicrobial resistance mechanisms. *Ann. Med.* 39, 162–176. doi: 10.1080/07853890701195262

Prudêncio, C., Vieira, M., Van der Auweraer, S., and Ferraz, R. (2020). Recycling old antibiotics with ionic liquids. *Antibiotics* 9, 1–16. doi: 10.3390/antibiotics9090578

Putman, M., Van Veen, H. W., and Konings, W. N. (2000). Molecular properties of bacterial multidrug transporters. *Microbiol. Mol. Biol. Rev.* 64, 672–693. doi: 10.1128/MMBR.64.4.672-693.2000

Qiao, M., Ying, G.-G., Singer, A. C., and Zhu, Y.-G. (2018). Review of antibiotic resistance in China and its environment. *Environ. Int.* 110, 160–172. doi: 10.1016/j.envint.2017.10.016

Rogers, R. D., and Seddon, K. R. (2003). Ionic Liquids-Solvents of the Future? *Science* 302, 792–793.

Ruegg, T. L., Kim, E.-M., Simmons, B. A., Keasling, J. D., Singer, S. W., Soon Lee, T., et al. (2014). An auto-inducible mechanism for ionic liquid resistance in microbial biofuel production. *Nat. Commun.* 5:3490. doi: 10.1038/ncomms4490

Shafaati, M., Boroumand, M., Nowroozi, J., Amiri, P., and Kazemian, H. (2016). Correlation between qacE and qacEΔ1 efflux pump genes, antibiotic and disinfectant resistant among clinical isolates of *E. coli*. *Recent Pat. Antiinfect. Drug Discov.* 11, 189–195. doi: 10.2174/1574891X11666160815094718

Szmolka, A., and Nagy, B. (2013). Multidrug resistant commensal Escherichia coli in animals and its impact for public health. *Front. Microbiol.* 4:258. doi: 10.3389/fmicb.2013.00258

Tarannum, A., Jonnalagadda, R. R., and Nishter, N. F. (2019). Stability of collagen in ionic liquids: ion specific Hofmeister series effect. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* 212, 343–348. doi: 10.1016/j.saa.2019.01.029

Tezel, U., and Pavlostathis, S. G. (2015). Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology. *Curr. Opin. Biotechnol.* 33, 296–304. doi: 10.1016/j.copbio.2015.03.018

Venkata Nancharaiah, Y., Reddy, G. K. K., Lalithamanasa, P., and Venugopalan, V. P. (2012). The ionic liquid 1-alkyl-3-methylimidazolium demonstrates comparable antimicrobial and antibiofilm behavior to a cationic surfactant. *Biofouling* 28, 1141–1149. doi: 10.1080/08927014.2012.736966

Wasserscheid, P., and Welton, T. (eds.) (2008). in *Ionic Liquids in Synthesis. Vol. 1* (Weinheim: Wiley-Vc).

Weyhing-Zerrer, N., Gundolf, T., Kalb, R., Oßmer, R., Rossmanith, P., and Mester, P. (2017). Predictability of ionic liquid toxicity from a SAR study on different systematic levels of pathogenic bacteria. *Ecotoxicol. Environ. Saf.* 139, 394–403. doi: 10.1016/j.ecoenv.2017.01.055

Yamasaki, S., Nagasawa, S., Fukushima, A., Hayashi-nishino, M., and Nishino, K. (2013). Cooperation of the multidrug efflux pump and lipopolysaccharides

in the intrinsic antibiotic resistance of salmonella enterica serovar Typhimurium. *J. Antimicrob. Chemother.* 68, 1066–1070. doi: 10.1093/jac/dks528

Yu, Z., Mo, L., Zhang, J., and Liu, S. (2016). Time- and anion-dependent stimulation on triphosphopyridine nucleotide followed by antioxidant responses in *Vibrio fischeri* after exposure to 1-ethyl-3-methylimidazolium salts. *Chemosphere* 163, 452–460. doi: 10.1016/j.chemosphere.2016.08.061

Conflict of Interest: RK is employed by Proionic GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 01 June 2022
doi: 10.3389/fmicb.2022.862080

Optimization of *Monascus purpureus* for Natural Food Pigments Production on Potato Wastes and Their Application in Ice Lolly

Hossam E. F. Abdel-Raheam[1], *Sulaiman A. Alrumman*[2], *Samir I. Gadow*[3], *Mohamed H. El-Sayed*[4], *Dalia M. Hikal*[5], *Abd El-Latif Hesham*[6]* *and Maysa M. A. Ali*[7]

[1] Department of Food Sciences, Faculty of Agriculture, Beni-Suef University, Beni Suef, Egypt, [2] Biology Department, College of Science, King Khalid University, Abha, Saudi Arabia, [3] Department of Agricultural Microbiology, National Research Centre, Agriculture and Biology Research Institute, Cairo, Egypt, [4] Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Cairo, Egypt, [5] Nutrition and Food Science, Home Economics Department, Faculty of Specific Education, Mansoura University, Mansoura, Egypt, [6] Department of Genetics, Faculty of Agriculture, Beni-Suef University, Beni Suef, Egypt, [7] Botany and Microbiology Department, Faculty of Science, Assuit University, Assuit, Egypt

OPEN ACCESS

Edited by:
Laurent Dufossé,
Université de La Réunion, France

Reviewed by:
Lourdes Morales-Oyervides,
Universidad Autónoma de Coahuila, Mexico
Farhan Binti Mohd Said,
Universiti Malaysia Pahang, Malaysia

***Correspondence:**
Abd El-Latif Hesham
hesham_egypt5@agr.bsu.edu.eg

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *27 January 2022*
Accepted: *04 April 2022*
Published: *01 June 2022*

Citation:
Abdel-Raheam HEF, Alrumman SA, Gadow SI, El-Sayed MH, Hikal DM, Hesham AE-L and Ali MMA (2022) Optimization of Monascus purpureus for Natural Food Pigments Production on Potato Wastes and Their Application in Ice Lolly. Front. Microbiol. 13:862080. doi: 10.3389/fmicb.2022.862080

During potato chips manufacturing, large amounts of wastewater and potato powder wastes are produced. The wastewater obtained at washing after cutting the peeled potatoes into slices was analyzed, and a large quantity of organic compounds and minerals such as starch (1.69%), protein (1.5%), total carbohydrate (4.94%), reducing sugar (0.01%), ash (0.14%), crude fat (0.11%), Ca (28 mg/L), Mg (245 mg/L), Fe (45.5 mg/L), and Zn (6.5 mg/L) were recorded; these wastes could be considered as valuable by-products if used as a fermentation medium to increase the value of the subsequent products and to exceed the cost of reprocessing. In this study, we used wastewater and potato powder wastes as a growth medium for pigment and biomass production by *Monascus purpureus* (Went NRRL 1992). The response surface methodology was used to optimize total pigment and fungal biomass production. The influence of potato powder waste concentration, fermentation period, and peptone concentration on total pigment and biomass production was investigated using the Box-Behnken design method with 3-factors and 3-levels. The optimal production parameters were potato powder waste concentration of 7.81%, fermentation period of 12.82 days, and peptone concentration of 2.87%, which produced a maximum total pigment of 29.86 AU/ml that include, respectively, a maximum biomass weight of 0.126 g/ml and the yield of pigment of 236.98 AU/g biomass. The pigments produced were used as coloring agents for ice lolly. This study has revealed that the ice lolly preparations supplemented with these pigments received high acceptability. Finally, we recommend using wastewater and potato powder wastes for pigment and biomass production, which could reduce the cost of the pigment production process on an industrial scale in the future.

Keywords: potato powder wastes, *Monascus purpureus*, pigment, response surface methodology, chips manufacturing wastewater, ice lolly

INTRODUCTION

Horticulture production has significantly increased to 23.7 million tons of food per day during the last 50 years. Some portion of biomass from agriculture products generates waste, which may not be used as food (Duque-Acevedo et al., 2020). In addition, industrial production consumes a significant amount of soil and water (Aguilera et al., 2020).

According to Kot et al. (2020), there is an increased need for biotechnological methods for biorefinery of the industrial waste to use as ingredients of culture media for microorganisms. This provides the complete biodegradation of organic compounds and the production of a new product with the added value. Moreover, the use of waste products as medium components reduces the total production costs. The sustainability of industrial production becomes concerned with the recovery and reuse of their wastes as a resource within the cycle of the circular economy (Günerhan et al., 2020).

Nowadays, there are intensive interests in environmental protection, which become one of the priorities of international politics. The accumulation of industrial wastes plays a significant role in the degradation of the environment, especially waste products of the food industry, as they contain a large amount of organic substances. Pathak et al. (2018) reported that ∼0.16 tons of solid waste are obtained per ton of processed potato.

Large amounts of water are necessary to manufacture potato chips for washing, peeling, and blanching the raw material (**Figure 1**). Liquid waste generated from these operations is characterized by high starch content ranging from 20 to 25% g/L (Haung et al., 2003). After settling this water, large amounts of starch and solids are produced. These sediments could be used as the culture medium to produce valuable products by microorganisms at a low cost.

Recently, microbial pigments have received intensive attention because they are characterized by many medicinal properties, nutritional effects, and controllable and predictable yield (Global Food Color Market Research Report, 2021), mainly produced from agro-industrial by-products. *Monascus purpureus* is one of the most important producers of microbial pigments. Various food industry wastes were used to produce *Monascus* pigments in the literature. These are hydrolyzed rice straw (Liu et al., 2020), waste beer (Atalay et al., 2020), orange peels (Kantifedaki et al., 2018), chicken feather (Orak et al., 2018), sugarcane bagasse (Hilares et al., 2018), and potato powder (Sharmila et al., 2013). It produces a complex mixture of six chemically defined colored compounds of polyketide origin (Campoy et al., 2006). These compounds are rubropunctatine, monascorubrine (orange), rubropunctamine, monascorubramine (red), monascine, and ankaflavine (yellow) (Zhou et al., 2009). The red pigment is in high demand, especially for its use in meat products to substitute nitrites (Fabre et al., 1993). *Monascus* pigments are used for the natural coloring of oriental foodstuffs in Asian countries and the textile dyeing process (Santis et al., 2005; Velmurugan et al., 2010).

The *Monascus* pigments have been of increasing interest to the food industry as food colorants because *Monascus* products are extracellular and water-soluble making them easy to use. *Monascus* pigments applications include the increased red coloring in meat, fish, and ketchup (Hamano and Kilikian, 2006). It can also be added to dairy products such as fruit-flavored yogurt (Abdel-Raheam et al., 2019) and flavored milk (Gomah et al., 2017) for enhancing the natural color of the fruit. Similarly, it can be used for poultry products such as poultry salami (Mal'a et al., 2010) and for sweet products such as lollipops, jelly beans (Darwesh et al., 2020), and drops sweets (Abdel-Raheam et al., 2021). The results by previous investigators reported that food products gain more intense and stable color and improved organoleptic characteristics when *M. purpureus* pigments were used (Hamano and Kilikian, 2006 and Mal'a et al., 2010; Gomah et al., 2017; Abdel-Raheam et al., 2019, 2021; Darwesh et al., 2020).

Production of *Monascus* pigments by submerged fermentation was preferred instead of the solid-state process due to its easy control of process parameters, high productivity, large volume processing, reduced fermentation time, and cost (Zhou et al., 2014). It can also solve the problems of low income, capital intensive, time-consuming, and the necessity of large surface area in the case of solid fermentation. Moreover, this method is more appropriate for large-scale industrial production than solid-state production (Yuliana et al., 2017).

Response surface methodology RSM is successfully applied to optimize medium components and process parameters in various bioprocesses (Kalaivani and Rajasekaran, 2014). It is well-known that optimization using one parameter study is high cost and expends time, whereas response surface methodology (RSM) being reliable and easy to use reduces the number of experiment runs, and it also details the interaction effect between the variables involved in the process. Also, it is fast, low cost, and statistically acceptable for carrying out research in comparison to the usual one variable study (Ajdari et al., 2013). It was reported that response surface methodology (RSM) is a practical statistical approach for the optimization of pigment production (Sen et al., 2019).

This study investigates the biorefinery of potato chips manufacturing wastewater obtained at washing after potatoes cutting into slices for pigment production during submerged fermentation by *M. purpureus* Went NRRL 1992. It also aimed to optimize pigment and biomass production conditions by using RSM. Separation of red, orange, and yellow *Monascus* pigments from optimized culture and individually applying the separated pigments in ice lolly was also mainly investigated.

MATERIALS AND METHODS

Wastes Used

First, potato chips manufacturing wastewater of cutting (slicing) and soaking was obtained from a potato chips factory at Assuit Governorate, Egypt.

Second, potato chips waste powder produced after settling wastewater and then dried under

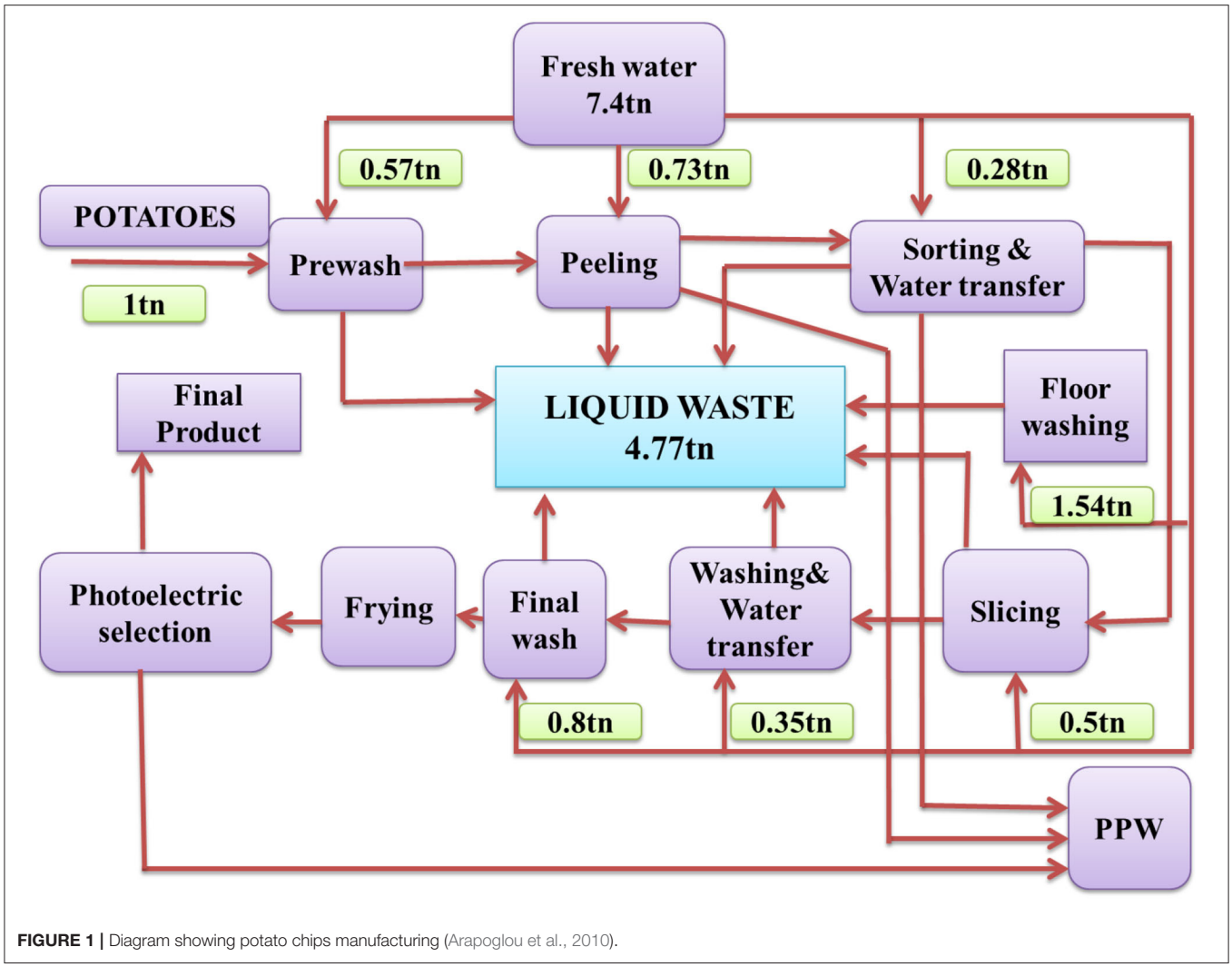


FIGURE 1 | Diagram showing potato chips manufacturing (Arapoglou et al., 2010).

sunlight was packed in polyethylene pages at 4°C until use.

Chemical Composition Analysis of Potato Chips Manufacturing Wastewater

The contents of crude protein, crude fat, ash, reducing sugar, and carbohydrate as a percentage (W/V) were determined using standard methods of the Association of Official Analytical Chemists (AOAC, 2005).

The mineral contents of potato chips manufacturing wastewater were determined by Perkin-Elmer Atomic Absorption Spectrophotometer 2,380 for calcium, iron, zinc, and magnesium. The determination was carried out in the Central Laboratory, Faculty of Agriculture, Assiut University, as described in AOAC (1995).

Microorganism

The strain used in this study was *M. purpureus* Went NRRL 1992 obtained from Microbiological Resources Center (MIRCEN), Ain Shams University Cairo, Egypt. It was maintained on a Yeast Extract–Peptone–Dextrose (YEPD) agar medium (1% yeast extract, 2% peptone, and 2% dextrose) at 4°C and subcultured periodically every 3 weeks. This strain was tested for its ability to produce citrinin as mycotoxin and found to be nonproducing (Abdel-Raheam, 2016). Inoculum preparation *M. purpureus* Went NRRL 1992 was grown on YEPD slant at 30°C. To fully sporulated (6–8 days old) agar slope culture, 10 ml of sterile distilled water was added and the spores were scraped under strict aseptic conditions. The spores suspension obtained was used as inoculum ($\sim 7 \times 10^5$ spores per ml) as described by Babitha et al. (2007).

Fermentation Medium

Submerged cultures were carried out at a 250-ml Erlenmeyer flask with a working volume of 100 ml; the fermentation medium was composed mainly of potato chips manufacturing wastewater. Different concentrations of potato waste powder as carbon source and different peptone concentrations as nitrogen source were used as supplements for wastewater. Flask contents were mixed well, fermentation medium was adjusted at pH 6.5, and

TABLE 1 | Values of independent variables in the Box-Behnken design.

Parameters (independent variables)	Units	Coded levels		
		−1	0	+1
Potato wastes (A)	%	5	7.5	10
Fermentation period (B)	day	7	10	13
Peptone concentration (C)	%	2	3.5	5

then autoclaved at 121°C for 15 min after cooling at room temperature; flasks were inoculated with 10 ml fungal spore suspension and incubated at 30°C in the dark for the different incubation periods.

Optimization of Pigment and Biomass Production and Experimental Design

The optimum conditions for total pigment production and biomass of *M. purpureus* Went NRRL 1992 were evaluated using response surface methodology (RSM) provided by Design Expert Software (2011) with a standard tool known as Box-Behnken design (BBD) (Box and Behnken, 1960) for testing the effect of independent variables and their interaction. Three factors were chosen based on preliminary tests for this work: potato powder (A), fermentation period (B), and peptone concentration (C). Three levels of each factor were included (**Table 1**).

The pigment production response and biomass are estimated by a model equation RSM second order as follows:

$$Y = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2. \quad (1)$$

Where Y is the dependent variable, and A, B, and C are independent variables.

Determination of Pigment

At the end of the fermentation process, the medium was filtered using Whatman No. 1 filter paper, and the mycelia were washed twice with distilled water. The extracellular pigments were determined at the filtrate, which was measured by using a UV visible spectrophotometer (Abilene 9400—SCHOTT Instruments, EU) at 400, 470, and 500 nm for yellow, orange, and red pigments, respectively (Carels and Shepherd, 1977; Lin et al., 1992; Orozco and Kilikian, 2008) (Equation 3).

The intracellular pigment was extracted from the washed mycelia by transferring (0.1 g) to a 50-ml Erlenmeyer flask and 10 ml of ethanol 95% was added. The suspension was allowed to stand at 30°C for 12 h (Tseng et al., 2000). The supernatant was then centrifuged (using Himac CR 22GII, Hitachi Koki Company Limited, Japan) at 4,000 rpm for 10 min. The absorbance of the clear solution was measured at 500, 470, and 400 nm using a UV visible spectrophotometer (Chen and Johns, 1993) (Equation 4). Extra- and intracellular pigments yield in absorbance unit (AU) per ml were calculated by using the following formula (Mekhael and Yousif, 2009) (Equation 2). The yield (AU/g) was defined as the total pigments produced by every gram of biomass.

$$AU_{\text{Total pigment}} = AU_{\text{Total extra pigment}} + AU_{\text{Total intra pigment}} \quad (2)$$

$$AU_{\text{Total extrapigment}} = AU_{\text{Extra}} \times df \quad (3)$$

$$AU_{\text{Total intrapigment}} = (AU_{\text{Intra}} \times df)/(\text{weight of sample, g}) \quad (4)$$

Determination of Biomass

The total fungal biomass was determined by measuring fresh weight per ml of fermentation medium after filtration through Whatman No. 1 filter paper, and the mycelia were washed two times with distilled water.

Separation of Pigments for Application in the Preparation of Ice Lolly Products

The red, orange, and yellow pigments were separated and purified individually from the submerged culture as follows:

First: Separation of Red and Yellow Pigments Individually

The red and yellow pigments were extracted successively with ethyl acetate from culture filtrate (1:1 v/v), which was adjusted to pH 3.0 with 2 N HCl as shown in **Figure 2**. The ethyl acetate layer contains a mixture of red and yellow pigments. The ethyl acetate layer was eluted by a separating funnel. The ethyl acetate extract was evaporated in vacuo at 50°C. The semidry residue was dissolved in n-hexan and filtered through a filter paper (Whatman, No. 1; International Ltd., Maidstone, England) to separate the yellow pigment (the filtrate) from the red pigment (the residue). Separately yellow and red pigments were redissolving in a small amount of water by adjusting the pH to 7.0 with 2 N NaOH.

In addition, the yellow pigment can be separated from the ethyl acetate extract by evaporating the extract in vacuo at 50°C. The semidry residue was re-dissolved in a small amount of water by adjusting the pH to 7.0 with 2 N NaOH. Then 50 ml of cold acetone was added and kept at 4*C* for 30 min to precipitate yellow pigment. The acetone layer containing red pigment was collected, filtered, and concentrated further, and then taken in 20 ml of water. The yellow crystals, which settled at the bottom of the flask, were dissolved in 20 ml of water and kept in a glass vial.

Second: The Separation of Orange Pigments

The orange pigment, which eluted from the separation funnel, was collected, filtered, and concentrated further by evaporated in vacuo at 50*C*, and then taken in 20 ml of water separated water-soluble red, orange, and yellow pigments were used separately to prepare red, orange, and yellow ice lolly.

Application of the Separated Pigments for the Coloring of Ice Lolly

The traditional methods manufactured ice lolly, and red, orange, and yellow pigments were added to the ingredients and then

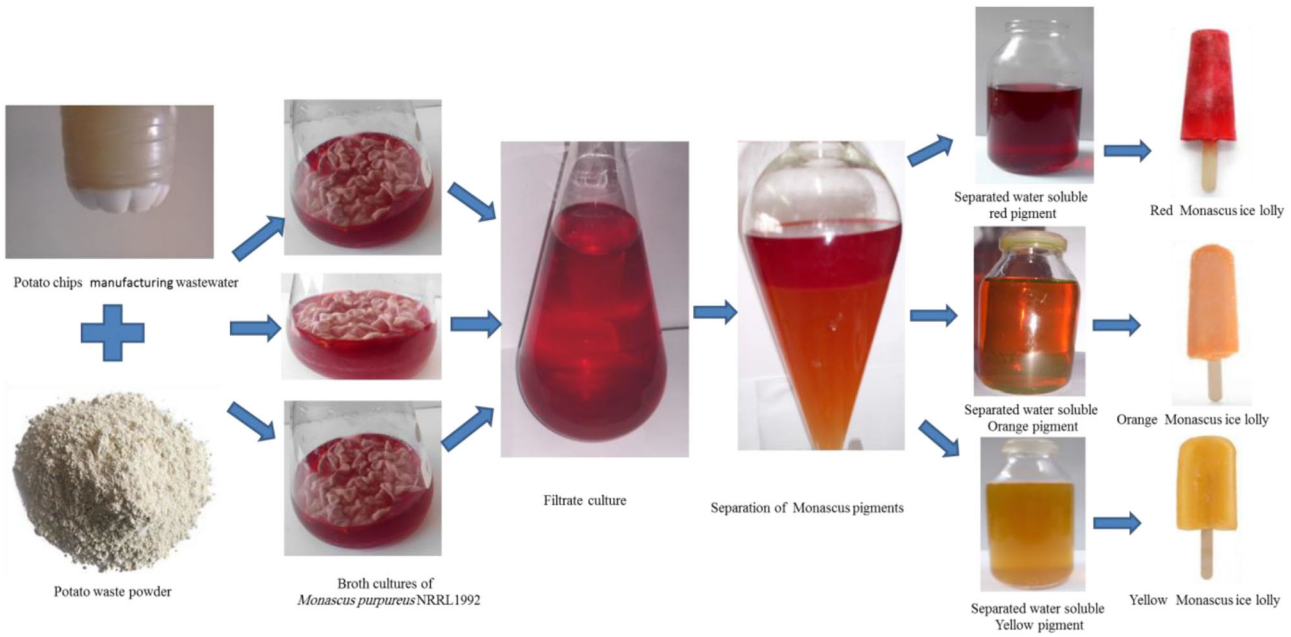


FIGURE 2 | Production and separation of *Monascus purpureus* NRRL 1992 pigments and their applications in ice lolly.

flavored with strawberry, orange, and banana, respectively. For ice lollies manufacturing, mix was standardized to sugar (20 %), stabilizer (0.5%), citric acid (0.3%), color (0.05%), flavor (0.45%), and water. Selecting ingredients, figuring the mix, blending, pasteurizing (68°C/30 min), transferring into molds, freezing (−18°C), and packing/storing until subjected to sensory evaluation (as described by Arbuckle, 1972).

Attributes of color, taste, odor, texture, and overall acceptability were tested using the slandered scorecard.

Sensory Evaluation

A total of 10 trained panelists conducted sensory analyses of the samples (red, orange, and yellow ice lolly) according to the method described by Reitmeier and Nonnecke (1991). Attribute ratings were analyzed by analysis of variance and organoleptic tests (taste, color, texture, odor, and overall acceptability) using 10-point hedonic scales.

RESULTS AND DISCUSSION

Chemical Composition Analysis

The wastewater used for experimental work was collected from the potato chips industry. The wastewater was obtained after the cutting (slicing) and soaking process, so this water contains a low microbial charge. The results obtained from the chemical analysis of the wastewater are presented in **Table 2**.

The results show that the wastewater of cutting (slicing) and soaking from the potato chips manufacturing process contains a high amount of calcium and magnesium minerals and organic compounds of crude protein, starch, and carbohydrate. It also has a low content of crude fat, ash, reducing sugar, and zinc and iron minerals. As a result, these wastes could be considered valuable by-products if used as a fermentation medium to increase the value of the subsequent products and exceed the cost of reprocessing.

This study results are rather different from the study described by Gautam et al. (2017) who reported that wastewater of cutting (slicing) and soaking in potato chips industry contains 1.5% starch and it has a pH of 6.54.

Pigment and Biomass Production

The results of BBD experimental design include 17 runs of 3 independent variables (potato wastes concentration, incubation period, and peptone concentration) for optimizing pigment production and biomass (dependent variables) by *M. purpureus* Went NRRL were listed in **Table 3**. The statistical test of total pigment and biomass produced was developed using analysis of variance (ANOVA) as shown in **Tables 3**, **4**, respectively.

Second-order quadratic model equation (Equation 1) (coded units) was used to express total pigment and biomass production as the following:

$$\begin{aligned} Y_{Pigment} &= 16.22 - 4.48A + 1.95B - 13.35C - 1.58AB \\ &\quad + 1.59AC + 1.25BC - 3.02A^2 + 8.29B^2 + 9.51C^2 \\ Y_{Biomass} &= 0.112 + 0.029A + 0.028B + 0.004C + 0.028AB \\ &\quad + 0.019AC - 0.011BC + 0.066A^2 - 0.014B^2 - 0.008C^2 \end{aligned}$$

$Y_{Pigment}$ is the total pigment concentration, $Y_{Biomass}$ is the biomass weight g/ml, (A) potato wastes %, (B) fermentation period days, and (C) peptone concentration %.

In the case of optimization of total pigment production, the model's performance is usually indicated by the plots of

TABLE 2 | Chemical composition of wastewater from potato chips manufacturing.

pH	Carbohydrate (%)	Starch (%)	Reducing sugars (%)	Crude protein (%)	Crude fat (%)	Ash (%)	Zn (mg/L)	Ca (mg/L)	Mg (mg/L)	Fe (mg/L)
7.30	4.94	1.96	0.01	1.50	0.11	0.14	6.50	280.00	245.00	45.5

TABLE 3 | A Box-Behnken experimental design of independent variables and actual results of response (1) total pigment and response (2) biomass produced by *Monascus purpureus* Went NRRL 1992.

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2
	A: potato wastes concentration	B: fermentation period	C: peptone concentration	Total pigment	biomass
	%	days	%	AU/ml	g/ml
1	7.5	10	3.5	13.61	0.11
2	7.5	10	3.5	15.024	0.12
3	10	10	5	10.28	0.213
4	7.5	10	3.5	16.15	0.12
5	5	10	2	44.33	0.168
6	7.5	7	5	16.64	0.081
7	7.5	10	3.5	20.26	0.114
8	10	10	2	25.94	0.17
9	7.5	13	2	48.89	0.12
10	5	13	3.5	29.48	0.128
11	10	13	3.5	17.61	0.262
12	7.5	13	5	22.84	0.11
13	10	7	3.5	16.68	0.144
14	7.5	7	2	47.7	0.046
15	7.5	10	3.5	16.072	0.098
16	5	7	3.5	22.21	0.123
17	5	10	5	10.31	0.132

TABLE 4 | ANOVA for the entire quadratic model of response (1) concentration of total pigment.

Source	Sum of squares	Mean square	*F*-value	*p*-value	
Model	2440.89	271.21	59.88	<0.0001	Significant
A-potato wastes concentration	160.38	160.38	35.41	0.0006	
B-fermentation period	30.38	30.38	6.71	0.0359	
C-peptone concentration	1425.51	1425.51	314.75	<0.0001	
AB	10.05	10.05	2.22	0.1800	
AC	84.27	84.27	18.61	0.0035	
BC	6.28	6.28	1.39	0.2776	
A^2	38.28	38.28	8.45	0.0227	
B^2	289.17	289.17	63.85	<0.0001	
C^2	380.57	380.57	84.03	<0.0001	
Residual	31.70	4.53			
Lack of fit	7.11	2.37	0.3857	0.7701	Not significant
Pure error	24.59	6.15			
Cor. total	2472.59				

the predicted vs. actual results (**Figure 3B**), which showed high correlation coefficients ($R^2 = 0.987$), indicating that the predicted and actual values were in reasonable agreement. The correlation coefficient (R_2) is a tool to check the "goodness of fit" between the experimental and the predicted results (Myers and Montgomery, 1995). In this case, R^2 value of 0.9872 demonstrated that there is 98.7% of fitting between experimental and the predicted results, whereas the remaining percentage of 1.3% may have been due to other uncontrollable variables. The residual analysis was performed to investigate the suitability of the model. This

was carried out by observing the normal probability plot of the residual in **Figure 3A**, where all the points lie on a straight line, suggesting that the errors were distributed evenly.

The adjusted coefficient of determination value R^2adj is equal to 97%. This indicates that the regression equation provides a suitable model for the BBD experiment.

The adequate precision for total pigment is 23.77. SO, these quadratic models were significant for the process. The adequate precision test is used to determine the ratio of the predicted values at the design points to the average prediction error, when the ratio is more than 4; it means the model is acceptable and it can be used to explore the design space.

According to ANOVA (**Table 4**), the model obtained has a significant influence on the significance level (α) of 5% because of the high *F*-value (9.64) and low *p*-value (<0.05). From **Table 4**, all independent variables (potato wastes concentration, incubation period, and peptone concentration) have a significant value ($p < 0.05$) for total pigment production. In addition, the value of the quadratic potato wastes concentration, incubation period, peptone concentration, and the interaction between potato wastes concentration and peptone concentration also showed a significant effect on the production of total pigment. Non-significant lack of fit means that the model is adequate to describe the observed data.

In the case of biomass production, R^2, R^2adj, and Adeq precision were 0.972, 0.937, and 21.59, respectively. The normal probability plot indicated the quality of the adequacy of the current model where all residual points were on a straight line (**Figure 4A**). The predicted vs. actual plot of biomass experiment showed that the data were closely related, indicating that the model was highly fit (**Figure 4B**).

Based on ANOVA, which is listed in **Table 5**, the model of biomass was significant with a *p*-value (0.0001). The effect of potato wastes concentration and fermentation period was significant. Also, the interactions between potato wastes concentration and fermentation period and potato wastes concentration and peptone concentration were significant. The quadric effect of potato wastes concentration and fermentation period was significant.

Total pigment concentration increased with increasing concentration of potato waste, and the maximum amount of total pigment was 48.89 AU/ml at 7.5% potato waste, while the maximum amount of biomass was 0.262 g/ml at 10% potato waste (**Table 3**); this is possible because potato waste was a suitable carbon source for pigment and biomass production, where the main role of carbon in fermentation medium was to produce cell biomass other than as an accelerator for the production of bioactive compounds through the secondary metabolism pathway (**Table 6**). De Carvalho et al. (2014) reported that some carbon sources that can be added include glucose, fructose, maltose, lactose, and galactose. Besides being able to increase pigment compounds and biomass, the right carbon source can produce high secondary metabolites. Also, starch is the main ingredient in rice, which is usually the major carbon source for *Monascus* growth and pigment production (Patakova, 2013; Chen et al., 2015). Abdel-Raheam et al. (2021) used potato chips manufacturing wastes as a suitable substrate for the production of pigment by *Monascus ruber* Went AUMC 5705 on solid-state fermentation. It was reported that the addition of a C source of 50 g/L can increase the growth rate of molds and increase the synthesis of pigment compounds, cell biomass, and ethanol production (Jones, 1998). By increasing the concentration of potato residues, more than 7.8% for the production of pigments, the productivity decreases, which could be explained by the accumulation of inhibitors and fermentation by-products in the fermentation medium. Wong et al. (1981) reported that high glucose concentration in the fermentation medium can be an advantage for mycelium growth, but as fermentation progresses, the fermentation medium becomes more acidic and this can lead to low pigment yields. But a further increase in glucose concentration progressively decreased the specific growth rate. This may have been an osmotic effect (Kim et al., 1997). Osmotic pressure increases with the increasing concentration of glucose and this reduces the water availability for microbial growth.

The presence of nitrogen in molds growing medium is essential, where it is mainly included in primary metabolism, which is essential for microbial living and growth. It is also involved in secondary metabolism, which produced different bioactive compounds. Vidyalakshmi et al. (2009) reported that the addition of nitrogen can increase the metabolic activity of molds and the quantity of pigment compounds can be increased. In this study, peptone was used as a nitrogen source and it affected total pigment and biomass production positively, where the maximum amount of total pigment was 48.89 AU/ml at 2% peptone while 3.5% was the best for maximum biomass production of 0.262 g/ml (**Table 3**).

Celestino et al. (2014) reported that peptone provides many nutrients such as peptides and amino acids to the broth and it seems to be easily metabolized by most fungi, which can lead to increased production of their metabolites, including pigments. Also, they found that the use of nitrogen sources is based on the main substrate and other external factors, each of which also has an important role in the biosynthesis of bioactive compounds by *Monascus* sp.

Mousa et al. (2018) found that peptone was the most suitable nitrogen source among tested nitrogen sources (peptone, monosodium glutamate, yeast extract, sodium nitrate, and malt extract) for red pigment production by *M. purpureus* ATCC16436, and 2.5% of peptone was the best concentration for maximum pigment production. This result was near to our results; it was mentioned in many literatures that the nitrogen in peptone was available as amino acids, which help in the production of more stable extracellular pigment (Zhang et al., 2013; Ahmad and Panda, 2014). Also, peptone as an organic nitrogen source may be used as a carbon source. It may accelerate the protein-bound dissolution of red pigments into the culture broth (Broder and Koehler, 1980) and other previous studies (**Table 6**).

Figure 5 shows that by increasing peptone concentration, the amount of pigment decrease. Mehri et al. (2021) found that the pigment production increased linearly up to 25 g/L of MSG concentration resulting in a maximum pigment synthesis of 45.7 UA_{510nm} and then declined at higher

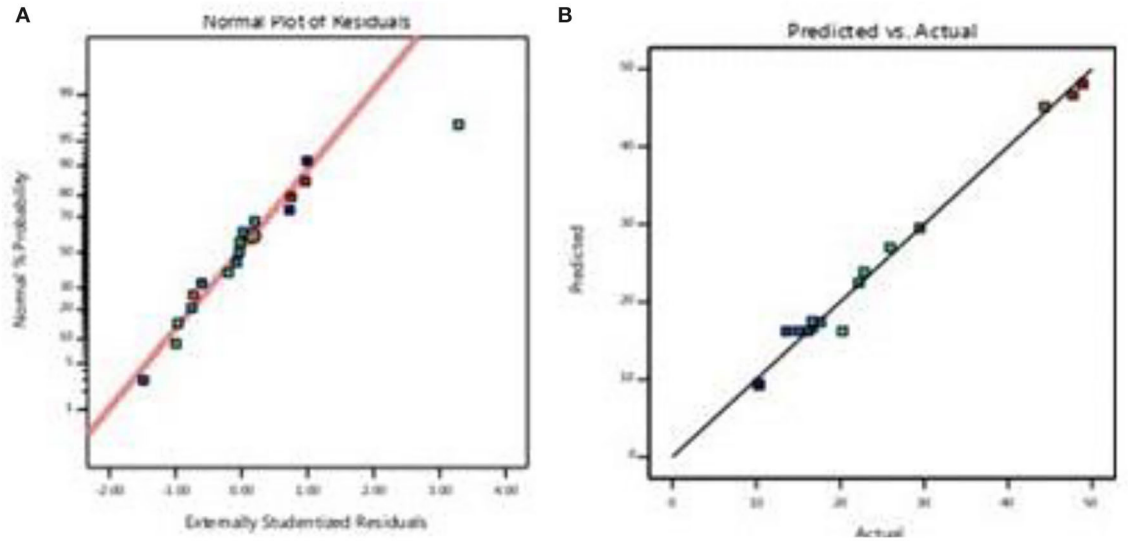


FIGURE 3 | (A) Normal probability and **(B)** predicted vs. actual values.

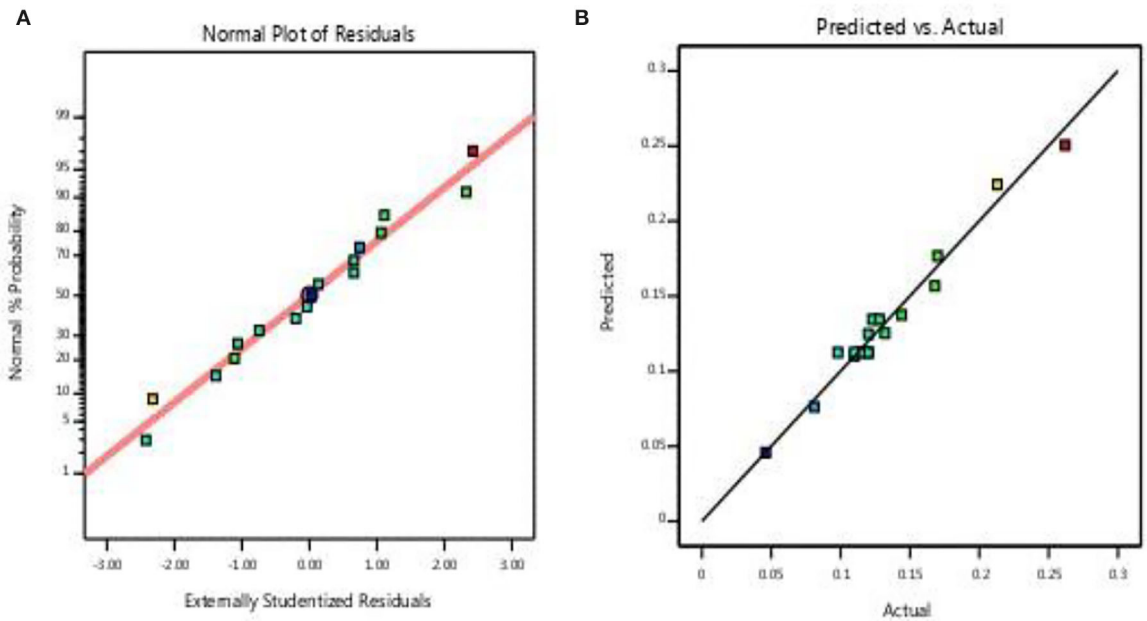


FIGURE 4 | (A) Normal probability and **(B)** predicted vs. actual values.

monosodium glutamate (MSG) concentrations. Raman et al. (2015) found that decreasing the concentration of nitrogen source in the production medium of melanin by *Aspergillus fumigatus* AFGRD105 has a positive effect on pigment production.

While **Figure 6** shows the amount of biomass increase with increasing peptone concentration, this may be because of increasing peptone concentration C/N ratio will decrease, which has an effect on pigment and biomass production. Cho et al. (2002) mentioned that the C/N ratio usually affects the rates of biosynthesis of many metabolites, its influence on mycelia growth, and pigment production in fungi. Horwath (2007) demonstrated that 10–15 C/N ratio was preferred for biomass production, while more than 20 C/N ratio is preferred for pigment production. According to Ajdari et al. (2011), *M. purpureus* DSM1379 strain had the highest radial growth at C/N ratio of 1.8, while the lowest was obtained at C/N ratio of 10.03. On the contrary, *M. purpureus* FTC5357 strain had the highest

TABLE 5 | ANOVA for the entire quadratic model of response (2) concentration of biomass.

Source	Sum of squares	Mean square	*F*-value	*p*-value	
Model	0.0382	0.0042	27.80	0.0001	Significant
A-potato wastes concentration	0.0071	0.0071	46.33	0.0003	
B-fermentation period	0.0064	0.0064	41.78	0.0003	
C-peptone concentration	0.0001	0.0001	0.8376	0.3905	
AB	0.0032	0.0032	20.89	0.0026	
AC	0.0016	0.0016	10.21	0.0152	
BC	0.0005	0.0005	3.31	0.1115	
A^2	0.0187	0.0187	122.49	<0.0001	
B^2	0.0009	0.0009	6.06	0.0434	
C^2	0.0003	0.0003	1.91	0.2095	
Residual	0.0011	0.0002			
Lack of fit	0.0007	0.0002	2.97	0.1600	Not significant
Pure error	0.0003	0.0001			
Cor. total	0.0393				

TABLE 6 | Comparison between current study and others.

Microorganism	Yield of pigment	Statistical analysis	References	Substrate
Monascus purpureus (Went NRRL 1992)	Total pigment 29.86 AU/ml	(RSM)	Current study	Potato wastes
Monascus purpureus CMU001	Red pigment (22.25 UA/ml)	(RSM)	Silbir and Goksungur (2019)	Brewer's spent grain hydrolysate
Monascus purpureus	Total pigment 71.25 (CVU) color value unit	(RSM)	Dikshit and Tallapragada (2017)	Potato dextrose broth
Monascus purpureus (PTCC 5303)	Total pigment 4.38 (ODU/ml)	(RSM)	Seyedin et al. (2015)	Synthetic medium
Monascus purpureus (MTCC 369)	Total pigment 7.18 ODU/ml	(RSM)	Sharmila et al. (2013)	Potato powder
Monascus anka mutant	Yellow pigment 87.24 OD	(RSM)	Zhou et al. (2009)	Synthetic medium

radial growth at C/N ratio of 10.03, while the lowest was observed at C/N ratio of 1.8. They also concluded that the effect of C/N ratio on cell production was strain-dependent.

Fermentation of any substrate is influenced by the time of incubation. A significant increase in total pigment and biomass production of *M. purpureus* Went NRRL 1992 was observed by increasing fermentation period, which reached 12.8 days then they decreased. Sehrawat et al. (2017) found that variations in incubation time to achieve maximum pigment production may be due to strain specificity. Non-availability of nutrients might have ceased the growth of fungus. Johns et al. (1982) suggested that the reduction of pigment production by increasing the fermentation period may be due to pigments being degraded by an enzymatic pathway, which may be induced by nutrient exhaustion where enzyme degradation of secondary metabolites is a common phenomenon in fungi. Chen and Johns (1993) reported that a decrease in pigment production by *M. purpureus* was observed, which might be due to the decomposition of pigments (degradation of the chromophore pigment group or changes in the pigment structure). The incubation period in this study was shorter than that of which reported by Dikshit and Tallapragada (2011), Tallapragada et al. (2013), and Mousa et al. (2018) who reported that 16 days was the best incubation period for pigment production on broth medium by different *Monascus* sp. While Abdel-Raheam et al. (2019) mentioned that 11-day fermentation period gives the maximum amount of pigment by *M. ruber* Went AUMC 5705.

To test the validity of the model, an experiment was repeated in triplicate with optimized values of independent variables suggested by the model for maximum total pigment and a suitable amount of biomass: potato wastes concentration of 7.81%, fermentation period of 12.82 days, and peptone concentration of 2.87%. The amount of total pigment produced was 29.86 ± 1.42 AU/ml, which consisted of red, orange, and yellow pigments were 12.62 ± 0.61, 9.49 ± 0.47, and 7.75 ± 0.44 AU/ml, respectively, and the maximum biomass weight was 0.126 ± 0.003 g/ml, and the yield of pigment was 236.98 AU/g biomass, with 95.92% validity of the predicted model.

Separation of the Produced Pigments

The pigment separation procedure utilized successfully resulted in the fractionation of crude *Monascus* pigments into three

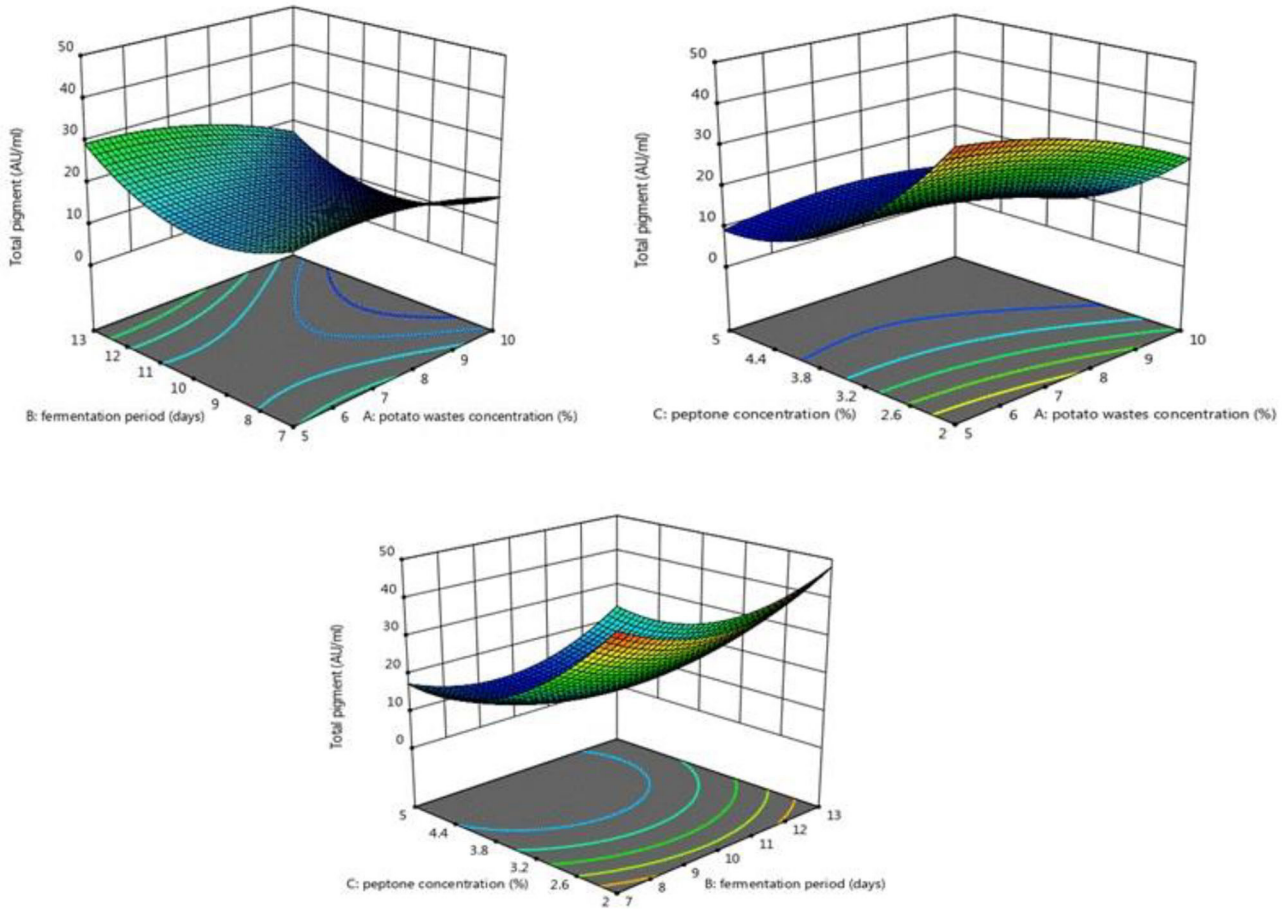


FIGURE 5 | Response surface plots indicated the effect of potato wastes concentration, peptone concentration, and fermentation period on total pigment production.

separate hues, such as red, orange, and yellow, ready for application as food colorants (**Figure 2**).

Sensory Evaluations of the Produced Pigments as Colorant Additives for Ice Lolly

Pigments extracted and separated from submerged *M. purpureus* NRRL 1992 culture were used as colorant additives for ice lolly to enhance its appearance and acceptability. The red-, orange-, and yellow-flavored ice lollies were developed by individually adding the separated *Monascus* pigments. In this study, *M. purpureus* NRRL 1992 pigments were directly mixed with the food products during their preparation to impart red, orange, and yellow pigments individually to these products (**Figure 2**) and improved the aesthetic value.

The prepared ice lolly using *Monascus* pigments as colorants was sensory evaluated for taste, color, texture, odor, and overall acceptability by 10 panelists.

Data in **Table 7** show the average sensory analysis scorecard and total scores for the separated *M. purpureus* NRRL 1992 pigments as natural colors for the butterscotch ice lolly. The overall average scores for the red, orange, and yellow ice lolly samples were 47.1, 46.4, and 46.5.

As indicated in **Table 1**, all produced food product samples colored with *M. purpureus* NRRL 1992 pigments received high ratings in all sensory assessed testing criteria (7).

The average scores for taste, color, texture, odor, and overall acceptability were between 8.8 and 9.9 scored as "like extremely" for all tested food products samples as described by Wang and Zhao (2008). The result showed that incorporating *M. purpureus* NRRL 1992 pigments for coloring prepared food products has proved to be excellent. The pigment is distributed evenly in the food product giving a pleasing appearance.

These results are in agreement with previous investigators who reported that food products gain more intense and stable color and improved organoleptic characteristics when *M. purpureus* pigments were used (Hamano and Kilikian, 2006 and Mal'a et al., 2010; Gomah et al., 2017; Abdel-Raheam et al., 2019, 2021; Darwesh et al., 2020).

Blanc et al. (1995) found that using *M. purpureus* pigment gave food items a more deep and persistent red hue and better organoleptic features. Furthermore, the use of natural pigments protects customers' health by lowering their salt intake and

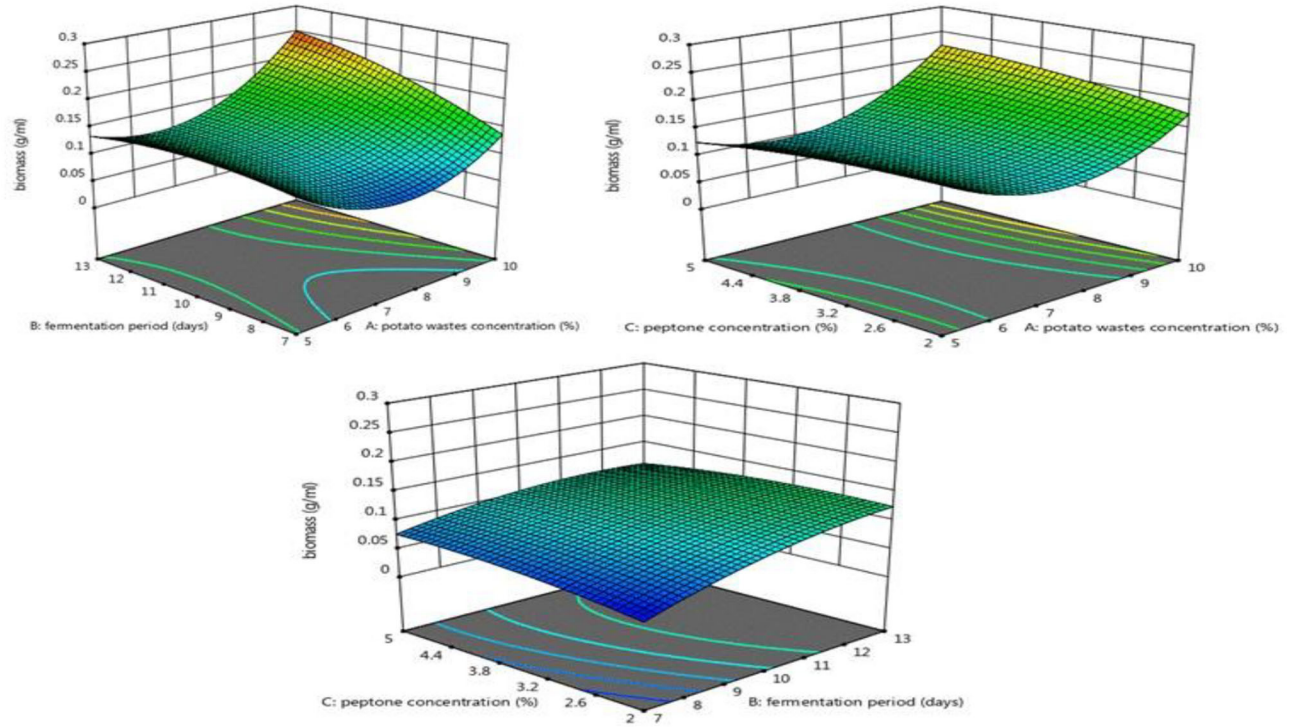


FIGURE 6 | Response surface plots indicated the effect of potato wastes concentration, peptone concentration, and fermentation period on biomass production.

TABLE 7 | Mean sensory scores of ice lolly samples colored with *Monascus purpureus* NRRL 1992 pigments.

Items and score	Taste (10)	Color (10)	Odor (10)	Texture (10)	Overall acceptability (10)	Total score (50)
Name of product						
Red ice lolly	9.2 ± 0.30	9.9 ± 0.10	9.3 ± 0.26	9.2 ± 0.10	9.5 ± 0.26	47.1 ± 0.3
Orange ice lolly	9.4 ± 0.2	8.8 ± 0.36	9.7 ± 0.26	9.5 ± 0.26	9 ± 0.26	46.4 ± 0.79
Yellow ice lolly	9.1 ± 0.46	9.6 ± 0.36	9.5 ± 0.44	9 ± 0.26	9.3 ± 5.29	46.5 ± 1.08

enables the production of totally natural foods without the use of synthetic chemicals (Su et al., 2005; Basuny and Abdel-Raheam, 2020). Traditionally, these pigments were used to make red rice, red wine, sausages, fish sauces, meat items, and soybean curd (Anonymous, 1999).

Vidyalakshmi et al. (2009) reported that *M. ruber* fermented rice (MFR) was employed as a colorant in the manufacturing of food items (Kesari) and had a very nice color and look. They also investigated the use of MFR for coloring flavored milk, which revealed that pigment dispersed uniformly, resulting in an attractive hue and acceptable look with higher acceptance.

CONCLUSION

The utilization of potato waste powder is beneficial in manufacturing pigment from *M. purpureus* Went NRRL 1992. The BoxBehnken (BBD) design was influential in modeling total pigment production as a function of changes in process variables, potato waste concentration, fermentation period, and concentration of peptones. The optimum conditions were potato wastes concentration of 7.81%, fermentation period of 12.82 days, and peptone concentration of 2.87%, which produce total pigments of 29.86 AU/ml. The produced pigments were applied as coloring agents for ice lolly (red, orange, and yellow ice lolly). Applications of these natural colorants in the manufacturing of these products were judged to be extremely acceptable based on sensory assessment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**,

further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

HA-R and MA: investigation, draft writing, and software and methodology. SG and AH: writing and reviewing. SA, MH and DM: reviewing. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.862080/full#supplementary-material

REFERENCES

Abdel-Raheam, H. E. F. (2016). *Production and evaluation of some natural food pigments from Monascus purpureus fungus* (Ph.D. Thesis). Faculty of Agriculture, Assiut University, Asyut, Egypt.

Abdel-Raheam, H. E. F., Abdel-Mageed, W. S., and Abd El-Rahman, M. A. M. (2019). Optimization of production of *Monascus ruber* pigments on broth medium and their application in flavored yogurts. *Egypt. J. Food Sci.* 47, 271–283.

Abdel-Raheam, H. E. F., Hassan, S. H. A., and Ali, M. M. A. (2021). Production and application of natural food pigments by *Monascus ruber* using potato chips manufacturing wastes. *Bull. Pharm. Sci.* 44, 551–563. doi: 10.21608/bfsa.2021.207182

Aguilera, E., Díaz-Gaona, C., García-Laureano, R., Reyes-Palomo, C., Guzmán, G. I., Ortolani, L., et al. (2020). Agroecology for adaptation to climate change and resource depletion in the Mediterranean Region. *A Review. Agric. Syst*. 181, 102809. doi: 10.1016/j.agsy.2020.102809

Ahmad, M., and Panda, B. P. (2014). Optimization of red pigment production by *Monascus purpureus* MTCC 369 under solid-state fermentation using response surface methodology. *Songklanakarin J. Sci. Technol.* 36, 439–444.

Ajdari, Z., Ebrahimpour, A., Abdul Manan, M., Hamid, M., Mohamad, R., and Ariff, A. B. (2011). Nutritional requirements for the improvement of growth and sporulation of several strains of *Monascus purpureus* on solid state cultivation. *J. Biomed. Biotechnol*. 2011, 487329 doi: 10.1155/2011/487329

Ajdari, Z., Ebrahimpour, A., Manan, M. A., Ajdari, D., Abbasiliasi, S., Hamid, M., et al. (2013). Nutrients interaction investigation to improve *Monascus purpureus* FTC5391 growth rate using response surface methodology and artificial neural network. *Malays. J. Microbiol.* 9, 68–83. doi: 10.21161/mjm.44912

Anonymous (1999). *Doosan Training and Technology Centre*. Available online at: http://www.dst.doosan.com

AOAC (1995). *Association of Official Analytical Chemists, 16th Edn*. Washington, DC: A.O.A.C International.

AOAC (2005). *Official Methods of Association of Agricultural Chemicals, 18th Edn*. Washington, DC: AOAC.

Arapoglou, D., Varzakas, T., Vlyssides, A., and Israilides, C. (2010). Ethanol production from potato peel waste (PPW). *Waste Manage.* 30, 1898–1902. doi: 10.1016/j.wasman.2010.04.017

Arbuckle, W. S. (1972), *Ice Cream*. Westport: The Avi Publishing Company, Inc.

Atalay, P., Sargin, S., and Goksungur, Y. (2020). Utilization of residual beer for red pigment production by *Monascus purpureus* in submerged fermantation. *Fresenius Environ. Bull.* 29, 1025–1034. doi: 10.1257/app.20190070

Babitha, S., Soccol, C. R., and Pandey, A. (2007). Solid-state fermentation for the production of Monascus pigments from jackfruit seed. *Bioresour. Technol*. 98, 1554–1560. doi: 10.1016/j.biortech.2006.06.005

Basuny, A. M., and Abdel-Raheam, H. E. F. (2020). Red and yellow Monascus pigments as potential natural antioxidants for fatty foods. *Plant Arch.* 20, 444–445.

Blanc, P. J., Laussac, J. P., Le Bars, J., Le Bars, P., Loret, M. O., Pareilleux, A., et al. (1995). Characterization of monascidin A from Monascus as citrinin. *Int. J. Food Microbiol.* 27, 201–213. doi: 10.1016/0168-1605(94)00167-5

Box, G. E. P., and Behnken, D. W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics* 2, 455–475. doi: 10.1080/00401706.1960.10489912

Broder, C. U., and Koehler, P. E. (1980). Pigments produced by *Monascus purpureus* with regard to quality and quantity. *J. Food Sci*. 45, 567–569. doi: 10.1111/j.1365-2621.1980.tb04102.x

Campoy, S., Rumbero, A., and Martin, J. F. (2006). Characterization of anhyperpigmenting mutant of *Monascus purpureus* IB1: identification of two novel pigment chemical structures. *Appl. Microbiol. Biotechnol.* 70, 488–496. doi: 10.1007/s00253-005-0090-y

Carels, M., and Shepherd, D. (1977). The effect of different nitrogen sources on pigment production and sporulation of Monascus species in submerged, shaken culture. *Can. J. Microbiol*. 24, 134611357. doi: 10.1139/m78-217

Celestino, J. d. R., de Carvalho, L. E., da Paz Lima, M., Lima, A. M., Ogusku, M. M., and de Souza, J. V. B. (2014). Bioprospecting of Amazon soil fungi with the potential for pigment production. *Process Biochem*. 49, 569–575. doi: 10.1016/j.procbio.2014.01.018

Chen, M. H., and Johns, M. R. (1993). Effect of pH and nitrogen source on pigment production by *Monascus purpureus*. *Appl. Microbiol. Biotechnol*. 40, 132–138. doi: 10.1007/BF00170441

Chen, W. P., He, Y., Zhou, Y. X., Shao, Y. C., Feng, Y. L., Li, M., et al. (2015). Edible filamentous fungi from the species Monascus: early traditional fermentations, modern molecular biology, and future genomics. *Compr. Rev. Food Sci. Food Saf.* 14, 555–567 doi: 10.1111/1541-4337.12145

Cho, Y. J., Park, J. P., Hwang, H. J., Kim, S. W., Choi, J. W., and Yun, J. W. (2002). Production of red pigment by submerged culture of *Paecilomyces sinclairii*. *Lett. Appl. Microbiol.* 35, 195–202. doi: 10.1046/j.1472-765X.2002.01168.x

Darwesh, O. M., Matter, I. A., Almoallim, H. S., Alharbi, S. A., and Oh, Y.-K. (2020). isolation and optimization of *Monascus ruber* OMNRC45 for red pigment production and evaluation of the pigment as a food colorant. *Appl. Sci.* 10, 67–88. doi: 10.3390/app10248867

De Carvalho, J. C., Cardoso, L. C., Ghiggi, V., Woiciechowski, A. L., de Souza Vandenberghe, L. P., and Soccol, C. R. (2014). "Bioactive secondary metabolites, microbial pigments," in *Biotransformation Waste Biomass into High Value Biochemicals*, Vol. 2, eds S. K. Brar, G. S. Dhillion, and C. R. Soccol (New York, NY: Springer), 73–97.

Dikshit, R., and Tallapragada, P. (2011). *Monascus purpureus*: a potential source for natural pigment production. *J. Microbiol. Biotechnol. Res*. 1, 164–174. Available online at: www.scholarsresearchlibrary.com

Dikshit, R., and Tallapragada, P. (2017). *Monascus purpureus*: a potential source for natural pigment production. *J. Microbiol. Biotechnol. Res.* 1, 164–174.

Duque-Acevedo, M., Belmonte-Ureña, L. J., Cortés-García, F. J., and Camacho-Ferre, F. (2020). Agricultural waste: review of the evolution, approaches and perspectives on alternative uses. *Global. Ecol. Conserv*. 22, 1–23. doi: 10.1016/j.gecco.2020.e00902

Fabre, C. E., Santerre, A. L., Loret, M. D., Baberian, R., Parailleux, A., and Goma, G. (1993). Production and food application of the red pigments of *Monascus ruber*. *J. Food Sci.* 58, 1099–1103. doi: 10.1111/j.1365-2621.1993.tb06123.x

Gautam, N., Sapkal, V. S., and Sapkal, R. S. (2017). Production of bio-ethanol from potato processing wastewater using membrane technology. *Int. J. Creative Res. Thoughts* 378–389. Available online at: www.ijcrt.org doi: 10.1727/IJCRT.17168

Global Food Color Market Research Report (2021). *Information by Source (Natural and Synthetic), Application (Bakery & Confectionery, Dairy & Frozen Desserts, Beverages, Sweet & Savory Snacks, Meat Products and Others) and Region (North America, Europe, Asia-Pacific and Rest of the World) - Forecast Till 2027 (2021)*. Fortune Business Insights Pvt. Ltd. ID: MRFR/F-B & N/1952-HCR, p. 164.

Gomah, N. H., Abdel-Raheam, H. E. F., and Mohamed, T. H. (2017). Production of natural pigments from *Monascus ruber* by solid state fermentation of broken

rice and its application as colorants of some dairy products. *J. Food and Dairy Sci.* 8, 37–43. doi: 10.21608/jfds.2017.37112

Günerhan, Ü., Us, E., Dumlu, L., Yilmaz, V., Carrère, H., and Perendeci, A. (2020). Impacts of chemical-assisted thermal pretreatments on methane production from fruit and vegetable harvesting wastes: process optimization. *Molecules* 25, 500. doi: 10.3390/molecules25030500

Hamano, P. S., and Kilikian, B. V. (2006). Production of red pigments by M. ruber in culture media containing corn steep liquor. *Braz. J. Chem. Eng.* 23, 443–449. doi: 10.1590/S0104-66322006000400002

Haung, L. P., Jin, B., Lant, P., and Zhou, J. (2003). Biotechnological production of lactic acid integrated with potato wastewater treatment by Rhizopus arrhizus. *J. Chem. Tech. Biotech*. 78, 899–906. doi: 10.1002/jctb.877

Hilares, R. T., de Souza, R. A., Marcelino, P. F., da Silva, S. S., Dragone, G., Mussatto, S. I., et al. (2018). Sugarcane bagasse hy-drolysate as a potential feedstock for red pigment production by *Monascus ruber*. *Food Chem*. 245, 786–791. doi: 10.1016/j.foodchem.2017.11.111

Horwath, W. (2007). "Carbon cycling and formation of soil organic matter," in *Soil Microbiology, Ecology, and Biochemistry, 3rd Edn*, ed E. A. Paul (Oxford: Elsevier), 303–339.

Johns, M. R., Chong, R., and Maddox, I. S. (1982). Hydrolysis of some natural and synthetic bile acid conjugates by *Cerospora melonis*. *Can. J. Microbiol.* 28, 457–461. doi: 10.1139/m82-069

Jones, D. A. (1998). Why are so many food plants cyanogenic? *Phytochemistry* 47, 155–162. doi: 10.1016/S0031-9422(97)00425-1

Kalaivani, M., and Rajasekaran, A. (2014). Improvement of monacolin K/citrinin production ratio in *Monascus purpureus* using UV mutagenesis. *Nutrafoods* 13, 79–82. doi: 10.1007/s13749-014-0021-6

Kantifedaki, A., Kachrimanidou, V., Mallouchos, A., Papanikolaou, S., and Koutinas, A. (2018). Orange processing waste valorisation for the production of bio-based pigments using the fungal strains *Monascus purpureus* and *Penicillium purpurogenum*. *J. Clean. Prod*. 185, 882–890. doi: 10.1016/j.jclepro.2018.03.032

Kim, S. Y., Lee, K. H., Kim, J. H., and Oh, D. K. (1997). Erythritol production by controlling osmotic pressure in *Trigonopsis variabilis*. *Biotechnol. Lett.* 19, 727–729. doi: 10.1023/A:1018371722456

Kot, A. M., Pobiega, k, Piwowarek, K., Kieliszek, M., Błazejak, S., and Małgorzata Gniewosz Edyta Lipińska, M. G. E. (2020). Biotechnological methods of management and utilization of potato industry waste-a review. *Potato Res.* 63, 431–447. doi: 10.1007/s11540-019-09449-6

Lin, T. F., Yalcushijin, K., Buchi, G. H., and Demain, A. L. (1992). Formation of watersoluble Monascus red pigments by biological and semi-synthetic processes. *J. Ind. Microbiol.* 9, 173–179. doi: 10.1007/BF01569621

Liu, J., Luo, Y., Guo, T., Tang, C., Chai, X., Zhao, W., et al. (2020). Cost-effective pigment production by *Monascus purpureus* using rice straw hydrolysate as substrate in submerged fermentation. *J. Biosci. Bioeng.*129, 229–236. doi: 10.1016/j.jbiosc.2019.08.007

Mal'a, P., Baranová, M., Marcinčáková, D., and Nagy, J. (2010). Organoleptic evaluation of poultry meat products with wheat protein seitan, colored by microbial natural pigment. *Assam Univ. J. Sci. Technol. Biol. Environ. Sci.* 5, 1–5. Available online at: https://www.researchgate.net/publication/277207492

Mehri, D., Perendeci, N. A., and Goksungur, Y. (2021). Utilization of whey for red pigment production by *Monascus purpureus* in submerged fermentation. *Fermentation*. 7, 75. doi: 10.21203/rs.3.rs-180649/v1

Mekhael, R., and Yousif, S. Y. (2009). The role of red pigment produced by Serratia marcescens as antibacterial and plasmid curing agent. *J. Duhok. Univ*. 12, 268–274.

Mousa, S. A., Abdou, D. A. M., Mohamed, G. A., Abo-El-Seoud, M. A., Karam Eldin, A. A., and El-mehalawy, A. A. (2018). Production of red pigment by *Monascus purpureus* NRRL1992 under submerged and solid-state fermentation, Egypt. *J. Microbiol*. 53, 83–94. doi: 10.21608/ejm.2018.3962.1059

Myers, R., and Montgomery, D. (1995). *Response Surface Methodology-Process and Product Optimization Using Designed Experimental*. New York, NY: John Wiley & Sons, Inc.

Orak, T., Caglar, O., Ortucu, S., Ozkan, H., and Taskin, M. (2018). Chicken feather peptone: a new alternative nitrogen source for pigment production by *Monascus purpureus*. *J. Biotechnol*. 271, 56–62. doi: 10.1016/j.jbiotec.2018.02.010

Orozco, S. F. B., and Kilikian, B. V. (2008). Effect of pH on citrinin and red pigments production by *Monascus purpureus* CCT 3802. *World J. Microbiol. Biotechnol*. 24, 263–268. doi: 10.1007/s11274-007-9465-9

Patakova, P. (2013). Monascus secondary metabolites: production and biological activity. *J. Ind. Microbiol. Biotechnol.* 40, 169–181 doi: 10.1007/s10295-012-1216-8

Pathak, P. D., Mandavgane, S. A., Puranik, N. M., Jambhulkar, S. J., and Kulkarni, B. D. (2018). Valorization of potato peel: a biorefinery approach. *Crit. Rev. Biotechnol.* 38, 218–230. doi: 10.1080/07388551.2017.1331337

Raman, N. M., Shah, P. H., Mohan, M., and Ramasamy, S. (2015). Improved production of melanin from Aspergillus fumigatus AFGRD105 by optimization of media factors. *AMB Express*. 5:72. doi: 10.1186/s13568-015-0161-0

Reitmeier, C. A., and Nonnecke, G. R. (1991). Objective and sensory evaluation of fresh fruit of day neutral strawberry cultivars. *J. Hor. Sci*. 26, 843–845. doi: 10.21273/HORTSCI.26.7.843

Santis, D. D., Moresi, M., Gallo, A. M., and Petruccioli, M. (2005). Assessment of the dyeing properties of pigments from *Monascus purpureus*. *J. Chem. Technol. Biotechnol*. 80, 1072–1079. doi: 10.1002/jctb.1285

Sehrawat, R., Panesar, P. S., Swer T. L., and Anit, K. (2017). Response surface methodology (RSM) mediated interaction of media concentration and process parameters for the pigment production by *Monascus purpureus* MTCC 369 under solid state fermentation. *Pigment Resin Technol.* 46, 14–20. doi: 10.1108/PRT-08-2015-0077

Sen, T., Barrow, C. J., and Deshmukh, S. K. (2019). Microbial pigments in the food industry-challenges and the way forward. *Front. Nutr*. 6, 7. doi: 10.3389/fnut.2019.00007

Seyedin, A., Yazdian, F., Hatamian-Zarmi, A., Rasekhi, B., and Mirderikvand, M. (2015). Natural red pigment production by *Monascus purpureus*: bioreactor yield improvement through statistical analysis. *J Appl Food Biotechnol*. 2, 23–30.

Sharmila, G., Nidhi, B., and Muthukumaran, C. (2013). Sequential statistical optimization of red pigment production by *Monascus purpureus* (MTCC 369) using potato powder. *Ind. Crops Prod*. 44, 158–164. doi: 10.1016/j.indcrop.2012.11.007

Silbir, S., and Goksungur, Y. (2019). Natural red pigment production by *Monascus purpureus* in submerged fermentation systems using a food industry waste: Brewer's spent grain. *Foods*. 8, 161. doi: 10.3390/foods8050161

Su, N.-W., Lin, Y.-L., Lee, M.-H., and Ho, C.-Y. (2005). Ankaflavin from Monascus -fermented red rice exhibits selective cytotoxic effect and induces cell death on Hep G2 cells. *J. Agric. Food Chem*. 53, 1949–1954. doi: 10.1021/jf048310e

Tallapragada, P., Dikshit, R., and Dessai, P. T. (2013). Effect of glycerol as a sole carbon source on Monascus sp. for pigment production. *Int. Food Res. J*. 20, 3265–3268. doi: 10.1016/j.jgeb.2017.02.003

Tseng, Y. Y., Chen, M. T., and Lin, C. F. (2000). Growth, pigment production and protease activity of *Monascus purpureus* as affected by salt, sodium nitrite, polyphosphate and various sugars. *J. Appl. Microbiol*. 88, 31–37. doi: 10.1046/j.1365-2672.2000.00821.x

Velmurugan, P., Kim, M. J., Park, J. S., and Karthikeyan, K. (2010). Dyeing of cotton yarn with five water soluble fungal pigments obtained from five fungi. *Fibers Polym.* 11, 598–605. doi: 10.1007/s12221-010-0598-5

Vidyalakshmi, R., Paranthaman, R., Murugesh, S., and Singaravadivel, K. (2009). Stimulation of Monascus pigments by intervention of different nitrogen sources. *Glob. J. Biotechnol. Biochem*. 4, 25–28.

Wang, E. X., and Zhao, J. (2008). Application of sensory evaluation in meats. *Meat Res.* 9, 78–82. doi: 10.1007/s10884-008-9111-8

Wong, H. C., Lin, Y. C., and Koehler, P. E. (1981). Regulation of growth and pigmentation of *Monascus purpureus* by carbon and nitrogen concentrations. *Mycologia* 73, 649–654. doi: 10.1080/00275514.1981.12021392

Yuliana, A., Singgih, M., Julianti, E., and Blanc, P. (2017). Derivates of azaphilone Monascus pigments: a review. *J. Biocatal. Agric. Biotechnol*. 9, 183–194. doi: 10.1016/j.bcab.2016.12.014

Zhang, Y. L., Kong, L. C., Yin, C. P., Jiang, D. H., Jiang, J. Q., He, J., et al. (2013). Extraction optimization by response surface methodology, purification and principal antioxidant metabolites of red pigments extracted

from bayberry (*Myrica rubra*) pomace. *LWT Food Sci. Technol.* 51, 343–347. doi: 10.1016/j.lwt.2012.09.029

Zhou, B., Wang, J., Pu, Y., Zhu, M., Liu, S., and Liang, S. (2009). Optimization of culture medium for yellow pigments production with Monascus anka mutant using response surface methodology. *Euro. Food Res. Technol.* 228, 895–901. doi: 10.1007/s00217-008-1002-z

Zhou, Z., Yin, Z., and Hu, X. (2014). Corncob hydrolysate, an efficient substrate for Monascus pigment production through submerged fermentation. *Biotechnol. Appl. Biochem.* 61, 716–723. doi: 10.1002/bab.1225

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 June 2022
doi: 10.3389/fmicb.2022.909987

Revealing Probiotic Potential of *Enterococcus* Strains Isolated From Traditionally Fermented *Chhurpi* and Healthy Human Gut

Kriti Ghatani[1]*, *Subarna Thapa*[1], *Shankar Prasad Sha*[2]*, *Sourav Sarkar*[3], *Debabrata Modak*[3] *and Soumen Bhattacharjee*[3]*

[1] Food Microbiology Laboratory, Department of Food Technology, University of North Bengal, Raja Rammohunpur, India, [2] Food Microbiology Laboratory, Department of Botany, Kurseong College, Kurseong, India, [3] Cell and Molecular Biology Laboratory, Department of Zoology, University of North Bengal, Raja Rammohunpur, India

OPEN ACCESS

Edited by:
Laurent Dufossé,
Université de la Réunion, France

Reviewed by:
Chun-Feng Guo,
Northwest A&F University, China
Yousef Nami,
Agricultural Biotechnology Research Institute of Iran, Iran

***Correspondence:**
Shankar Prasad Sha
shankarprasadsha@gmail.com
Kriti Ghatani
ghatanik@nbu.ac.in
Soumen Bhattacharjee
soumenb@nbu.ac.in

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 31 March 2022
Accepted: 11 May 2022
Published: 16 June 2022

Citation:
Ghatani K, Thapa S, Sha SP, Sarkar S, Modak D and Bhattacharjee S (2022) Revealing Probiotic Potential of Enterococcus Strains Isolated From Traditionally Fermented Chhurpi and Healthy Human Gut. Front. Microbiol. 13:909987. doi: 10.3389/fmicb.2022.909987

In this study, the two lactic acid bacterial strains *Enterococcus durans* and *Enterococcus lactis* previously isolated from soft *chhurpi*, a traditionally fermented milk product prepared by the indigenous community of Sikkim Himalayas and healthy human gut were used. In this study, we attempted to investigate the probiotic attributes, safety, and health beneficial role, and hypercholesterolemia of *Enterococcus durans* and *Enterococcus lactis.* Both probiotic potential strains showed good hypocholesterolemic activity *in vitro* along with tolerance to acid pH (2 and 2.5), tolerance to three bile salts, oxbile, cholic acid, and taurocholic acid (0.5 and 1%), presence of BSH enzyme and its activity, and cell surface adherence. On assessing for safety, both LAB strains were sensitive to antibiotics and exhibited no hemolytic activity. The probiotic strains were tested *in vivo* in the Sprague–Dawley rats which were divided into five experimental groups: Normal Control (ND), probiotic strain *Enterococcus durans* HS03 (BSH-negative) and high-cholesterol diet (HCD1), probiotic strain *Enterococcus lactis* YY1 (BSH-positive) and high-cholesterol diet (HCD2), and a combination of both strains and high-cholesterol diet (HCD3) and Negative Control (HCD). The probiotic-treated groups HCD1, HCD2, and HCD3 showed a decrease in serum cholesterol levels up to 22.55, 6.67, and 31.06%; the TG and VLDL concentrations were 25.39, 26.3, and 33.21%; reduction in LDL-cholesterol was 33.66, 28.50, and 35.87%; and increase of HDL was 38.32, 47.9, and 41.92%. Similarly, the effects of total cholesterol and TG in the liver, kidney and liver histopathology, liver and body lipid index, and oxidative stress in rat liver were also studied. The fecal lactobacilli were more in the samples of the probiotic-treated groups and their fecal coliform and *E. coli* counts decreased relatively as compared to the control groups in 0, 7, 14, and 21 days. This is the first report on the probiotic potential of *Enterococcus durans* HS03 and *Enterococcus lactis* YY1 strains that gives a new insight into the cholesterol-lowering and probiotic product development with wide health attributes.

Keywords: *chhurpi*, human gut, probiotic, *Enterococcus*, blood lipids, histopathology

INTRODUCTION

Over the last decades, the ubiquity of hyperlipidemia among people throughout the world has inflated and attained an epidemic level (Zhang et al., 2021). Hyperlipidemia is a condition in which the plasma or serum contains elevated levels of total cholesterol (TC) and/or triglycerides (TG) and/or low-density lipoprotein (LDL); and low levels of high-density lipoprotein (HDL) (Wang et al., 2021). Numerous aspects like environmental factors, unhealthy food habits, and heredity may be the cause of hyperlipidemia. Whatever be the causative factors of hyperlipidemia, long-term exposure to such a condition as hyperlipidemia may be followed by various health issues like cardiovascular diseases, cerebrovascular diseases, atherosclerosis, metabolic syndrome, and many chronic diseases (Skrypnik et al., 2018; Wang et al., 2021). Although the use of drug therapy (statins, fibrate, etc.) has proved promising for the treatment of hyperlipidemia. These drugs are associated with various side effects such as diarrhea, abdominal discomfort, flatulence, dementia, low levels of vitamin D in the plasma, and sleep deprivation and are also very expensive (Wang et al., 2012; Ma et al., 2017; Zhang et al., 2021). Further, the continuous use of statins may lead to liver damage and muscle toxicity (Zhang et al., 2021). Considering such effects of hyperlipidemia and also the side effects of drug therapy, today, much focus has been given to the ability of probiotics as an alternative strategy to curb the incidence of hyperlipidemia *via* various mechanisms (Tomaro-Duchesneau et al., 2014). The mechanisms involved in the hypolipidemic activity of probiotics have been suggested to involve the deconjugation of bile acid *via* bile salt hydrolase activity, cholesterol binding to the probiotic cell wall, transformation of the cholesterol into coprostanol, redistribution of the plasma cholesterol to the liver, inhibition of hepatic cholesterol and triglyceride synthesis by short-chain fatty acids (e.g., propionate), and assimilation of cholesterol (Salaj et al., 2013).

Probiotics are "live microorganisms", mostly belonging to the lactic acid bacterial group, and include species of *Lactobacillus*, *Bifidobacterium, and Enterococcus*, that have become propitious agents to confer health benefits to the host (Hotel and Cordoba, 2001; Zhu et al., 2019). Probiotics have proven as promising agents for decreasing the blood LDL and TC concentrations (Skrypnik et al., 2018; Nami et al., 2019). The earliest documented case of serum cholesterol reduction by probiotic *Lactobacillus* strain was reported by Shaper et al. (1963), Mann and Spoerry (1974) and Vinderola and Reinheimer (2003). An *in vivo* study conducted by Guo et al. (2016) on the effect of oral feeding of *Enterococcus durans* in hypercholesterolemic Sprague–Dawley rats suggested the potential of *E. durans* to lower total serum cholesterol and LDL (Guo et al., 2016). *In vitro* study by Albano et al. (2018) on the hypocholesterolemic effect of *Enterococcus lactis* BT161 has reported a 42–55% decrease in the cholesterol level in the broth (Albano et al., 2018).

Although numerous strains of *Bifidobacterium* and *Lactobacillus* species have been reported to alleviate cholesterol both *in vivo* and *in vitro*, very limited reports are available for the hypocholesterolemic effect of the *Enterococcus* strains (Tomaro-Duchesneau et al., 2014; Shehata et al., 2016; Hyrslova et al., 2021; Marras et al., 2021; Yang et al., 2021; Kouhi et al., 2022). Thus, this study has aimed to determine the beneficial effects of probiotic *Enterococcus durans* HS03 (BSH-negative) and *Enterococcus lactis* YY1 (BSH-positive) on lipid metabolism in Sprague–Dawley rats fed with a high-cholesterol diet.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

Enterococcus durans HS03 (accession no. KX274030) and *Enterococcus lactis* YY1 (accession no. KU 601443) were the two strains used in the study. These isolates were previously isolated from the human gut and *chhurpi* (fermented yak milk product) and examined for cholesterol-lowering property in the laboratory (Ghatani and Tamang, 2017). The *chhurpi* samples were collected from North Sikkim. Yak (*Bos gruinnens*), known as *gyag* in the Bhutia language, is a multipurpose "sure-footed animal" of the Himalayan region. Several value-added products are made from yak milk, meat, skin, hair, and horns (Ghatani and Tamang, 2016). Yak milk and its products are popular foods in high-altitude regions and it plays a major role in providing essential nutrients and minerals to the herdsman and the local people. The most common products made from milk are *marr* (Ghew in Nepali), *chhurpi, thara* (Mohi in Nepali), *shyow* (Dahi in Nepali), *chilu* (yak fat), *philu* (Dewan and Tamang, 2007), and *tema* (yak cream) (Ghatani and Tamang, 2016). There are three different types of *chhurpi*: hard type, soft type, and *dudh chhurpi*. The strain *Enterococcus lactis* YY1 has been isolated from the soft variety that is prepared by boiling buttermilk or whey to form a white mass that is sieved out from the remaining liquid (Ghatani and Tamang, 2017).

The strains were subcultured in de Man, Rogosa, and Sharpe (MRS) broth (Hi Media, Mumbai, India) followed by incubation at 37°C in an Anaerobic Gas Pack system (Hi Media LE002, Mumbai, India) for 72 h.

Tolerance to Low pH/Acid

The overnight-grown culture 1% was inoculated in MRS broth supplemented with 0.30% oxgall, pH adjusted to 2.5 with HCl, and incubated at 37°C for 2 h (Liong and Shah, 2005). Serial dilutions were prepared and plated every 30 min until 2 h in MRS agar. The strains were tested for acid tolerance at pH 2 determined by plate count methods.

Tolerance to Bile Salt

The strains were tested for tolerance to three different types of bile salts—oxgall, cholic acid, and taurocholic acid (Liong and Shah, 2005) with slight modifications. MRS broths containing 0.5 and 1% (w/v) of oxgall, cholic acid, or taurocholic acid were inoculated with each strain and incubated at 37°C. MRS broth without bile salt was used as a control according to the method of Gilliland and Walker (1990). Bacterial growth was monitored by measuring the turbidity with a spectrophotometer (Lambda UV-VIS spectrophotometer, Perkin Elmer, Wokingham, United Kingdom) at 620 nm at 0 and 8 h, respectively.

Cell Surface Hydrophobicity

The strains were grown in MRS broth at 37°C for 24 h and centrifuged at 8,000 g for 5 min (Rosenberg, 1984; Pérez et al., 1998), the pellet was washed three times with Ringer solution (Merck, Darmstadt, Germany), and thoroughly mixed in a vortex; 1 ml of the suspension was taken and the absorbance at 580 nm was measured. Then 1.5 ml of the suspension was mixed with an equal volume of n-hexadecane (RM 2238, HiMedia, Mumbai, India) in duplicates and mixed thoroughly in a vortex. The phases were allowed to separate for 30 min at room temperature, after which the aqueous phase was carefully removed and absorbance at 580 nm was measured (Nami et al., 2020; Kiani et al., 2021).

Direct Plate Assay for Screening BSH Activity

The strains were checked for bile salt hydrolase activity by the direct plate assay technique with slight modifications by Nguyen et al. (2007). The cultures were streaked on MRS agar plates containing 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA: Sigma) and incubated in an anaerobic jar at 37°C for 72 h. Only MRS agar plates were streaked for comparison. After incubation, the presence of precipitated bile acid surrounding colonies (opaque halo) or the formation of opaque granular white colonies with a silvery-white shine was considered as the presence of BSH enzyme.

BSH Assay

BSH activity was measured by determining the number of amino acids liberated from conjugated bile salts by lactobacilli strains as described by Tanaka et al. (1999), with few modifications.

Safety Evaluation of Probiotics for Human Use

Probiotic bacteria must be studied for the following parameters to be generally recognized as safe. So, the strains were tested for safety before *in vivo* study.

Determination of antibiotic resistance patterns for any risk of transferable antibiotic resistance. Antibiotic resistance patterns were studied using antibiotic disk of penicillin (*P*) 10 units, erythromycin (E) 15 μg, vancomycin (VA) 30 μg, teicoplanin (TE1) 30 μg, clindamycin (CD) 2 μg, ofloxacin (OF) 5 μg, azithromycin (AZM) 15 μg, and tetracycline (TE) 30 μg (Kumar et al., 2017). The *E coli* MTCC 1098 and *Staphylococcus aureus* MTCC 7443 were used as control strains for antibiotic susceptibility.

Hemolytic activity was investigated as described by Gerhardt et al. (1913) by incubating blood agar plates (48 h at 37°C) and examining them for the signs of β-hemolysis (clear zones around colonies), α-hemolysis (green zones around colonies), or γ hemolysis (no clear zones around colonies).

In vivo Study

Experimental Animals

A total of 30 adult male Sprague–Dawley (SD) rats (200 ± 10 gm) were purchased from CSIR-CDRI (Central Drug Research Institute), Lucknow, India. All animals were housed in well-ventilated polypropylene cages (Tarsons, India) with paddy husk as bedding material in the Animal House of Department of Zoology, University of North Bengal. All the animals were maintained under a constant 12 h light−12 h dark cycle. Room temperature was controlled at 22–25°C with about 56–60% relative humidity. The animals were provided with sufficient food and water *ad libitum*. The animals undergo acclimatization for 1 week to the laboratory environment before the start of the experiment. The approval of the Institute's Animal Ethics Committee (IAEC/NBU/2019/17) was obtained before conducting the experimental trial from CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) of the University of North Bengal, West Bengal, India.

TABLE 1 | Composition of experimental high- cholesterolemic diet.

Constituents			In %
High-cholesterolemic diet	AIN-76 semi purified diet	Corn starch	15
		Casein purified high Nitrogen	20
		Corn oil	5
		Vitamin mixture	1
		Mineral mixture	3.5
		DL-methionine	0.30
		Cholesterol	0.50
		Sucrose	50
		Choline bitartrate	0.2
		Alphacel, non-nutritive bulk	5
	Cholesterol	Cholesterol	1

Animal Diet

AIN-76 mineral mixture (MP Biomedicals, France) with 1% cholesterol was used as the high-cholesterolemic diet. The base composition of the experimental AIN-76 diet is recorded in **Table 1**. However, the normal diet used in the experiment does not include cholesterol in its high-cholesterolemic diet (Kumar et al., 2011).

Animal Grouping

All the 30 rats were divided into 5 experimental groups having 6 rats each ($n = 6$). The experimental groups included are summarized in **Table 2**. The HCD group contained 1% (w/w) supplemental cholesterol.

Preparation of Bacterial Cultures

The probiotic strains were all grown in an MRS medium under anaerobic conditions. The overnight-grown test cultures were pelleted at 5,000 rpm for 30 min at 4°C and washed two times with saline. The strains were resuspended at 1 × 10^8 CFU/ml concentration in neutral saline. The number of CFU administered was routinely verified by plating. The culture was concentrated to 20 ml (Stock) and kept at 8°C until

TABLE 2 | Experimental grouping used in this study.

SL. No.	Group	Experimental diets
1.	Normal control (ND)	Normal diet without cholesterol (AIN76)
2.	Probiotic strain 1 (HCD1)	High-cholesterol diet (AIN76+ 1% cholesterol) + *Enterococcus durans* HS03 ($\sim10^8$ cfu/g)
3.	Probiotic strain 2 (HCD2)	High-cholesterol diet (AIN76+ 1% cholesterol) + *Enterococcus lactis*YY1 ($\sim10^8$ cfu/g)
4.	Probiotic strain 1 and 2 (HCD3)	High-cholesterol diet (AIN76+ 1% cholesterol) + *Enterococcus durans* HS03 and *Enterococcus lactis*YY1 ($\sim10^8$ cfu/g)
5.	Negative control (HCD)	High-cholesterol diet (AIN76+ 1% cholesterol)

feeding; 0.3 ml bacterial solution was administered to each rat by intragastric gavage (Kumar et al., 2011).

Feeding Schedule

The rats were fed with standard pellets for 7 days to remove the effect of stress during the acclimatization phase. At the end of the acclimatization phase, all the groups were fed their respective experimental diets for the next 21 days. During the entire course of the experiment, the rats had free access to water. Each rat was fed with 10 gm/day of diet from the 1st week, which was then increased by 2 gm/day per week. Feed intake was recorded daily and body weight was measured weekly.

Blood Sampling and Serum Lipid Profile Analysis

After the treatment schedule, all animals were anesthetized with sodium pentobarbital (60 mg/kg; i.p.) and euthanized by cervical dislocation (Modak et al., 2021). Thereafter, blood samples were obtained through the cardiac puncture placed in sterile microfuge tubes. For serum analyses, blood samples were centrifuged at 5,000 rpm for 10 min at 4°C and serums were collected. The samples were analyzed for total cholesterol (TC), triglycerides (TG), LDL-cholesterol (LDL-C), and HDL-cholesterol (HDL-C) using commercial Coral Kits (Coral clinical systems, India) following the manufacturers' protocol with a spectrophotometer (Lambda UV-VIS spectrophotometer, Perkin Elmer, Wokingham, United Kingdom). The Atherogenic index (AI) and VLDL-cholesterol were calculated as per Friedewald's equation (Friedewald et al., 1972).

Assay for Liver Lipids

Liver tissues (100 mg) were pulverized in liquid nitrogen to prepare 10% tissue homogenate. These homogenates were extracted under 4°C with 5 ml of Folch solution (chloroform: methanol ratio = 2:1) for 48 h, then centrifuged at 10,000 rpm for 15 min at 4°C. The concentrations of liver TG and TC in the supernatant were determined according to the protocols (Yin et al., 2010).

Liver and Kidney Histopathology

After euthanization, the liver and kidney tissues of each rat from each group were immediately removed and washed in chilled phosphate buffer saline (pH 7.4). The middle lobe of the liver was standardized as the sampling region. Both the tissues were sectioned and soaked in 4% formalin for fixation and were dehydrated using serial dilutions of ethanol and subsequently embedded in paraffin wax according to previous protocols (Modak et al., 2021). The tissues were cut into transverse sections at 5 μm thickness and stained with hematoxylin and eosin. Thereafter, microscopic examinations were done under a light microscope (Nikon Eclipse E200, Nikon, Tokyo, Japan) with 10X and 40X objectives and, respectively, scale bar had been attached (100 and 25 μm). Each histologic section was observed for 5 fields of high-power fields.

Assay for Liver and Body Lipid Index

After euthanization, the liver and white adipose tissue (WAT) of the mesenteric fat-pad and epididymal fat-pad of each rat from each group were immediately removed and weighed. Measurements were (1) liver index: liver weight/body weight ratio and (2) body lipid index: viscera fat weight/body weight ratio [viscera fat includes retroperitoneal (RET) and epididymal (EPI) white adipose tissues] (Yin et al., 2010).

Preparation of Liver Homogenate and Analysis of Oxidative Stress Markers

The liver homogenate was prepared following standard protocol. Briefly, liver tissue was removed from each rat at the time of sacrifice and 10% of liver homogenates was obtained by homogenizing liver tissues (1 gm) in 10 ml cold phosphate buffer (pH 7.4) solution (4°C). The homogenates were centrifuged at 12,000 rpm for 10 min at 4°C and the resulting supernatant was collected and used to detect oxidative stress markers.

Determination of Lipid Peroxidation

Lipid peroxidation in the liver was estimated colorimetrically by measuring the thiobarbituric acid reactive substrates (TBARS) using a previously described method (Draper and Hadley, 1990). The absorbance of the clear supernatant was measured at 535 nm against a blank reference.

Determination of Catalase

CAT activities were determined using a previously described method (Aebi, 1974). Changes in absorbance of the reaction solution at 240 nm were determined after 1 min. One unit of CAT activity was defined as a change in the absorbance of 0.01 unit/min.

Determination of Reduced Glutathione

Reduced glutathione levels were estimated using the method reported by Ellman (1959). After the yellow color developed, the absorbance of the mixture was immediately recorded at 412 nm against a blank reference (Ellman, 1959).

Microbiological Analysis of Fecal Samples

Fecal samples from distinctly grouped, experimental SD rats were collected in distinct sterile vials to analyze their microbial

TABLE 3 | Probiotic attributes of *Enterococcus durans* HS03 and *Enterococcus lactis* YY1.

Sl. no.	Probiotic characters	Conditions	Duration	Strains	
				***Enterococcus durans* HS03**	***Enterococcus lactis* YY1**
1.	Acid tolerance (viable count log CFU/ml)	pH 2.5	0 h	9.75 ± 0.30	10.13 ± 0.23
			1 h	5.11 ± 0.35	7.60 ± 0.23
			2 h	2.65 ± 0.21	5.17 ± 0.12
		pH 2	0 h	9.60 ± 0.34	9.67 ± 0.007
			1 h	5.01 ± 0.12	3.2 ± 0.01
			2 h	2.05 ± 0.31	1.5 ± 0.24
2.	Tolerance to bile (turbidity or tolerance)	Ox bile (0.5%)	0 h	0.13 ± 0.23	0.16 ± 0.01
			4 h	0.33 ± 0.34	0.19 ± 0.01
			8 h	0.49 ± 0.45	0.31 ± 0.11
		Taurocholic acid (0.5%)	0 h	0.14 ± 0.12	0.12 ± 0.25
			4 h	0.47 ± 0.11	0.34 ± 0.13
			8 h	1.56 ± 0.23	1.15 ± 0.02
		Cholic acid (0.5%)	0 h	0.13 ± 0.11	0.14 ± 0.12
			4 h	0.15 ± 0.23	0.17 ± 0.26
			8 h	0.21 ± 0.25	0.29 ± 0.34
		Ox bile (1%)	0 h	0.13 ± 0.34	0.17 ± 0.02
			4 h	0.23 ± 0.25	0.25 ± 0.19
			8 h	0.35 ± 0.23	0.33 ± 0.30
		Taurocholic acid (1%)	0 h	0.11 ± 0.23	0.13 ± 0.59
			4 h	0.33 ± 0.12	0.36 ± 0.56
			8 h	0.80 ± 0.11	1.40 ± 0.03
		Cholic acid (1%)	0 h	0.12 ± 0.54	0.12 ± 0.04
			4 h	0.16 ± 0.34	0.14 ± 0.06
			8 h	0.24 ± 0.14	0.22 ± 0.08
3.	Cell surface hydrophobicity (%)			65 ± 0.34	70 ± 0.25

content. Each sample was homogenized in sterile PBS using a stomacher blender. This was followed by a ten-fold serial dilution of each sample in peptone water; 1 ml of each diluent was placed on nutrient agar, eosin methylene blue agar, violet red bile agar, and MRS agar. Nutrient agar, eosin methylene blue agar, violet red bile agar, and MRS agar were used for total plate count, *Escherichia coli*, coliforms, and total lactobacilli count, respectively. All the plates except those for the enumeration of total lactobacilli were incubated at 37°C for 48 h. The MRS agar plates for the enumeration of total lactobacilli were incubated under anaerobic conditions using a desiccator at 37°C for 48 h (Kumar et al., 2011).

Statistical Analysis

For *in vitro* experiments, all parameters were repeated six times and were presented as mean ± SD. For *in vivo*, all data were presented as the mean ± SD ($n = 6$). However, the body and organ weight was measured as ±SEM. Univariate analysis of variance test was applied to determine the statistical significance of the difference among the groups, using the using Graph Pad Prism Version 7.00 (San Diego, United States of America). Comparison between more than two groups was done using one-way analysis of variance (ANOVA) or by two-way ANOVA following the *post-hoc* analysis with a Dunnett's multiple comparisons test. Values of $p \leq 0.05$ were taken to indicate a statistical difference.

RESULTS

Lowering of cholesterol was observed in both the strains up to 70 and 65% for *Enterococcus durans* HS03 and *Enterococcus lactis* YY1, respectively (Ghatani and Tamang, 2017). The two strains were then tested for probiotics attributes like tolerance to acid, tolerance to bile, hydrophobic nature, and antibiotic sensitivity tests.

On checking *Enterococcus durans* HS03 and *Enterococcus lactis* YY1 for the ability to tolerate acidic conditions, first at pH 2.5 and then at pH 2.0, was found to be satisfactory. The pH of gastric juice secreted in the stomach is about 2 and many microorganisms are destroyed at this and lower pH. So, tolerance to acidic conditions is a very important criterion for the selection of probiotic bacteria. The pH 2.0 was regarded as a strong discriminative pH for the selection of high acid-tolerant strains. The viable cell counts (log CFU/ml) and survival percentages of selected LAB to acid conditions at pH 2 after 2 h incubation is presented in **Table 3**.

The strain *Enterococcus lactis* YY1 was more acid-tolerant than *Enterococcus durans* HS03 showing 6.47–7.15 log cycles in

TABLE 4 | BSH activity of *Enterococcus lactis* YY1.

Sodium glycocholate			Sodium taurocholate		
Total protein mg/ml	**Total activity U/ml**	**Specific activity U/mg**	**Total protein mg/ml**	**Total activity U/ml**	**Specific activity U/mg**
1.21 ± 0.11	1.30 ± 0.10	1.07 ± 0.11	1.22 ± 0.11	0.93 ± 0.11	0.76 ± 0.23

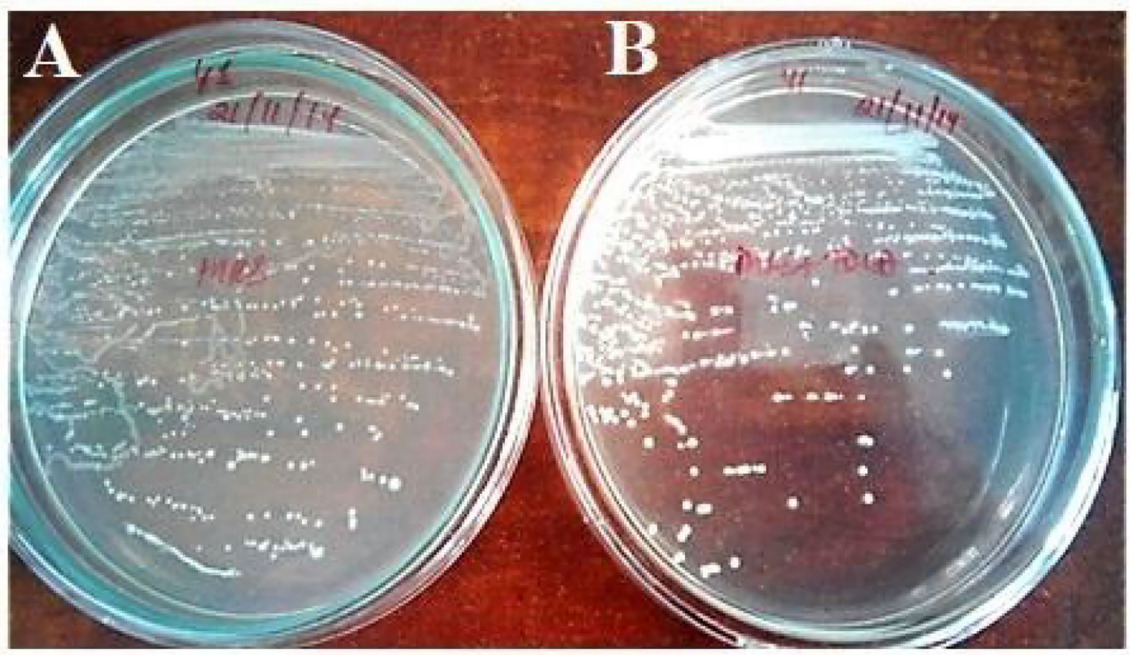


FIGURE 1 | Bile salt hydrolase activity of *Enterococcus lactis* YY1, **(A)** On MRS plate and **(B)** MRS plate supplemented with TDCA.

comparison to 6.6 log cycle at pH 2.5 for 2 h. Then, on testing the isolate pH 2, it was observed that both strains could tolerate the acidic condition for up to 2 h. Tolerance to three bile salts: ox bile, cholic acid, and taurocholic acid at 0.5 and 1% concentrations were studied. The results were calculated as the percentage increase in turbidity, in the case of *Enterococcus lactis* YY1 94, 892, and 107% in 0.5% ox bile, cholic acid, and taurocholic acid and 94, 977, and 83% in 1% bile salts were observed, respectively. In *Enterococcus durans* HS03 percentage increase in turbidity at 0.5% bile concentration was 277, 1,014, and 62% and 169, 627, and 100% at 1%. Both the strains showed good cell surface hydrophobicity. *Enterococcus lactis* YY1 was BSH-positive and showed excellent BSH enzyme activity *(**Table 4**) in sodium taurocholate and sodium glycocholate (**Figure 1**). *Enterococcus durans* HS03 was BSH-negative although it is a cholesterol-lowering strain that tolerates bile acid concentration.

Safety Evaluation of Probiotics for Human Use

The strains were resistant to penicillin, however, the highest zone of inhibition was seen in both the strains against vancomycin and azithromycin antibiotics (**Table 5**; **Figure 2**).

The strains gave no reaction on sheep blood agar, hence both strains gave a negative test for hemolytic activity (**Figure 3**).

Bodyweight and Feed Efficiency

All the experimental rats used in the study remained healthy, and their body weight gain and feed intake after 21 days were calculated and recorded for all the groups in **Table 6**. The statistical analysis showed a significant ($p < 0.05$) difference in final body weight and gain in weight among the experimental groups when compared to the hypercholesterolemic diet groups. After 3 weeks of treatment, the final bodyweight of the normal diet control group (ND) and the probiotic-treated groups (HCD1, HCD2, and HCD3) was 236.5 ± 1.708, 242.5 ± 3.096, 252.5 ± 3.819, and 236 ± 4.726 gm, respectively. The hypercholesterolemic diet control (HCD) group which was fed with a cholesterol-enriched diet, showed a significantly ($p < 0.05$) higher body weight (272.5 ± 2.141 gm) in comparison to other experimental groups (**Figure 4**). The highest body weight gain was also recorded in the HCD group (63.75 ± 5.42 gm), while the body weight gain of all the other experimental groups seems to be recorded significantly lower ($p < 0.05$) than the HCD group.

TABLE 5 | Antibiotic sensitivity test.

Strain	Inhibition zone in mm							
	P	E	VA	TEI	CD	OF	AZM	TE
Enterococcus lactis YY1	–	25-S	23 - S	17- I	17-I	16-I	17-I	32-S
Enterococcus durans HS03	–	25-S	23-S	16-I	16-I	16-I	10-I	30-S

Results were expressed as sensitive, S (≥21 mm), intermediate, I (16–20 mm) and Resistant, R (≤15 mm), respectively, according to that described by Charteris et al. (1998) and Volkova et al. (2013). The strains were sensitive to penicillin. Penicillin (P) 10 units, erythromycin (E) 15 μg, vancomycin (VA) 30 μg, teicoplanin (TEI) 30 μg, clindamycin (CD) 2 μg, ofloxacin (OF) 5 μg, azithromycin (AZM) 15 μg and tetracycline (TE) 30 μg.

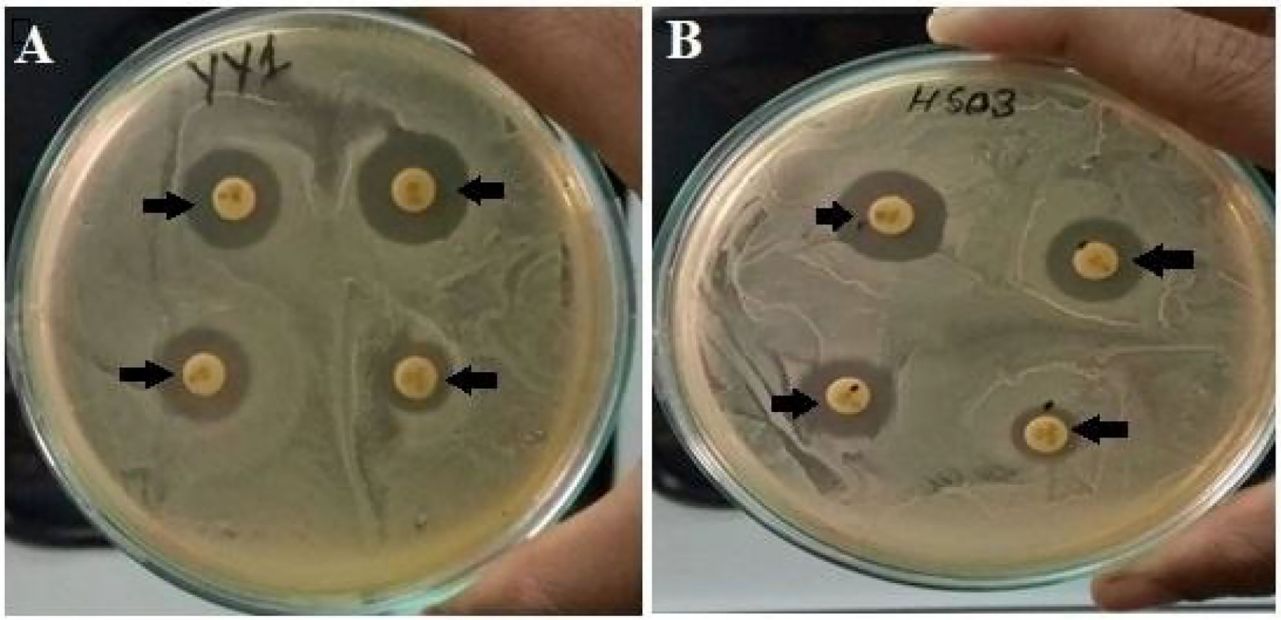


FIGURE 2 | (A) Antibiotic sensitivity pattern of *Enterococcus lactis* YY1 and **(B)** *Enterococcus durans* HS03.

Similarly, feed intake was higher in the HCD group (208.2 ± 2.643 gm) than in probiotic-treated groups (HCD1, HCD2, and HCD3) and the normal diet control group (ND) group. The feed intake of the normal diet control group (ND) and the probiotic-treated groups (HCD1, HCD2, and HCD3) was 180.8 ± 6.193, 186 ± 3.813, 202.3 ± 3.163, and 182 ± 5.366 gm, respectively, which was significantly lower ($p < 0.05$) than the HCD group.

Analysis of Serum Lipid Profile

The effect of dietary treatments on serum lipid profile (serum total cholesterol, TG, LDL-cholesterol, HDL-cholesterol, VLDL-cholesterol, and AI) has been recorded in **Table 7** and **Figure 5**.

Total serum cholesterol values for each dietary treatment group differed significantly ($p < 0.05$) and were recorded as 46.4 ± 5.45, 54.6 ± 4.77, 65.8 ± 2.86, 48.6 ± 0.827, and 70.5 ± 5.67 mg/dl for the ND, HCD1, HCD2, HCD3, and HCD dietary groups, respectively (**Figure 5A**). The dietary treated groups HCD1, HCD2, and HCD3 showed decreases in serum cholesterol levels up to 22.55, 6.67, and 31.06%, respectively, when compared with the HCD group after 21 days of dietary treatment.

The **serum TG** and **VLDL-cholesterol** concentrations also differed significantly ($p < 0.05$) among all the groups throughout the experiment. After dietary treatment, TG levels for all the five groups were recorded as 55.5 ± 6.68, 65.8 ± 3.66, 65 ± 5.74, 58.9 ± 0.659, and 88.2 ± 5.62 mg/dl for the ND, HCD1, HCD2, HCD3, and HCD groups, respectively (**Figures 5B,E**). The reduction in TG and VLDL concentrations was 25.39, 26.3, and 33.21% in the HCD1, HCD2, and HCD3 groups, respectively, when compared with the HCD group.

LDL-cholesterol values for each dietary treatment group also differed significantly ($p < 0.05$) and were recorded as 24.6 ± 2.09, 27 ± 1.27, 29.1 ± 1.13,26.1 ± 1.76, and 40.7 ± 2.98 mg/dl for the ND, HCD1, HCD2, HCD3, and HCD groups, respectively (**Figure 5C**). The reduction in LDL-cholesterol was 33.66, 28.50, and 35.87% in the HCD1, HCD2, and HCD3 groups, respectively, when compared with the HCD group.

The **HDL-cholesterol** values for all the five groups were recorded as 28 ± 0.712, 23.1 ± 1.6, 24.7 ± 2.76, 23.7 ± 0.871, and 16.7 ± 0.20 mg/dl for the ND, HCD1, HCD2, HCD3, and HCD groups, respectively (**Figure 5D**). There is a significant

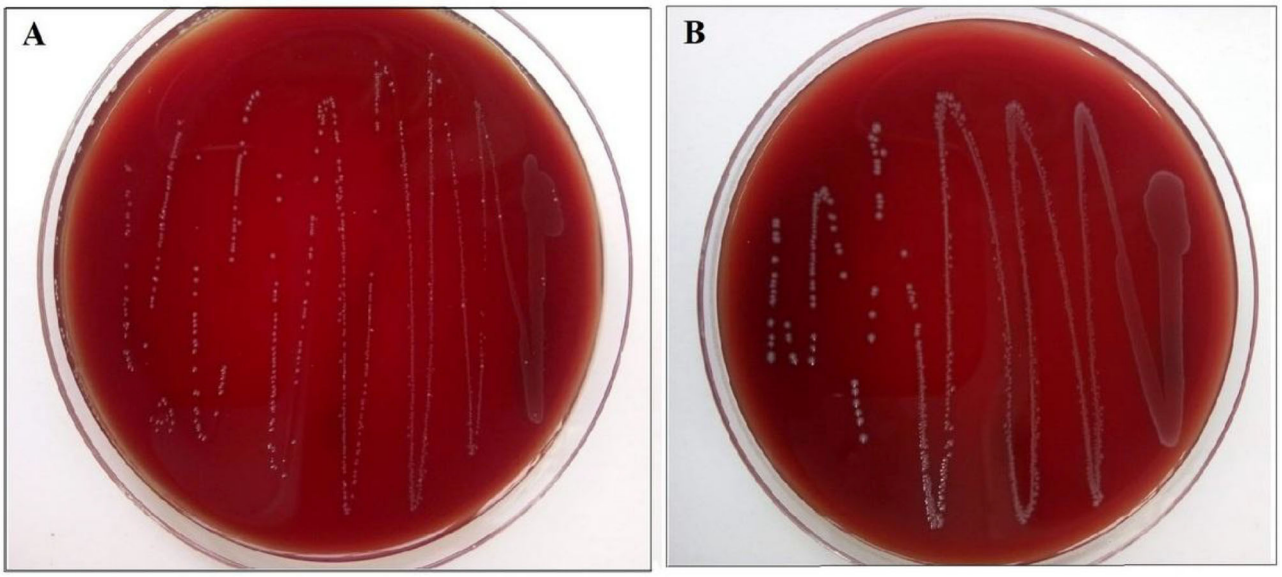


FIGURE 3 | (A) *Enterococcus lactis* YY1 and **(B)** *Enterococcus durans* HS03 showing negative hemolytic activity on sheep blood agar.

TABLE 6 | Bodyweight, weight gain, and feed intake of rats fed with experimental diets (mean values, $n = 6$).

Group	Initial weight (g)	Final weight (g)	Weight gain (g)	Feed intake (g)
ND	205 ± 1.826	236.5 ± 1.708***	31.5 ± 2.432***	180.8 ± 6.193***
HCD1	208.75 ± 1.25	242.5 ± 3.096***	33.75 ± 3.146***	186 ± 3.813**
HCD2	204 ± 2.38	252.5 ± 3.819***	48.5 ± 4.89**	202.3 ± 3.163^{ns}
HCD3	204 ± 3.109	236 ± 4.726***	32 ± 7.113***	182 ± 5.366***
HCD	208.75 ± 1.25	272.5 ± 2.141	63.75 ± 5.42	208.2 ± 2.643

*Analysis was done by One-way ANOVA followed by Dunnett's post hoc test where ***indicates $p \leq 0.001$, **indicates $p \leq 0.01$, and ^{ns}indicates non-significant value.*

difference ($p < 0.05$) among the HDL-cholesterol values of different experimental groups. The resulting increase of HDL-cholesterol in the probiotic treatment groups, i.e., HCD1, HCD2, and HCD3 was 38.32, 47.9, and 41.92%, respectively, when compared with the HCD group.

The **Atherogenic Index (AI) score** of the dietary treatment groups of the rats also differed significantly ($p < 0.05$). The AI score of the ND group was 0.659 ± 0.224. However, the AI score of the probiotic-treated groups was found to decrease after 21 days of treatment as compared with the HCD group (3.21 ± 0.372). The maximum reduction in AI score was recorded in the HCD3 group (1.05 ± 0.0514) than in the HCD1 (1.38 ± 0.304) and HCD2 groups (1.67 ± 0.185) (**Figure 5F**).

Changes in Liver Total Cholesterol and Triglyceride

The **total cholesterol level** significantly ($p < 0.05$) differed among the supernatants of liver homogenate among different experimental groups. The **total cholesterol level** of different experimental groups was recorded as 63.1 ± 2.76, 64 ± 2.83, 67.8 ± 0.527, 64.2 ± 4.13, and 77.6 ± 4.35 mg/dl for the ND, HCD1, HCD2, HCD3, and HCD dietary groups, respectively (**Figure 6A**). The dietary treated groups HCD1, HCD2, and HCD3 showed decreases in serum cholesterol levels up to 17.52, 12.63, and 17.16%, respectively, when compared with the HCD group after 21 days of dietary treatment.

The **triglyceride (TG)** concentrations from the supernatants of liver homogenate also differed significantly ($p < 0.05$) among all the groups throughout the experiment. After dietary treatment, **TG levels** for all the five groups were recorded as 21.2 ± 2.97, 32.1 ± 2.47, 30.8 ± 2.71, 32.4 ± 2.92, and 44.8 ± 3.62 mg/dl for the ND, HCD1, HCD2, HCD3, and HCD groups, respectively (**Figure 6B**). The reduction in TG concentrations was 28.35, 31.25, and 27.68% in the HCD1, HCD2, and HCD3 groups, respectively, when compared with the HCD group.

Effects on Liver and Kidney Histopathology

From the histopathology, no observable structural changes were found in kidney sections between the different experimental groups. The sections of kidneys (**Figures 7A–E**) were found to have well-organized Bowman's capsule cells (Black arrow). There was no significant structural change observed between the experimental groups. A moderate degree of fatty vacuolization was observed in the HCD treated group (**Figure 7J**). However, the liver sections (**Figures 7F–I**) of all the experimental groups including the normal control group showed well-organized cellular (lobule) structure and no abnormalities were seen in any groups than the HCD-treated group (**Figure 7J**).

Effect on Liver and Body Lipid Index

The liver index was found to be significantly low in the ND, HCD1, HCD2, and HCD3 groups than the HCD group (2.74 ±

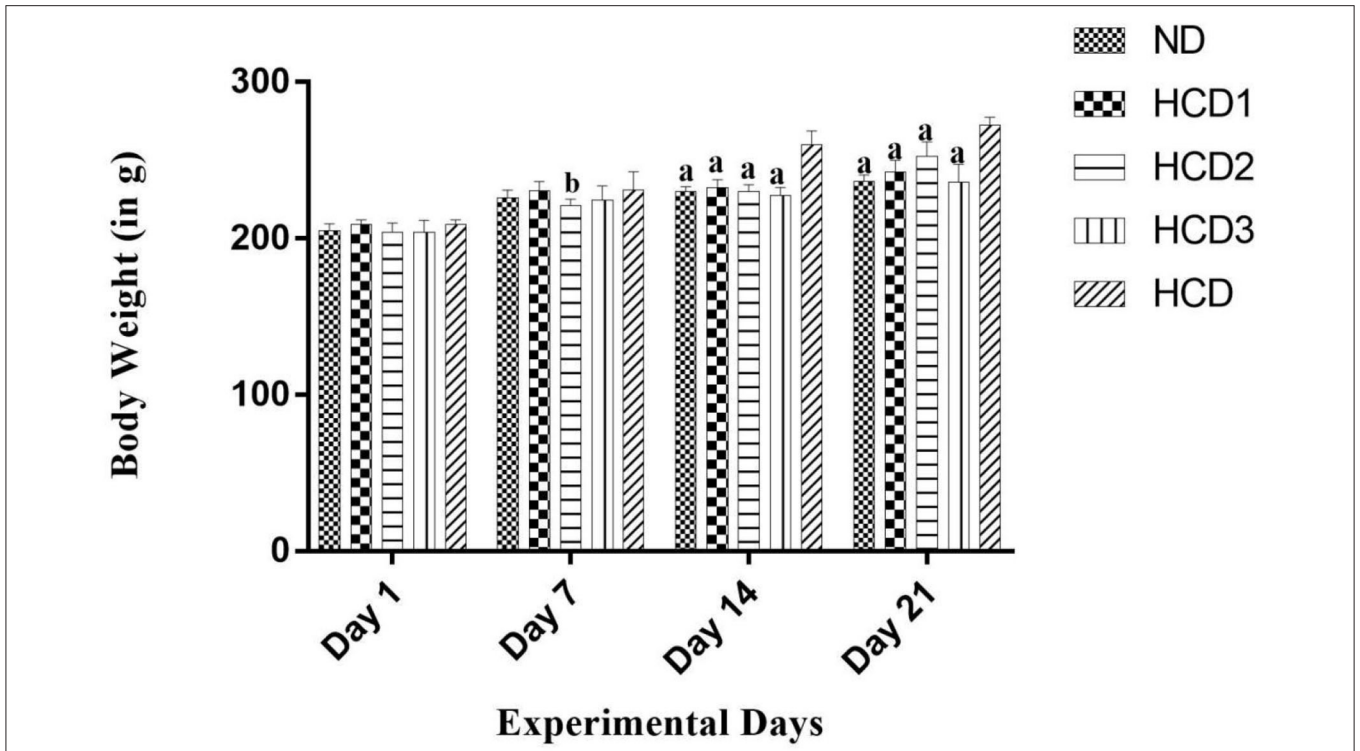


FIGURE 4 | Bodyweight increment in all experimental groups after 21 days. Results are shown as mean ± SEM ($n = 6$). "a" denotes $p \leq 0.0001$; "b" denotes $p \leq 0.001$ vs. Hypercholesterolemic diet control (HCD) group.

TABLE 7 | Serum total cholesterol, triglyceride, LDL-, HDL-D-cholesterol, VLDL and AI score in experimental rats.

Group	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	LDL-cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	VLDL (mg/dl)	Atherogenic index (AI) score
ND	46.4 ± 5.45***	55.5 ± 6.68***	24.6 ± 2.09***	28 ± 0.736***	11.1 ± 1.34***	0.659 ± 0.224***
HCD1	54.6 ± 4.77**	65.8 ± 3.66***	27 ± 1.27***	23.1 ± 1.6**	13.2 ± 0.732***	1.38 ± 0.304***
HCD2	65.8 ± 2.86^{ns}	65 ± 5.74***	29.1 ± 1.13***	24.7 ± 2.76***	13 ± 1.15***	1.67 ± 0.185***
HCD3	48.6 ± 0.827***	58.9 ± 0.659***	26.1 ± 1.76***	23.7 ± 0.871***	11.8 ± 0.132***	1.05 ± 0.0514***
HCD	70.5 ± 5.67	88.2 ± 5.62	40.7 ± 2.98	16.7 ± 0.20	17.6 ± 1.12	3.21 ± 0.372

*Analysis was done by One-way ANOVA followed by Dunnett's post hoc test where ***indicates p ≤ 0.001, **indicates p ≤ 0.01, and ^{ns}indicates non-significant value.*

0.429%, 2.93 ± 0.141%, 3.22 ± 0.13%, 3.19 ± 0.271% vs. 3.62 ± 0.158%, $p < 0.05$) (**Figure 8A**). The body lipid index also showed significant difference among the experimental groups ($p < 0.05$). The highest percentage of body lipid index was recorded in the HCD group (2.34 ± 0.177%), while the lowest percentage was recorded in the HCD3 group (1.85 ± 0.326%, $p = 0.007$) (**Figure 8B**).

Effects of Probiotic Strains on Oxidative Stress in the Liver of Rats

Long-term intake of hypercholesterolemic diet results in obesity and increased oxidative stress in the liver. Oxidative stress is assessed by measuring the malondialdehyde (MDA), reduced glutathione (GSH), and catalase (CAT) activities in the liver. The effects of the probiotic strains on the level of MDA as well as the activities of the other antioxidant enzymes in the liver are presented in **Figure 9**.

The MDA levels in the hypercholesterolemic diet (HCD) group were significantly ($p < 0.05$) higher than that of the normal control group (by a 3.42-fold increase) and other probiotic strain-fed groups, indicating the over-peroxidation of liver injury (**Figure 9A**). The lowest MDA level was recorded in the HCD1 and HCD3 groups which were fed with probiotic strain 1 and a combination of both probiotic strains 1 and 2, respectively, as compared with the negative control group (HCD).

At the same time, the liver GSH level showed a significant ($p < 0.05$) decrease in the hypercholesterolemic diet (HCD)

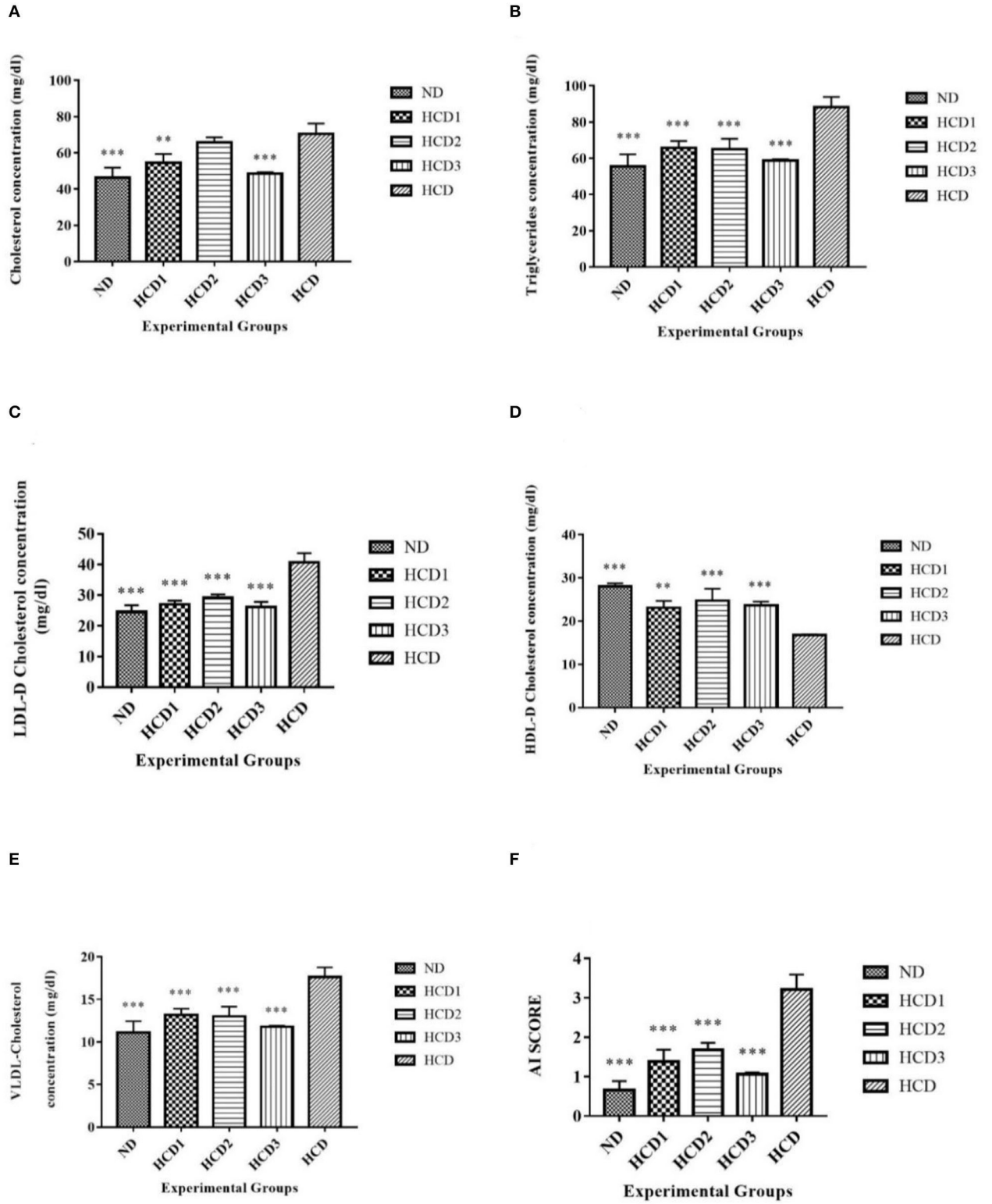


FIGURE 5 | Serum lipid parameters of different experimental groups. Analysis was done by One-way ANOVA followed by Dunnett's *post-hoc* test where ***indicates $p \leq 0.001$ and **indicates $p \leq 0.01$. All data represented as mean ± SD of 6 animals. **(A)** Cholesterol **(B)** Triglycerides. **(C)** LDL-D cholesterol. **(D)** HDL-D cholesterol. **(E)** VLDL-D cholesterol. **(F)** Atherogenic Index (AI).

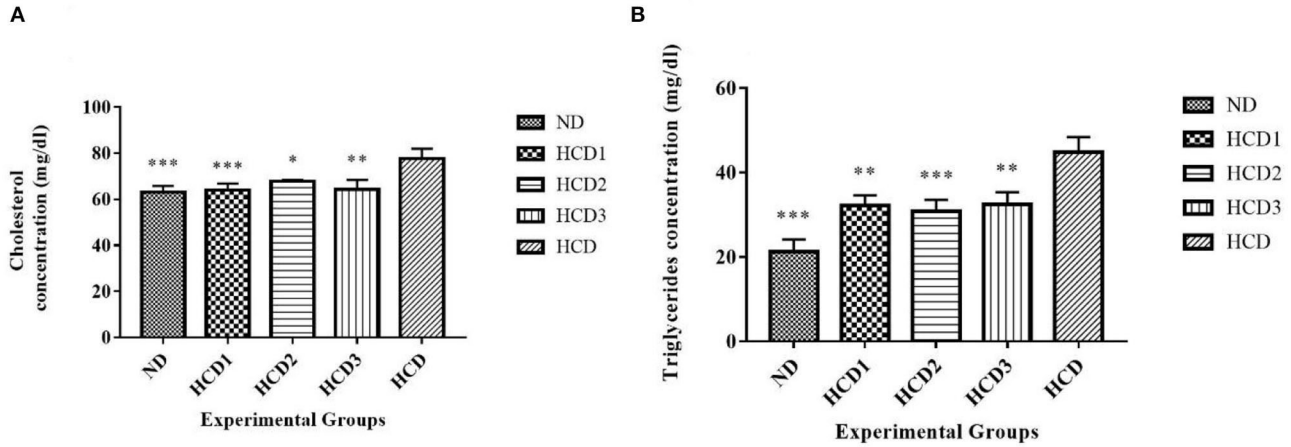


FIGURE 6 | Liver lipid parameters of different experimental rats. Analysis was done by One-way ANOVA followed by Dunnett's *post-hoc* test where ***indicates $p \leq 0.001$, **indicates $p \leq 0.01$, and *indicates $p \leq 0.05$. All data represented as mean ± SD of 6 animals. **(A)** Cholesterol (liver). **(B)** Triglycerides (liver).

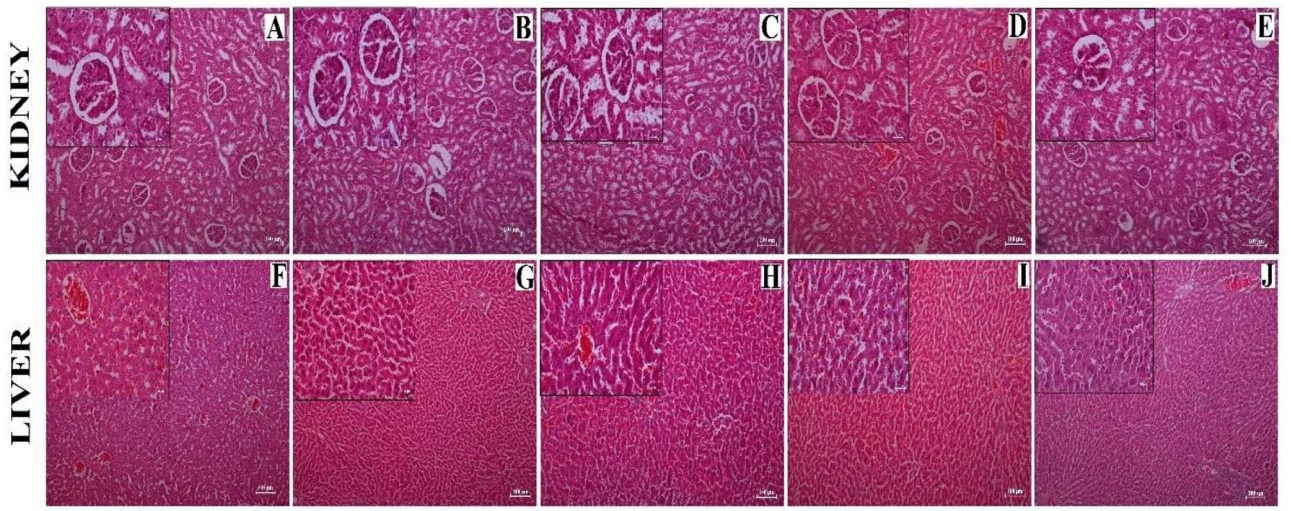


FIGURE 7 | Histological sections showing kidney **(A–E)** and liver **(F–J)** obtained from different experimental groups. **(A,F)** = Normal diet control (ND) group, **(B,G)** = HCD1 group, **(C,H)** = HCD2 group, **(D,I)** = HCD3 group, and **(E,J)** = Hypercholesterolemic diet control (HCD) group. The main images represent sections observed under 10X magnification; the inset represents 40X magnified images. Scale bars of 100 and 25 μm are allotted for 10X and 40X magnifications, respectively.

group when compared to those of the normal control (ND) group as well as the probiotic strain-fed groups (**Figure 9B**). In the liver, supplementation of probiotic strains significantly ($p < 0.05$) increased the concentration of reduced-GSH. However, the reduced-GSH level recorded in the HCD1 and HCD3 groups (which were fed with probiotic strain 1 and a combination of both probiotic strains 1 and 2, respectively), showed the best results when compared with the hypercholesterolemic diet (HCD) group.

In this study, the catalase activity was significantly decreased in the hypercholesterolemic diet (HCD) group (**Figure 9C**) compared to the normal control rats. Probiotic supplement significantly ($p < 0.05$) restored the catalase activity in high-fat diet-fed rats to the levels of normal control rats (ND). The catalase activity recorded in the HCD1 group (fed with probiotic strain 1) showed the best results when compared with the hypercholesterolemic diet (HCD) group.

All these results suggest that treatment with probiotic strains could improve the hepatic antioxidant status.

Fecal Sample Analysis

SD rats fed with probiotic supplements showed comparatively higher total bacterial counts in their feces than that of the

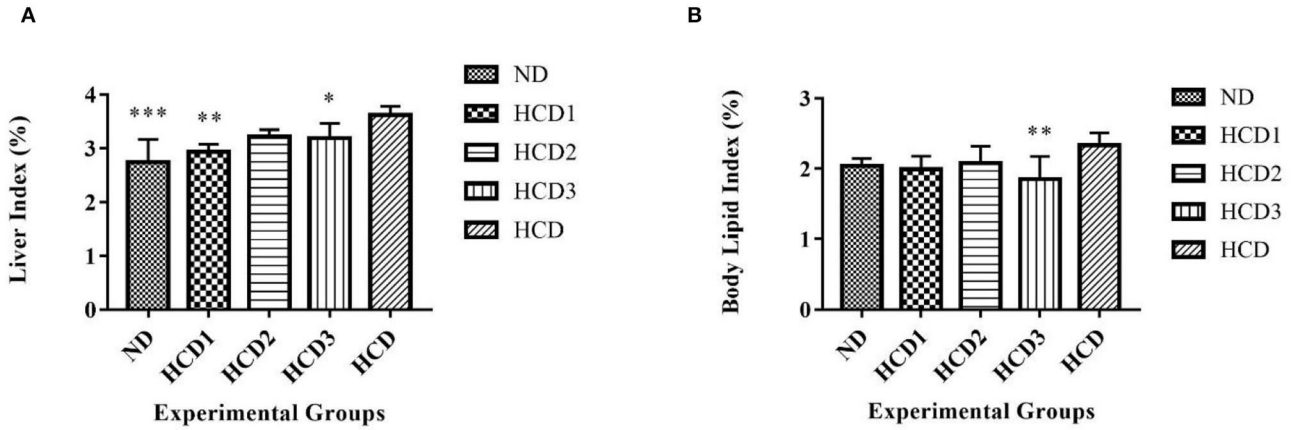


FIGURE 8 | Liver and organ lipid indexes in different experimental rat groups. Analysis was done by One-way ANOVA followed by Dunnett's *post-hoc* test where ***indicates $p \leq 0.001$, **indicates $p \leq 0.01$, and *indicates $p \leq 0.05$. All data represented as mean ± SD of 6 animals. **(A)** Liver index. **(B)** Body lipid index.

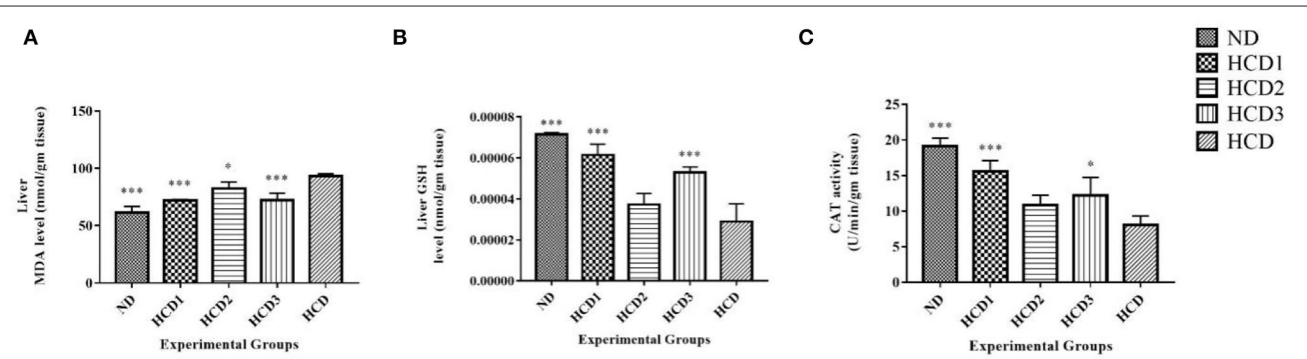


FIGURE 9 | Effect of probiotic strains on MDA, GSH, and CAT levels in the liver of high-fat diet fed rats. **(A)** Liver MDA level. **(B)** Liver GSH level. **(C)** Liver CAT activity. Analysis was done by One-way ANOVA followed by Dunnett's *post-hoc* test where ***indicates $p \leq 0.001$ and *indicates $p \leq 0.05$. All data represented as mean ± SD of 6 animals.

control groups. Their fecal total bacterial count ranged from 8.273 to 8.900 CFU $\log_{10}$/g. Also, a higher number of lactobacilli counts, ranging from 8.263 to 8.830 CFU $\log_{10}$/g was recorded in the feces of the SD rats fed with probiotic supplements in comparison to the control groups. Further, the experimental groups HCD1 and HCD3 showed maximum lactobacilli count in their feces among all the other groups. In contrast to the total bacterial counts and the lactobacilli counts in the feces of the SD rats fed with probiotics, their fecal coliform and *E. coli* counts decreased relative to that of the control groups. Alternatively, the maximum fecal coliform and *E. coli* count were observed in the HCD group. The HCD fecal coliform and *E. coli* count ranged from 7.633 to 8.100 and 7.653 to 8.350, respectively. The overall mean value and the ±SEM of the bacterial counts in the experimental SD rat feces on days 0, 7, 14, and 21 procured from distinct experimental groups have been tabulated in **Table 8**.

DISCUSSION

Enterococcus sp. are highly diverse and are present as common microbiota in the environment, the intestine of humans, mammals, and fermented foods (Giraffa, 2002; Li et al., 2018; Zommiti et al., 2018). A similar type of lactic acid bacteria such as *Enterococcus, Lactococcus*, *Lactobacillus*, *Leuconostoc* were also reported in ethnic starter cultures of Sikkim by Sha et al. (2017, 2018, 2019). There has been research on the probiotic properties of lactic acid bacteria like *Lactobacillus* spp. and *Bifidobacterium* but only limited studies have been undertaken on the probiotic attributes of *Enterococcus* spp. Therefore, in this study, the *Enterococcus* strains, namely *Enterococcus durans* HS03 (accession no. KX274030) and *Enterococcus lactis* YY1 (accession no. KU 601443) (Ghatani and Tamang, 2017) were studied for their probiotic properties, especially cholesterol-lowering, as one of the important health benefits by *in vivo* studies, which makes it one of the first kind of studies conducted so far. The strains

TABLE 8 | Effect of feeding probiotics on fecal bacterial population ($n = 3$).

Log CFU/g of feces	Day	ND		HCD1		HCD2		HCD3		HCD	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Total aerobes	0	8.273	0.018	8.273	0.018	8.273	0.018	8.273	0.018	8.273	0.018
	7	8.233^{ns}	0.038	8.657^{a}	0.038	8.720^{a}	0.035	8.827^{a}	0.043	8.207	0.032
	14	8.227^{ns}	0.037	8.673^{a}	0.058	8.723^{a}	0.034	8.823^{a}	0.039	8.250	0.025
	21	8.187^{ns}	0.026	8.647^{a}	0.026	8.710^{a}	0.023	8.900^{a}	0.055	8.150	0.017
Lactobacillus	0	8.270	0.072	8.270	0.072	8.270	0.072	8.270	0.072	8.270	0.072
	7	8.110^{a}	0.023	8.603^{a}	0.033	8.263^{a}	0.071	8.633^{a}	0.041	7.627	0.041
	14	8.247^{ns}	0.032	8.647^{a}	0.044	8.460^{b}	0.070	8.750^{a}	0.017	8.237	0.035
	21	8.260^{c}	0.023	8.830^{a}	0.012	8.470^{a}	0.029	8.757^{a}	0.015	8.060	0.029
Coliforms	0	7.633	0.035	7.633	0.035	7.633	0.035	7.633	0.035	7.633	0.035
	7	7.660 ^{ns}	0.064	7.453^{b}	0.049	7.563^{ns}	0.045	7.420^{a}	0.031	7.633	0.041
	14	7.560^{a}	0.035	6.830^{a}	0.040	8.030^{ns}	0.029	7.437^{a}	0.023	8.100	0.029
	21	7.600^{a}	0.029	6.360^{a}	0.023	7.570^{a}	0.017	6.450^{a}	0.017	8.450	0.017
Escherichia coli	0	7.653	0.047	7.653	0.047	7.653	0.047	7.653	0.047	7.653	0.047
	7	7.660^{ns}	0.069	7.130^{a}	0.029	7.583^{c}	0.009	7.400^{a}	0.042	7.733	0.073
	14	7.467^{a}	0.009	6.437^{a}	0.034	7.597^{a}	0.026	7.423^{a}	0.035	8.010	0.040
	21	7.670^{a}	0.017	7.090^{a}	0.035	7.520^{a}	0.023	7.427^{a}	0.044	8.350	0.029

ND, normal diet; HCD1, hypercholesterolemic diet containing Enterococcus; HCD2, hypercholesterolemic diet containing Enterococcus; HCD3, hypercholesterolemic diet containing Enterococcus durans; HS03, Enterococcus lactis YY1.

$^{a}p < 0.0001$ and <0.001.

$^{b}p < 0.01$.

$^{c}p < 0.05$.

^{ns}indicates non-significant value.

were previously isolated from fermented yak milk, a product of the Sikkim Himalayas known as *chhurpi* and healthy human gut, and then tested for cholesterol reduction and were identified by 16s rDNA sequencing (Ghatani and Tamang, 2017). A probiotic bacterium should have at least one health benefit. In human blood, higher than 1 mmol of cholesterol (i.e., 18 mg/dl) than the normal level is reported to increase the risk of coronary heart disease and coronary death. There is growing public attention to healthy food due to increasing knowledge in society as statins that lower high cholesterol are expensive and are reported to cause severe side effects (Golomb and Evans, 2008).

For a strain to be considered as a potential probiotic candidate, it should be able to tolerate or resist the pH conditions, bile salt concentrations, and cell surface hydrophobicity. The beneficial bacteria through the oral route pass through the highly acidic gastric juice in the stomach (approximately pH 3) and a weakly basic juice in the intestine (pH of 7.8–8.4) containing 0.3–2% (w/v) bile salts in the upper part of the intestinal tract where they can stay for 1–2 h (Chen et al., 2010). The strains were first tested at pH 2.5 and pH 2 which is considered to be a strong differential pH range (Turchi et al., 2013). Both the strains were observed to tolerate pH up to 2 h in both pH ranges as in accordance to the tolerance reported in the case of *Lactobacillus strains* (Liong and Shah, 2005). However, similar to our study, a decrease in pH with time has also been reported by Raghavendra et al. (2010).

The ability to tolerate bile concentrations studied by different researchers varies in concentration, however, we had used oxgall, taurocholic acid, and cholic acid at 0.5 and 1% for 0, 4, and 8 h, in accordance to 0.5% (Mathara et al., 2008), 0.1–0.3% (Dunne et al., 2001) and the prevailing time is suggested to be 4 h (Mishra and Prasad, 2005). The two strains showed variability concerning acid and bile tolerance which may be due to species and strain specificity.

Cell surface hydrophobicity is one of the important criteria as probiotics need to be adhered to the intestinal mucosa to avoid being removed from the peristaltic movement of the colon. *Enterococcus durans* HS03 and *Enterococcus lactis* YY1 gave 65 and 70% cell surface hydrophobicity percentage, respectively, which was as per the report where hydrophobic index > 40% was considered as hydrophobic (Boris et al., 1998) and Nostro et al. (2004) recommended hydrophobicity index > 70% for adherence to the cell surfaces; 38.1–67.8% hydrophobicity was observed by *L. acidophilus* (Vinderola and Reinheimer, 2003) which was in accordance with our study shown by *Enterococcus durans* HS03.

Several mechanisms for cholesterol-lowering have been reported like cholesterol assimilation, cholesterol co-precipitation with deconjugated bile, binding of cholesterol to the bacterial cell wall, and enzymatic deconjugation of bile acids due to the presence of BSH enzyme. The mechanism for cholesterol-lowering in our study was the presence of the BSH enzyme which was similar to Liong and Shah, 2005 and Lye et al. (2010). Numerous lactic acid bacterial species like *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp., and *Pediococcus* spp. with bile salt hydrolase (BSH) activity have been studied and thereby recommended for their cholesterol-lowering effects (Lim et al., 2004; Zhang et al., 2017). One strain *Enterococcus lactis* YY1 was BSH-positive when checked in direct assay containing TDCA similar to those reported by Archer and Halami (2015)

on screening the isolates from dairy and human fecal matter. The BSH enzyme-specific enzyme activity U/mg was more in the case of sodium glycocholate than sodium taurocholate as in earlier reports of BSH enzymes of *Lactobacillus* showing more affinity toward glycine-conjugated bile salts (Liong and Shah, 2005; Pavlović et al., 2012).

Antibiotic susceptibility of LAB is one of the important criteria for the safety of probiotics. *Enterococcus* species have been reported to become resistant to various antibiotics in some studies (Vidhyasagar and Jeevaratnam, 2013). Some probiotic enterococci transfer the antibiotic resistance to the nearby microbes in the gut and thereby disrupt the original intestinal flora (Taheur et al., 2016). Both the strains were susceptible to all antibiotics except penicillin which may be plasmid-borne or associated with the chromosomal DNA. According to Danielsen and Wind (2003), the resistance is due to the presence of D-Ala-D-lactate in their peptidoglycan instead of D-Ala-D-Ala dipeptide which is the target of the antibiotic.

The strains showed no hemolytic activity; thus, they were γ hemolytic, and the absence of hemolytic activity is considered a safety prerequisite for the selection of a probiotic strain. None of the strains showed α and β hemolytic activity when grown on sheep blood agar. Our study was similar to the observations made for the strains of *L. paracasei* subsp. *paraccasei*, *Lactobacillus* spp., and *L. casei* isolated from the dairy products (Maragkoudakis et al., 2006) and foods (Kumar and Murugalatha, 2012).

To determine the relationship between the administration of different strains of *Enterococcus* and the status of serum lipid profiling, *in vivo* study using the high-fat diet-induced obesity model in SD rats was done. Obesity is typically associated with body weight and dyslipidemia, which encompasses elevated levels of TC, TG, and LDL-C as well as lowered HDL-C levels. All of these are risk factors for cardiovascular disease (Skrypnik et al., 2018; Wang et al., 2021). The long-term exposure of high-fat diet seems to have a significant relation to the onset of obesity from various rat model studies (Yin et al., 2010; Lasker et al., 2019). From our results, the onset of obesity was also supported by the body weight and feed efficiency data. The hypercholesterolemic diet control (HCD) group which was fed with a cholesterol-enriched diet recorded the highest body weight gain increase due to the increased feed consumption. However, upon treatment with the probiotic strain, the HCD3 group, which was fed with both the *Enterococcus durans* HS03 and *Enterococcus lactis* YY1 strains significantly lowered its body weight as well as its feed intake when compared to the hypercholesterolemic diet control (HCD) group. The body lipid index data also follows a similar trend. The HCD3 group records the lowest body lipid index, when compared to the HCD group, indicating the effect of both *Enterococcus durans* HS03 and *Enterococcus lactis* YY1 strains in the amelioration of obesity in these experimentally induced obese rats. It was evident that different strains of *Enterococcus* might act as a new therapeutic probiotic candidate in controlling body weight gain.

Under normal conditions, the liver, which is an important site of lipid metabolism in the body, maintains a balance of lipid synthesis and decomposition, whereas a high-fat diet disrupts this balance, causing excessive lipid accumulation (i.e., hepatic steatosis) and oxidative stress in the liver, indicating the presence of liver injury (Skrypnik et al., 2018; Wang et al., 2021). Accumulation of lipids in the liver is the main cause of non-alcoholic fatty liver disease (NAFLD), which is a common complication of obesity (Skrypnik et al., 2018). Furthermore, multiple studies have shown that obesity is directly connected with body-liver weight, liver TC and liver TG, and serum lipid profiles in high-fat diet-induced obese rats (Skrypnik et al., 2018; Wang et al., 2021; Yang et al., 2021). In our experiments, it was evident that the high-fat diet-fed rats (HCD rats) showed an increased TC and TG levels from both the serum and liver tissue homogenate, which further triggered the lipid accumulation and lipotoxicity in the liver. The levels of TC, TG, LDL-C, and HDL-C in the serum of the different experimental groups of SD rats were evaluated after 3 weeks of animal feeding to assess their blood lipid metabolism. The serum levels of TC, TG, and LDL-C were dramatically raised in the HCD-treated group compared to the control group, while serum levels of HDLC were significantly lowered, as expected (**Table 6**). However, the *Enterococcus* treatment on the experimental rat groups, on the other hand, restored these abnormalities in SD rats fed on a high-fat diet, suggesting a reduction in metabolic dysfunction. This reduction of serum lipid profile may be due to the assimilation and reduced reabsorption of cholesterol by bacterial strain, deconjugation of bile acids by *Enterococcus* bile salt acid hydrolase, and increase cholesterol uptake by low-density lipoprotein receptor pathway in the liver tissues (Gill and Guarner, 2004). Moreover, as evidenced by liver histology, the degree of fatty accumulation in the liver was significantly reduced in all intervention experimental groups.

Long-term high-fat diets are an inappropriate dietary structure, and oxidative stress may result from increased reactive oxygen species (ROS) formation in the mitochondria of liver tissues due to fatty acid oxidation. Although oxidation activities are critical to life for energy and metabolism, excessive ROS can cause hepatic oxidative damage and may also trigger fibrosis in liver tissue, posing a serious threat to physical health, particularly in obese patients (Lasker et al., 2019). However, recent reports suggest that treatment with various probiotic strains, such as *Bifidobacterium animalis*, *Lactobacillus rhamnosus*, and *Bacillus* LBP32 significantly induce potent antioxidant capacity and reduce the oxidative damages *in vivo* models (Wu et al., 2019). MDA is a major byproduct of lipid peroxidation and also serves as an efficient reliable biomarker for oxidative stress-induced liver injury (Lin et al., 2018). The liver is a major organ involved in lipid metabolism. It was reported that various *Lactobacillus* strains can reduce the increase of MDA levels in the liver induced by D-gal (Lin et al., 2018). In this study, the levels of lipid peroxidation in the liver of hypercholesterolemic diet control (HCD) group were significantly higher than the normal (ND) group. However, upon the treatment with *Enterococcus durans* HS03 and *Enterococcus lactis* YY1 strains, both the experimental groups (HCD1 and HCD2) significantly reduced the MDA levels in the liver of the experimental rats.

The animal body has been demonstrated to have an effective mechanism for preventing free radical-induced tissue

cell damage, which is accomplished *via* a set of endogenous antioxidant enzymes and proteins such as GSH and CAT. GSH and CAT formed a defense against ROS that was mutually helpful. Studies have demonstrated that the antioxidant mechanisms of probiotics include chelating metal ion, possessing their own antioxidant enzymatic systems, and producing metabolites with antioxidative activity, such as GSH, butyrate, and folate (Wang et al., 2017). A recent report suggests that the yogurt supplementation increased the GSH and CAT levels in experimental rat models (Lasker et al., 2019). A similar pattern was also observed in our study. Supplementation of *Enterococcus durans* HS03 in the HCD1 group and both the strains of *Enterococcus durans* HS03 and *Enterococcus lactis* YY1 in HCD3 significantly increased the activities of the GSH, and CAT levels. This indicated that *Enterococcus* strains could keep scavenging free radicals and could improve the antioxidant defense system of hyperlipidemic rats.

Also, various pieces of literature have recorded the effects of feeding probiotic supplements on the fecal bacterial counts of animal models. However, the cholesterol-lowering effects of BSH-active *Enterococcus* spp. have scarcely been studied (Zhang et al., 2017). Further, quite a few studies have recorded the effects of feeding probiotic enterococci on the fecal bacterial count of animal models. As per our study, the microbial analysis of the SD rat feces revealed a significantly higher count of lactobacilli in the probiotic-fed SD rat in comparison to the control group rats. Lactobacillus has been considered the normal inhabitants of the intestinal microbiota in both humans as well as in animals. Also, they have been linked to various health benefits. In this study, SD rats supplemented with probiotic treatment showed higher total fecal lactobacilli counts as compared to that of the control SD rat groups, which is suggestive of the fact that feeding of the *Enterococcus* supplement has favored the intestinal stability of the lactobacilli. However, the lower coliform and *E. coli* count in the SD rats fed with probiotic supplements are on par with the findings previously recorded due to the probiotic feeding of experimental rats (Kumar et al., 2011). This finding is also on par with the study conducted by Zhu et al. (2019) and is suggestive of the fact that *E. durans* and *E. lactis* had a remarkable effect on the stability of the gut microflora, thereby indirectly assisting in cholesterol absorption and excretion (Zhu et al., 2019). The significant higher lactobacilli count in the probiotic-fed SD rats may also be attributed to the fact that lactic acid bacteria used in our study survived the low pH and high bile salt concentrations in the gastrointestinal (GI) tract, colonized the GI tract, and enhanced the intestinal probiotics (Kumar et al., 2011; Zhu et al., 2019). However, like in our findings, many previous reports have also revealed lower counts of *E. coli* and coliform in the feces of probiotic-fed rats (Kumar et al., 2011).

CONCLUSION

Although the effects of probiotic bacteria have been investigated previously by different researchers, the literature specifically on the effect of BSH-positive and BSH-negative bacterial strains on fecal bacteria counts in animal models is quite less. Results of this study showed that both the *Enterococcus durans* HS03 and *Enterococcus lactis* YY1 strains possess probiotic properties *in vitro*. The supplementation of both the *Enterococcus durans* HS03 and *Enterococcus lactis* YY1 can improve the high-fat diet-related complications in experimental rat model based on a 21-day experimental schedule. Further, the serum lipid profiles, liver cholesterol, and triglycerides from all the experimental rat groups show the hypolipidemic properties of both the strains. The potential mechanism of this effect might be related to decreasing oxidative stress *via* several antioxidant indices. In this study, fecal microbial analysis showed significantly higher fecal lactobacilli counts in probiotic-treated groups compared to the control groups. This could be because of the ability of the strains to survive at low pH and high bile concentration as described previously *in vitro* experiments. Hence, in this study, *Enterococcus durans* HS03 and *Enterococcus lactis* YY1 which were investigated to explore probiotic properties reveal that these two LAB strains have the potential to be developed as probiotic foods for better health attributes. In this study, microencapsulation had not been conducted although microencapsulated probiotic strain reducing plasma cholesterol levels in rats had been reported by others. The result of this study reveals that consumption of *Enterococcus durans* HS03 and *Enterococcus lactis* YY1 as a probiotic dietary adjunct might be useful in reducing serum cholesterol levels in humans. However, placebo-controlled clinical trials need to be conducted to validate the efficacy and safety of the strain and its use in the management of high cholesterol.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by IAEC/NBU/2019/17 from CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) of University of North Bengal, India.

AUTHOR CONTRIBUTIONS

KG conducted major experiments, analysis of data, and drafting of manuscript. ST has assisted in microbial analysis and drafting of the manuscript. DM and SS assisted with animal experiments, data analysis, and drafting of the manuscript. SPS was involved in developing the hypothesis, animal procurement, manuscript drafting, and manuscript finalizing. SB was involved in developing hypotheses, designing animal experiments, and approved of the final manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The work was funded by the North Bengal University Research Grant 2019 and DST- SERB project (EEQ/2019/000738). The authors gratefully acknowledge the financial support of the University of North Bengal and DST- SERB project sanctioned to KG.

REFERENCES

Aebi, H. (1974). "Catalase," in *Methods of Enzymatic Analysis*, ed H. U. Bergmeyer (New York, NY: Academic Press), 673–684.

Albano, C., Morandi, S., Silvetti, T., Casiraghi, M. C., Manini, F., and Brasca, M. (2018). Lactic acid bacteria with cholesterol-lowering properties for dairy applications: *In vitro* and *in situ* activity. *J. Dairy Sci.* 101, 10807–10818. doi: 10.3168/jds.2018-15096

Archer, A. C., and Halami, P. M. (2015). Probiotic attributes of *Lactobacillusfermentum* isolated from human feces and dairy products. *Appl. Microbiol. Biotechnol.* 99, 8113–8123. doi: 10.1007/s00253-015-6679-x

Boris, S., Suárez, J. E., Vázquez, F., and Barbés, C. (1998). Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infect. Immun.* 66, 1985–1989. doi: 10.1128/IAI.66.5.1985-1989.1998

Charteris, W. P., Kelly, P. M., Morelli, L., and Collins, J. K. (1998). Antibiotic susceptibility of potentially probiotic Lactobacillus species. *J. Food Prot.* 61, 1636–1643. doi: 10.4315/0362-028X-61.12.1636

Chen, W., Tan, H. Z., Hu, B., and Liu, X. M. (2010). The mechanism of acid and bile tolerance of probiotics in GI-trac. *J. Chin. Inst. Food Sci. Technol.* 12, 1–6.

Danielsen, M., and Wind, A. (2003). Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *Int. J. Food Microbiol.* 82, 1–11. doi: 10.1016/S0168-1605(02)00254-4

Dewan, S., and Tamang, J. P. (2007). Dominant lactic acid bacteria and their technological properties isolated from the Himalayan ethnic fermented milk products. *Antonie van Leeuwenhoek* 92, 343–352. doi: 10.1007/s10482-007-9163-5

Draper, H. H., and Hadley, M. (1990). Malondialdehyde determination as index of lipid Peroxidation. *Meth. Enzymol.* 186, 421–431. doi: 10.1016/0076-6879(90)86135-I

Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., and O'Halloran, S., et al. (2001). *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *Am. J. Clin. Nutr.* 73, 386s−392s. doi: 10.1093/ajcn/73.2.386s

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*82, 70–77. doi: 10.1016/0003-9861(59)90090-6

Friedewald, W. T., Levy, R. I., and Fredrickson, D. S. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* 18, 499–502. doi: 10.1093/clinchem/18.6.499

Gerhardt, P., Krieg, N. R., Nester, E. W., and Phillips, G. B. (1913). *General Bacteriology*. Washington, D.C.: American Society for Microbiology.

Ghatani, K., and Tamang, B. (2016). Indigenous rearing practices of Yak and its multipurpose uses in the Sikkim Himalayas. *IOSR J. Agric. Vet. Sci* 9, 1–8. doi: 10.9790/2380-09310108

Ghatani, K., and Tamang, B. (2017). Assessment of probiotic characteristics of lactic acid bacteria isolated from fermented yak milk products of Sikkim, India: chhurpi, shyow, and khachu. *Food Biotechnol.* 31, 210–232. doi: 10.1080/08905436.2017.1335212

Gill, H. S., and Guarner, F. (2004). Probiotics and human health: a clinical perspective. *Postgrad. Med. J.* 80, 516–526. doi: 10.1136/pgmj.2003.008664

Gilliland, S. E., and Walker, D. K. (1990). Factors to consider when selecting a culture of *LactobacillUnited Statescidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *J. Dairy Sci.* 73, 905–911. doi: 10.3168/jds.S0022-0302(90)78747-4

Giraffa, G. (2002). Enterococci from foods. *FEMS Microbiol. Rev.* 26, 163–171. doi: 10.1111/j.1574-6976.2002.tb00608.x

Golomb, B. A., and Evans, M. A. (2008). Statin adverse effects. *Am. J. Cardiovasc. Drugs.* 8, 373–418. doi: 10.2165/0129784-200808060-00004

Guo, L., Li, T., Tang, Y., Yang, L., and Huo, G. (2016). Probiotic properties of *Enterococcus* strains isolated from traditional naturally fermented cream in China. *Microb. Biotechnol.* 9, 737–745. doi: 10.1111/1751-7915.12306

Hotel, A. C. P., and Cordoba, A. (2001). Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria: report of a joint FAO WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. *Prevention* 5, 1–10.

Hyrslova, I., Krausova, G., Smolova, J., Stankova, B., Branyik, T., and Malinska, H., et al. (2021). Prebiotic and immunomodulatory properties of the microalga *Chlorellavulgaris* and its synergistic triglyceride-lowering effect with bifidobacteria. *Fermentation* 7, 125. doi: 10.3390/fermentation7030125

Kiani, A., Nami, Y., Hedayati, S., Jaymand, M., Samadian, H., and Haghshenas, B. (2021). Tarkhineh as a new microencapsulation matrix improves the quality and sensory characteristics of probiotic *Lactococcuslactis* KUMS-T18 enriched potato chips. *Sci. Rep.* 11, 1–13. doi: 10.1038/s41598-021-92095-1

Kouhi, F., Mirzaei, H., Nami, Y., Khandaghi, J., and Javadi, A. (2022). Potential probiotic and safety characterisation of *Enterococcus* bacteria isolated from indigenous fermented Motal cheese. *Int. Dairy J.* 126, 105247. doi: 10.1016/j.idairyj.2021.105247

Kumar, A. M., and Murugalatha, N. (2012). Isolation of *Lactobacillusplantarum* from cow milk and screening for the presence of sugar alcohol producing gene. *J. Microbiol. Antimicrob.* 4, 16–22. doi: 10.5897/JMA11.083

Kumar, R., Grover, S., and Batish, V. K. (2011). cholesterolemicHypocholesterolemic effect of dietary inclusion of two putative probiotic bile salt hydrolase-producing *Lactobacillusplantarum* strains in Sprague–Dawley rats. *Br. J. Nutr.* 105, 561–573. doi: 10.1017/S0007114510003740

Kumar, V., Kumari, A., Angmo, K., and Bhalla, T. C. (2017). Isolation and characterization of lactic acid bacteria from traditional pickles of Himachal Pradesh, India. *J. Food Sci. Technol.* 54, 1945–1952. doi: 10.1007/s13197-017-2629-1

Lasker, S., Rahman, M. M., Parvez, F., Zamila, M., Miah, P., Nahar, K., et al. (2019). High-fat diet-induced metabolic syndrome and oxidative stress in obese rats are ameliorated by yogurt supplementation. *Sci. Rep.* 9, 1–15. doi: 10.1038/s41598-019-56538-0

Li, B., Zhan, M., Evivie, S. E., Jin, D., Zhao, L., Chowdhury, S., et al. (2018). Evaluating the safety of potential probiotic *Enterococcusdurans* KLDS6. 0930 using whole genome sequencing and oral toxicity study. *Front. Microbiol.* 9, 1943. doi: 10.3389/fmicb.2018.01943

Lim, H. J., Kim, S. Y., and Lee, W. K. (2004). Isolation of cholesterol-lowering lactic acid bacteria from human intestine for probiotic use. *J. Vet. Sci.* 5, 391–395. doi: 10.4142/jvs.2004.5.4.391

Lin, X., Xia, Y., Wang, G., Yang, Y., Xiong, Z., Lv, F., et al. (2018). Lactic acid bacteria with antioxidant activities alleviating oxidized oil induced hepatic injury in mice. *Front. Microbiol.* 9, 2684. doi: 10.3389/fmicb.2018.02684

Liong, M. T., and Shah, N. P. (2005). Acid and bile tolerance and cholesterol removal ability of lactobacilli strains. *J. Dairy Sci.* 88, 55–66. doi: 10.3168/jds.S0022-0302(05)72662-X

Lye, H. S., Rahmat-Ali, G. R., and Liong, M. T. (2010). Mechanisms of cholesterol removal by lactobacilli under conditions that mimic the human gastrointestinal tract. *Int. Dairy J.* 20, 169–175. doi: 10.1016/j.idairyj.2009.10.003

Ma, N., Liu, X., Kong, X., Li, S., Jiao, Z., and Qin, Z., et al. (2017). Feces and liver tissue metabonomics studies on the regulatory effect of aspirin eugenol eater in hyperlipidemic rats. *Lipids Health Dis.* 16, 1–15. doi: 10.1186/s12944-017-0633-0

Mann, G. V., and Spoerry, A. (1974). Studies of a surfactant and cholesteremia in the Maasai. *Am. J. Clin. Nutr.* 27, 464–469. doi: 10.1093/ajcn/27.5.464

Maragkoudakis, P. A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., and Tsakalidou, E. (2006). Probiotic potential of *Lactobacillus*

strains isolated from dairy products. *Int. Dairy J.* 16, 189–199. doi: 10.1016/j.idairyj.2005.02.009

Marras, L., Caputo, M., Bisicchia, S., Soato, M., Bertolino, G., Vaccaro, S., et al. (2021). The role of bifidobacteria in predictive and preventive medicine: a focus on eczema and hypercholesterolemia. *Microorganisms* 9, 836. doi: 10.3390/microorganisms9040836

Mathara, J. M., Schillinger, U., Kutima, P. M., Mbugua, S. K., Guigas, C., Franz, C., et al. (2008). Functional properties of *Lactobacillusplantarum* strains isolated from Maasai traditional fermented milk products in Kenya. *Curr. Microbiol.* 56, 315–321. doi: 10.1007/s00284-007-9084-6

Mishra, V., and Prasad, D. N. (2005). Application of *in vitro* methods for selection of *Lactobacilluscasei* strains as potential probiotics. *Int. J. Food Microbiol.* 103, 109–115. doi: 10.1016/j.ijfoodmicro.2004.10.047

Modak, D., Paul, S., Sarkar, S., Thakur, S., and Bhattacharjee, S. (2021). Validating potent anti-inflammatory and anti-rheumatoid properties of *Drynariaquercifolia* rhizome methanolic extract through *in vitro*, *in vivo*, *in silico* and GC-MS-based profiling. *BMC Complement. Med. Ther.* 21, 1–20. doi: 10.1186/s12906-021-03265-7

Nami, Y., Bakhshayesh, R. V., Manafi, M., and Hejazi, M. A. (2019). cholesterolemicHypocholesterolemic activity of a novel autochthonous potential probiotic *Lactobacillusplantarum* YS5 isolated from yogurt. *LWT* 111, 876–882. doi: 10.1016/j.lwt.2019.05.057

Nami, Y., Panahi, B., Jalaly, H. M., Bakhshayesh, R. V., and Hejazi, M. A. (2020). Application of unsupervised clustering algorithm and heat-map analysis for selection of lactic acid bacteria isolated from dairy samples based on desired probiotic properties. *LWT* 118, 108839. doi: 10.1016/j.lwt.2019.108839

Nguyen, T. D. T., Kang, J. H., and Lee, M. S. (2007). Characterization of *Lactobacillusplantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. *Int. J. Food Microbiol.* 113, 358–361. doi: 10.1016/j.ijfoodmicro.2006.08.015

Nostro, A., Cannatelli, M. A., Crisafi, G., Musolino, A. D., Procopio, F., and Alonzo, V. (2004). Modifications of hydrophobicity, *in vitro* adherence and cellular aggregation of *Streptococcusmutans* by *Helichrysumitalicum* extract. *Lett. Appl. Microbiol.* 38, 423–427. doi: 10.1111/j.1472-765X.2004.01509.x

Pavlović, N., Stankov, K., and Mikov, M. (2012). Probiotics—interactions with bile acids and impact on cholesterol metabolism. *Appl. Biochem. Biotechnol.* 168, 1880–1895. doi: 10.1007/s12010-012-9904-4

Pérez, P. F., Minnaard, Y., Disalvo, E. A., and De Antoni, G. L. (1998). Surface properties of bifidobacterial strains of human origin. *Appl. Environ. Microbiol.* 64, 21–26. doi: 10.1128/AEM.64.1.21-26.1998

Raghavendra, P., Rao, T., and Halami, P. (2010). Evaluation of beneficial attributes for phytate-degrading *Pediococcuspentosaceus* CFR R123. *Benef. Microbes.* 1, 259–264. doi: 10.3920/BM2009.0042

Rosenberg, M. (1984). Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. *FEMS Microbiol. Lett.* 22, 289–295. doi: 10.1111/j.1574-6968.1984.tb00743.x

Salaj, R., Štofilová, J., Šoltesová, A., Hertelyová, Z., Hijová, E., Bertková, I., et al. (2013). The effects of two *Lactobacillusplantarum* strains on rat lipid metabolism receiving a high fat diet. *Sci. World J.* 2013, 135142. doi: 10.1155/2013/135142

Sha, S. P., Jani, K., Sharma, A., Anupma, A., Pradhan, P., Shouche, Y., et al. (2017). Analysis of bacterial and fungal communities in Marcha and Thiat, traditionally prepared amylolytic starters of India. *Sci. Rep.* 7, 10967. doi: 10.1038/s41598-017-11609-y

Sha, S. P., Suryavanshi, M. V., Jani, K., Sharma, A., Shouche, Y. S., and Tamang, J. P. (2018). Diversity of yeasts and molds by culture-dependent and culture-independent methods for mycobiome surveillance of traditionally prepared dried starters for the production of Indian alcoholic beverages. *Front. Microbiol.* 9, 2237. doi: 10.3389/fmicb.2018.02237

Sha, S. P., Suryavanshi, M. V., and Tamang, J. P. (2019). Mycobiome diversity in traditionally prepared starters for alcoholic beverages in India by high-throughput sequencing method. *Front. Microbiol.* 10, 348. doi: 10.3389/fmicb.2019.00348

Shaper, A. G., Jones, K. W., Jones, M., and Kyobe, J. (1963). Serum lipids in three nomadic tribes of northern Kenya. *Am. J. Clin. Nutr.* 13, 135–146. doi: 10.1093/ajcn/13.3.135

Shehata, M. G., El Sohaimy, S. A., El-Sahn, M. A., and Youssef, M. M. (2016). Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. *Ann. Agric. Sci.* 61, 65–75. doi: 10.1016/j.aoas.2016.03.001

Skrypnik, K., Bogdański, P., Łoniewski, I., Reguła, J., and Suliburska, J. (2018). Effect of probiotic supplementation on liver function and lipid status in rats. *Acta. Sci. Pol. Technol. Aliment.* 17, 185–192. doi: 10.17306/J.AFS.0554

Taheur, F. B., Kouidhi, B., Fdhila, K., Elabed, H., Slama, R. B., Mahdouani, K., et al. (2016). Anti-bacterial and anti-biofilm activity of probiotic bacteria against oral pathogens. *Microb. Pathog.* 97, 213–220. doi: 10.1016/j.micpath.2016.06.018

Tanaka, H., Doesburg, K., Iwasaki, T., and Mierau, I. (1999). Screening of lactic acid bacteria for bile salt hydrolase activity. *J. Dairy Sci.* 82, 2530–2535. doi: 10.3168/jds.S0022-0302(99)75506-2

Tomaro-Duchesneau, C., Jones, M. L., Shah, D., Jain, P., Saha, S., and Prakash, S. (2014). Cholesterol assimilation by *Lactobacillus* probiotic bacteria: an *in vitro* investigation. *BioMed Res. Int.* 2014, 380316. doi: 10.1155/2014/380316

Turchi, B., Mancini, S., Fratini, F., Pedonese, F., Nuvoloni, R., Bertelloni, F., et al. (2013). Preliminary evaluation of probiotic potential of *Lactobacillusplantarum* strains isolated from Italian food products. *World J. Microbiol. Biotechnol.* 29, 1913–1922. doi: 10.1007/s11274-013-1356-7

Vidhyasagar, V., and Jeevaratnam, K. (2013). Evaluation of *Pediococcuspentosaceus* strains isolated from idly batter for probiotic properties *in vitro*. *J. Funct. Foods.* 5, 235–243. doi: 10.1016/j.jff.2012.10.012

Vinderola, C. G., and Reinheimer, J. A. (2003). Lactic acid starter and probiotic bacteria: a comparative "*in vitro*" study of probiotic characteristics and biological barrier resistance. *Food Res. Int.* 36, 895–904. doi: 10.1016/S0963-9969(03)00098-X

Volkova, V. V., Lu, Z., Lanzas, C., and Grohn, Y. T. (2013). Evaluating targets for control of plasmid-mediated antimicrobial resistance in enteric commensals of beef cattle: a modelling approach. *Epidemiol. Infect.* 141, 2294–2312. doi: 10.1017/S0950268812002993

Wang, J., Zhang, H., Chen, X., Chen, Y., and Bao, Q. (2012). Selection of potential probiotic lactobacilli for cholesterol-lowering properties and their effect on cholesterol metabolism in rats fed a high-lipid diet. *J. Dairy Sci.* 95, 1645–1654. doi: 10.3168/jds.2011-4768

Wang, M., Wang, R., Li, L., Yan, Y., Jia, S., Jiang, H., et al. (2021). Quantitative proteomics of plasma and liver reveals the mechanism of turmeric in preventing hyperlipidemia in mice. *Food Funct.* 12, 10484–10499. doi: 10.1039/D1FO01849C

Wang, Y., Wu, Y., Wang, Y., Xu, H., Mei, X., Yu, D., et al. (2017). Antioxidant properties of probiotic bacteria. *Nutrients* 9, 521. doi: 10.3390/nu9050521

Wu, Y., Wang, B., Xu, H., Tang, L., Li, Y., Gong, L., et al. (2019). Probiotic *Bacillus* attenuates oxidative stress-induced intestinal injury via p38-mediated autophagy. *Front. Microbiol.* 10, 2185. doi: 10.3389/fmicb.2019.02185

Yang, L., Xie, X., Li, Y., Wu, L., Fan, C., Liang, T., et al. (2021). Evaluation of the cholesterol-lowering mechanism of *Enterococcusfaecium* strain 132 and *Lactobacillus paracasei* strain 201 in hypercholesterolemia rats. *Nutrients* 13, 1982. doi: 10.3390/nu13061982

Yin, Y. N., Yu, Q. F., Fu, N., Liu, X. W., and Lu, F. G. (2010). Effects of four *Bifidobacteria* on obesity in high-fat diet induced rats. *World J. Gastroenterol.* 16, 3394. doi: 10.3748/wjg.v16.i27.3394

Zhang, F., Qiu, L., Xu, X., Liu, Z., Zhan, H., Tao, X., et al. (2017). Beneficial effects of probiotic cholesterol-lowering strain of *Enterococcus faecium* WEFA23 from infants on diet-induced metabolic syndrome in rats. *J. Dairy Sci.* 100, 1618–1628. doi: 10.3168/jds.2016-11870

Zhang, Q., Fan, X. Y., Cao, Y. J., Zheng, T. T., Cheng, W. J., Chen, L. J., et al. (2021). The beneficial effects of *Lactobacillusbrevis* FZU0713-fermented *Laminariajaponica* on lipid metabolism and intestinal microbiota in hyperlipidemic rats fed with a high-fat diet. *Food. Funct.* 12, 7145–7160. doi: 10.1039/D1FO00218J

Zhu, Y., Li, T., Din, A. U., Hassan, A., Wang, Y., and Wang, G. (2019). Beneficial effects of *Enterococcus faecalis* in hypercholesterolemic mice on cholesterol transportation and gut microbiota. *Appl. Microbiol. Biotechnol.* 103, 3181–3191. doi: 10.1007/s00253-019-09681-7

Zommiti, M., Cambronel, M., Maillot, O., Barreau, M., Sebei, K., Feuilloley, M., et al. (2018). Evaluation of probiotic properties and safety of *Enterococcus faecium* isolated from artisanal Tunisian meat "Dried Ossban". *Front. Microbiol.* 9, 1685. doi: 10.3389/fmicb.2018.01685

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 17 June 2022
doi: 10.3389/fmicb.2022.867278

Clustered Regularly Interspaced Short Palindromic Repeats Genotyping of Multidrug-Resistant *Salmonella* Heidelberg Strains Isolated From the Poultry Production Chain Across Brazil

Daniel F. M. Monte[1,2], Matthew A. Nethery[3,4], Hanna Berman[5], Shivaramu Keelara[5], Nilton Lincopan[6,7], Paula J. Fedorka-Cray[5], Rodolphe Barrangou[3,4] and Mariza Landgraf[1]**

[1] Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, Food Research Center, University of São Paulo, São Paulo, Brazil, [2] Laboratory of Avian Pathology, Department of Pathology, Theriogenology, and One Health, São Paulo State University (FCAV-Unesp), São Paulo, Brazil, [3] Genomic Sciences Graduate Program, North Carolina State University, Raleigh, NC, United States, [4] Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, Raleigh, NC, United States, [5] Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, United States, [6] Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil, [7] Department of Clinical Analysis, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil

OPEN ACCESS

Edited by:
Laurent Dufossé,
Université de La Réunion, France

Reviewed by:
Nikki W. Shariat,
University of Georgia, United States
Amal Awad,
Mansoura University, Egypt

***Correspondence:**
Daniel F. M. Monte
monte_dfm@alumni.usp.br
Mariza Landgraf
landgraf@usp.br

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 31 January 2022
Accepted: 25 April 2022
Published: 17 June 2022

Citation:
Monte DFM, Nethery MA, Berman H, Keelara S, Lincopan N, Fedorka-Cray PJ, Barrangou R and Landgraf M (2022) Clustered Regularly Interspaced Short Palindromic Repeats Genotyping of Multidrug-Resistant Salmonella Heidelberg Strains Isolated From the Poultry Production Chain Across Brazil. Front. Microbiol. 13:867278. doi: 10.3389/fmicb.2022.867278

Salmonella enterica subsp. *enterica* serovar Heidelberg has been associated with a broad host range, such as poultry, dairy calves, swine, wild birds, environment, and humans. The continuous evolution of *S*. Heidelberg raises a public health concern since there is a global dispersal of lineages harboring a wide resistome and virulome on a global scale. Here, we characterized the resistome, phylogenetic structure and clustered regularly interspaced short palindromic repeats (CRISPR) array composition of 81 *S*. Heidelberg strains isolated from broiler farms (n = 16), transport and lairage (n = 5), slaughterhouse (n = 22), and retail market (n = 38) of the poultry production chain in Brazil, between 2015 and 2016 using high-resolution approaches including whole-genome sequencing (WGS) and WGS-derived CRISPR genotyping. More than 91% of the *S*. Heidelberg strains were multidrug-resistant. The total antimicrobial resistance (AMR) gene abundances did not vary significantly across regions and sources suggesting the widespread distribution of antibiotic-resistant strains from farm to market. The highest AMR gene abundance was observed for *fosA7*, *aac(6′)-Iaa*, *sul2*, *tet(A)*, *gyrA*, and *parC* for 100% of the isolates, followed by 88.8% for bla_{CMY-2}. The β-lactam resistance was essentially driven by the presence of the plasmid-mediated AmpC (pAmpC) bla_{CMY-2} gene, given the isolates which did not carry this gene were susceptible to cefoxitin (FOX). Most *S*. Heidelberg strains were classified within international lineages, which were phylogenetically nested with *Salmonella* strains from European countries; while CRISPR genotyping analysis revealed that the spacer content

was overall highly conserved, but distributed into 13 distinct groups. In summary, our findings underscore the potential role of *S*. Heidelberg as a key pathogen disseminated from farm to fork in Brazil and reinforce the importance of CRISPR-based genotyping for salmonellae. Hence, we emphasized the need for continuous mitigation programs to monitor the dissemination of this high-priority pathogen.

Keywords: antibiotic resistance, CRISPR, phylogeny, *Salmonella* Heidelberg, foodborne disease, WGS

INTRODUCTION

Salmonella enterica subsp. *enterica* serovar Heidelberg is most often associated with eggs and poultry (Hennessy et al., 2004; Chittick et al., 2006; Foley et al., 2008; Folster et al., 2012; Jackson et al., 2013). However, this scenario has changed since *Salmonella* Heidelberg has been associated with other sources from a broad host range, such as dairy calves (Centers for Disease Control and Prevention [CDC], 2017), swine (Cabral et al., 2017), wild birds (Liakopoulos et al., 2016a), environmental sources (Antony et al., 2018), human-derived clinical specimens (Abdullah et al., 2021), and outbreaks (Antony et al., 2018), which denotes their importance as a high-priority pathogen.

One of the most important risk factors surrounding foodborne illness is the international food trade that has been circumstantially accompanied by Salmonellae dispersal beyond borders. In this context, there is a global dispersal of multidrug-resistant lineages of serovar *S*. Heidelberg, reaching various countries in North America (Andrysiak et al., 2008; Centers for Disease Control and Prevention, 2014; Public Health Agency of Canada, 2014; Deblais et al., 2018; Cox et al., 2021), South America (Kipper et al., 2021), Europe (Liakopoulos et al., 2016b; Campos et al., 2018), and Asia (Wu et al., 2013). Therefore, the simultaneous increase and extended protraction of *S*. Heidelberg in many parts of the world have favored their genetic acquisition of virulence and antimicrobial resistance (AMR) genes through horizontal gene transfer (HGT), which has ultimately led to one of the most pressing global concerns.

Owing to their importance as a key poultry producer globally, Brazil quickly became the hotspot of *S*. Heidelberg and urgent actions were needed from the food safety authorities to mitigate this pathogen in order to reduce the economic losses in the poultry sector. In this context, most investigations to detect *Salmonella enterica* serovars in the poultry sector still had important methodological gaps, since the food industry focuses especially on *Salmonella* Typhimurium and *Salmonella* Enteritidis, which demonstrate the need for a combined approach between classical microbiology and high-resolution methods such as whole-genome sequencing (WGS) and clustered regularly interspaced short palindromic repeats (CRISPR) genotyping (Barrangou and Dudley, 2016; Thompson et al., 2018; Yousfi et al., 2020). Indeed, the use of these high-throughput sequencing analyses exemplifies a useful means, not only for identifying *Salmonella* serovars but also to trace back the origin of the contamination conferring a substantial aid in decision-making to the poultry sector. In this regard, we demonstrated the usefulness of WGS-based identification in our previous study for genotyping rare *Salmonella enterica* serovars isolated from food and related sources (Monte et al., 2021). This previous survey demonstrated that the CRISPR arrays were highly conserved, and this genomic inspection provides high-resolution genotyping of *Salmonella* serovars. Hence, we performed a genomic study by combining WGS and CRISPR genotyping to characterize *S*. Heidelberg isolates from different sources at broiler farms, slaughterhouses, transport, lairages, and retail markets in Brazil.

MATERIALS AND METHODS

Salmonella Heidelberg Strains and Antimicrobial Susceptibility Testing

A total of 79 non-duplicate *Salmonella enterica* subsp. *enterica* serovar Heidelberg from our collection that included isolates obtained from broiler farms (n = 16), transport and lairage (n = 5), slaughterhouses (n = 22), and retail markets (n = 38) in Brazil between 2015 and 2016 were used in this study (refer **Table 1**). We also included two *S*. Heidelberg strains (SH159 and SSc139) from our previous work (Monte et al., 2019) for comparative purposes, totalizing eighty-one isolates. The *Salmonella* isolation was performed according to the International Organization for Standardization (Anonymous, 2007, 2017). These isolates were serotyped on the basis of somatic O, phase 1, and phase 2 of H flagellar antigens by agglutination tests with antisera as specified in the Kauffmann–White–Le Minor scheme (Grimont and Weil, 2007; Guibourdenche et al., 2010).

Minimum inhibitory concentrations (MICs) were determined by broth microdilution using Sensititre® Gram-Negative Plates (Trek Diagnostic Systems, OH), such as 14 antimicrobials: cefoxitin (FOX), ceftriaxone (AXO), amoxicillin/clavulanic acid 2:1 ratio (AUG2), ceftiofur (XNL), ampicillin (AMP), nalidixic acid (NAL), ciprofloxacin (CIP), chloramphenicol (CHL), tetracycline (TET), gentamicin (GEN), sulfisoxazole (FIS), trimethoprim/sulfamethoxazole (SXT), streptomycin (STR), and azithromycin (AZI). MIC values were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute [CLSI], 2021) and the National Antimicrobial Resistance Monitoring System (US Food and Drug Administration [FDA], 2015). Multidrug resistance was defined as resistant to three or more classes of antimicrobials (Magiorakos et al., 2012).

Genomic Analysis

All *S*. Heidelberg isolates (n = 81) underwent DNA extraction performed by using a commercial kit (QiAmp tissue, Qiagen,

TABLE 1 | Features of *S*. Heidelberg strains ($n = 81$) isolated from different sources.

Strain ID	Location*/year	Origin	Source	Resistance profile	Resistance genes	Sequence type
SH018 GCA_002270265.1	SP/2016	Farm	Broiler chicken	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH019 GCA_002260805.1	SP/2016	Farm	Broiler chicken	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH10211124 GCA_006332685.1	SC/2016	Farm	Broiler chicken	TET-CIP-NAL	*fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH10227492 GCA_006291695.1	SC/2016	Farm	Broiler chicken	TET-CIP-NAL-STR	*fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH10230633 GCA_004161895.1	MS/2016	Farm	Broiler chicken	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *aph(3′)-Ia*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH10190712 GCA_011157915.1	PR/2016	Farm	Broiler chicken	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH10201911 GCA_011519745.1	SC/2016	Farm	Broiler chicken	TET-CIP-NAL	*fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH10206799 GCA_011520545.1	SC/2016	Farm	Broiler chicken	TET-CIP-NAL	*fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH10225532 GCA_007640935.1	SC/2016	Farm	Broiler chicken	TET-CIP-NAL	*fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
STy012 GCA_011606045.1	SP/2015	Farm	Broiler chicken	TET-CIP-NAL	*fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SI015 GCA_011598585.1	SP/2015	Farm	Broiler chicken	TET-CIP-NAL	*fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH134 GCA_011158435.1	SP/2016	Farm	Chicken cage after cleaning	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH159 GCA_011157595.1	MG/2016	Farm	Chicken cage after cleaning	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH415 GCA_006332505.1	SC/2016	Farm	Chicken cage after cleaning	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH434 GCA_006291935.1	SC/2016	Farm	Chicken cage after cleaning	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH715 GCA_003874535.1	SP/2016	Farm	Chicken cage after cleaning	TET-CIP-GEN-NAL-STR	*fosA7*, *aac(3)-VIa*, *aadA1*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH264 GCA_010933975.1	PR/2016	Transport and lairage	Truck after cleaning	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH265 GCA_010884255.1	PR/2016	Transport and lairage	Truck after cleaning	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH414 GCA_003877275.1	SC/2016	Transport and lairage	Truck after cleaning	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15

(Continued)

TABLE 1 | (Continued)

Strain ID	Location*/year	Origin	Source	Resistance profile	Resistance genes	Sequence type
SH433 GCA_006332565.1	SC/2016	Transport and lairage	Truck after cleaning	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH435 GCA_006291875.1	SC/2016	Transport and lairage	Truck after cleaning	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH122 GCA_011616265.1	SP/2016	Slaughterhouse	Chicken carcass	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH125 GCA_011544755.1	SP/2016	Slaughterhouse	Chicken carcass	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH128 GCA_010956115.1	SP/2016	Slaughterhouse	Chicken carcass	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH129 GCA_011591705.1	SP/2016	Slaughterhouse	Chicken carcass	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH258 GCA_011533705.1	PR/2016	Slaughterhouse	Chicken carcass	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH266 GCA_011157875.1	PR/2016	Slaughterhouse	Chicken carcass	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH283 GCA_011516545.1	SP/2016	Slaughterhouse	Chicken carcass	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH284 GCA_010005265.1	SP/2016	Slaughterhouse	Chicken carcass	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH285 GCA_006291795.1	SP/2016	Slaughterhouse	Chicken carcass	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SSc148 GCA_003877035.1	DF/2016	Slaughterhouse	Chicken carcass	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SSc155 GCA_006209245.1	DF/2016	Slaughterhouse	Chicken carcass	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH268 GCA_010979095.1	PR/2016	Slaughterhouse	Chicken carcass after chiller	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH269 GCA_011157135.1	PR/2016	Slaughterhouse	Chicken carcass after chiller	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH270 GCA_010977655.1	PR/2016	Slaughterhouse	Chicken carcass after chiller	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH1 GCA_011149295.1	SP/2016	Slaughterhouse	Mechanically recovered chicken meat	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH131 GCA_006211165.1	SP/2016	Slaughterhouse	Mechanically recovered chicken meat	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15
SH296 GCA_006292135.1	SP/2016	Slaughterhouse	Mechanically recovered	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A), aac(6′)-Iaa, gyrA:p.S83F, parC:p.T57S*	ST15

(Continued)

TABLE 1 | (Continued)

Strain ID	Location*/year	Origin	Source	Resistance profile	Resistance genes	Sequence type
SH297 GCA_003877075.1	SP/2016	Slaughterhouse	Mechanically recovered chicken meat	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH697 GCA_003874475.1	SC/2016	Slaughterhouse	Mechanically recovered chicken meat	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH700 GCA_006291975.1	SC/2016	Slaughterhouse	Mechanically recovered chicken meat	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH712 GCA_006210745.1	SP/2016	Slaughterhouse	Mechanically recovered chicken meat	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH164 GCA_010875785.1	SP/2016	Slaughterhouse	Viscera	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH118 GCA_011163895.1	SP/2016	Retail market	Chicken breast	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH276 GCA_011571185.1	SP/2016	Retail market	Salted chicken breast	TET-CIP-NAL	*fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH405 GCA_006332425.1	SC/2016	Retail market	Chicken breast fillet	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH410 GCA_006209405.1	SC/2016	Retail market	Chicken breast fillet	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH694 GCA_006291675.1	SC/2016	Retail market	Chicken breast fillet	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH120 GCA_011590585.1	SP/2016	Retail market	Chicken thigh and drumstick	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH286 GCA_006291895.1	SP/2016	Retail market	Chicken thigh and drumstick	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH411 GCA_006209285.1	SC/2016	Retail market	Chicken thigh and drumstick	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH692 GCA_006211665.1	SC/2016	Retail market	Chicken thigh and drumstick	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH121 GCA_010946195.1	SP/2016	Retail market	Chicken fillet sassami	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *lnu(G)*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH127 GCA_011146395.1	SP/2016	Retail market	Chicken fillet sassami	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *lnu(G)*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH135 GCA_011146615.1	SP/2016	Retail market	Whole chicken	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH427 GCA_003877155.1	SC/2016	Retail market	Whole chicken	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH138 GCA_010980075.1	SC/2016	Retail market	Leg quarter	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15

(Continued)

TABLE 1 | (Continued)

Strain ID	Location*/year	Origin	Source	Resistance profile	Resistance genes	Sequence type
SH158 GCA_010902135.1	MG/2016	Retail market	Fiesta boneless	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR	*bla*$_{CMY-2}$, *fosA7*, *aadA1*, *aadA2*, *aac(6′)-Iaa*, *cmlA1*, *dfrA12*, *sul2*, *sul3*, *tet(A)*, *qacL*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH287 GCA_004158845.1	SP/2016	Retail market	Chicken skin	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH289 GCA_004159315.1	SP/2016	Retail market	Seasoned chicken fillet	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH403 GCA_006292115.1	SC/2016	Retail market	Seasoned chicken fillet	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH290 GCA_006332625.1	SP/2016	Retail market	Chicken liver	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH402 GCA_006332585.1	SC/2016	Retail market	Chicken liver	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F,parC:p.T57S*	ST15
SH408 GCA_006291855.1	SC/2016	Retail market	Chicken liver	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH422 GCA_006291955.1	SC/2016	Retail market	Chicken liver	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH423 GCA_006209445.1	SC/2016	Retail market	Chicken liver	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH429 GCA_004160665.1	SC/2016	Retail market	Chicken liver	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH430 GCA_006291835.1	SC/2016	Retail market	Chicken liver	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH431 GCA_006210515.1	SC/2016	Retail market	Chicken liver	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH674 GCA_006332645.1	SC/2016	Retail market	Chicken liver	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH687 GCA_006211605.1	SC/2016	Retail market	Chicken liver	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH707 GCA_006211425.1	SC/2016	Retail market	Chicken liver	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH412 GCA_004159355.1	SC/2016	Retail market	Chicken wing	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH680 GCA_003877135.1	SC/2016	Retail market	Retail meat	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6′)-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15

(Continued)

TABLE 1 | (Continued)

Strain ID	Location*/year	Origin	Source	Resistance profile	Resistance genes	Sequence type
SH681 GCA_006292015.1	SC/2016	Retail market	Chicken wing	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH685 GCA_004161515.1	SC/2016	Retail market	Chicken neck	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH691 GCA_006291915.1	SC/2016	Retail market	Chicken wing	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH693 GCA_006210725.1	SC/2016	Retail market	Chicken wing	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SSc139 GCA_011578645.1	SP/2016	Retail market	Chicken wing	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SH716 GCA_006332605.1	SC/2016	Retail market	Chicken wing paddle	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15
SSC136 GCA_010932755.1	SP/2016	Retail market	Chicken wing	FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP	*bla*$_{CMY-2}$, *fosA7*, *sul2*, *tet(A)*, *aac(6')-Iaa*, *gyrA:p.S83F*, *parC:p.T57S*	ST15

Germany) per manufacturer's guidelines. Genomic DNA of eighty-one *Salmonella* isolates was sequenced at a 300-bp paired-end-read using the Nextera XT library preparation kit at the MiSeq platform (Illumina, San Diego, CA, United States).

Resulted raw sequence reads underwent strict quality control by using default settings in CLC workbench 10.1.1 (Qiagen) as per Monte et al. (2019), while assemblies were annotated with PROKKA version 1.14-dev (Seemann, 2014). A core genome phylogeny was constructed with an alignment of the core genes determined by the software version 3.11.2; the BlastP threshold was set to 95% (Page et al., 2015). A pan-genome genes presence–absence information from Roary was visualized with Phandango (Hadfield et al., 2018). The single nucleotide polymorphisms were extracted from the alignment using SNP-sites version 2.3.3 (Page et al., 2016). The phylogeny was reconstructed using RAxML version 8.2.12, using a General Time Reversible Model and Gamma distribution for rate heterogeneity (Stamatakis, 2014). The resulting phylogeny was tested against 1,000 bootstrap replications, as determined by implementing the majority rule, autoMR convergence criteria in the RAxML software (Pattengale et al., 2010). The phylogeny was visualized and annotated using iTol version 3 (Letunic and Bork, 2016).

Lastly, the assemblies were analyzed for acquired AMR genes and chromosomal point mutations using default settings of ResFinder 4.1 database available at the Center for Genome Epidemiology.[1] In addition, we used MLST 2.0 to detect multilocus sequence typing (MLST), and the PlasmidFinder software version 2.0.1 was run with database version 2018-11-20 (Carattoli et al., 2014). A minimum identity threshold of 95% was used as a filter for identification.

Clustered Regularly Interspaced Short Palindromic Repeats Genotyping and Phylogenetic Analysis

An automated high-throughput processing pipeline previously described by Nethery and Barrangou (2019) was used to identify the CRISPR loci within each strain. Using CRISPR Visualizer, we extracted and imported CRISPR loci into the web interface for visualization and alignment of all CRISPR spacer and repeat sequences.[2]

RESULTS

Salmonella Heidelberg Strains Harbored a Wide Resistome Against Critically Important Antimicrobials

A total of 81 (100%) *S.* Heidelberg strains were both phenotypically and genotypically resistant, whereas 91.3% (n = 74) were multidrug-resistant, defined as resistant to three or more classes of antimicrobial compounds (Magiorakos et al., 2012; **Table 1**). Results of the antimicrobial susceptibility testing are presented in **Table 2**. MICs vary among *S.* Heidelberg strains.

[1] http://www.genomicepidemiology.org/

[2] https://github.com/CRISPRlab/CRISPRviz

TABLE 2 | Minimum inhibitory concentration values for *Salmonella* Heidelberg strains (n = 81).

Antimicrobials	Resistance (%)	Intermediate resistance (%)	Distribution of *S.* Heidelberg strains (n = 81) among MIC values (μ g/ml)[a]														
			0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	≥512
Cefoxitin	88.8	0								6	2	1		72				
Azithromycin	12.3	0									22	49	10					
Chloramphenicol	1.23	12.3									7	63	10	1				
Tetracycline	100	0												81				
Ceftriaxone	88.8	0			9							2	45	20	5			
Amoxicillin/clavulanic acid	88.8	0			6					3				72				
Ciprofloxacin	100	0					35	36	10									
Gentamicin	1.23	0						64	16				1					
Nalidixic acid	100	0												81				
Ceftiofur	88.8	0			1				8			72						
Sulfisoxazole	100	0															81	
Trimethoprim/sulfamethoxazole	1.23	0			72			7	1		1							
Ampicillin	88.8	0			2				1	5	1			72				
Streptomycin	16.0	0											68	11	2			

[a]*Blue MIC values indicate intermediate resistance, while red MIC values in gray squares indicate resistance profiles, which were determined by broth microdilution method using CLSI interpretative breakpoints (Clinical and Laboratory Standards Institute [CLSI], 2021).*

All *S.* Heidelberg strains were resistant to TET, NAL, CIP, and FIS with MIC values ranging from 0.25 to ≥ 256 μg/ml (**Table 2**). The high MIC values observed in this study for β-lactams (AMP, amoxicillin/clavulanic acid, AXO, XNL, and FOX), TET, FIS, NAL, and STR (**Table 2**), confirm the high frequency of AMR genes and mutations predicted by genomic analysis. Yet, based on the MIC distribution, all *S.* Heidelberg strains displayed susceptibility to AZI (**Table 2**).

The total AMR gene abundances did not vary significantly across regions and sources suggesting pervasive distribution of antibiotic resistant strains from farm to market in six different States of Brazil (**Figure 1**). The highest AMR gene abundances were observed for fosfomycin (*fosA7*; 100%), sulfonamide (*sul2*; 100%), tetracycline [*tet(A)*; 100%], and aminoglycoside [*aac(6′)-Iaa*; 100%]. Seventy-two (88.8%) *S.* Heidelberg strains harbored the plasmid-mediated AmpC β-lactamase (bla_{CMY-2}), encoding resistance to third-generation cephalosporin (3GC). Unlike, *Inu(G)* (n = 2), *aadA1* (n = 2), *aph(3′)-Ia* (n = 1), *aac(3)-Via* (n = 1), *aadA2* (n = 1), *cmlA1* (n = 1), *dfrA12* (n = 1), *sul3* (n = 1), and *qacL* (n = 1) AMR genes were detected at very low levels (**Table 1**). On the other hand, chromosomal point mutations in *gyrA* [p. Ser83Phe (tcc → ttc)] and *parC* [p. Thr57Ser (acc → agc)] were identified in 100% of the strains. This quinolone resistance-determining region (QRDR) among *S.* Heidelberg strains was sufficient to promote high-level resistance at > 32 μ g/ml for NAL.

All 81 *Salmonella* genomes were analyzed for the content of plasmid replicons by using the Center for Genomic Epidemiology (CGE) web-tool PlasmidFinder 2.1, with 100% of the genomes containing at least two replicons, like ColpVC and IncA/C2. The remaining plasmids replicons such as IncX1 (n = 80; 98.7%), Incl1 (n = 56; 69.1%), IncFII (n = 3; 3.7%), IncFII(29) (n = 2; 2.4%), IncY (n = 2; 2.4%), Col156 (n = 1; 1.2%), IncFIA (n = 1; 1.2%), IncQ1(n = 1; 1.2%), and IncX4 (n = 1; 1.2%) were identified within *S.* Heidelberg genomes (**Figure 1**).

Spacer Composition and Sequence Type Were Highly Conserved Within *Salmonella* Heidelberg Strains

Next, we visualized CRISPR loci extracted from WGS data to analyze the pattern of repeats and spacers distributed among *S.* Heidelberg strains (n = 81). In doing so, we observed 13 unique CRISPR array patterns [P1 (n = 13), P2 (n = 3), P3 (n = 1), P4 (n = 3), P5 (n = 26), P6 (n = 1), P7 (n = 16), P8 (n = 1), P9 (n = 1), P10 (n = 8), P11 (n = 5), P12 (n = 2), and P13 (n = 1)] as shown in **Table 3**. Overall, we observed a maximum of 44 spacers across *S.* Heidelberg strains (P7), spread across two loci. SH265 and SH268, belonging to profile P12, contained 37 spacers, the lowest number presented here. Spacer composition was highly conserved across strains, which shared 43 (P1, P2, P3, P4, P5, and P6), 42 (P7 and P8), 40 (P9 and P10), 39 (P11), and 36 (P12) identical spacers, reflecting a common ancestral origin (**Figure 2**). Next, we performed a comparative analysis of the architecture of the type I-E CRISPR-Cas system present in these strains and observed 100% amino acid identity across all strains—further evidence of shared ancestral origin (**Figure 3**).

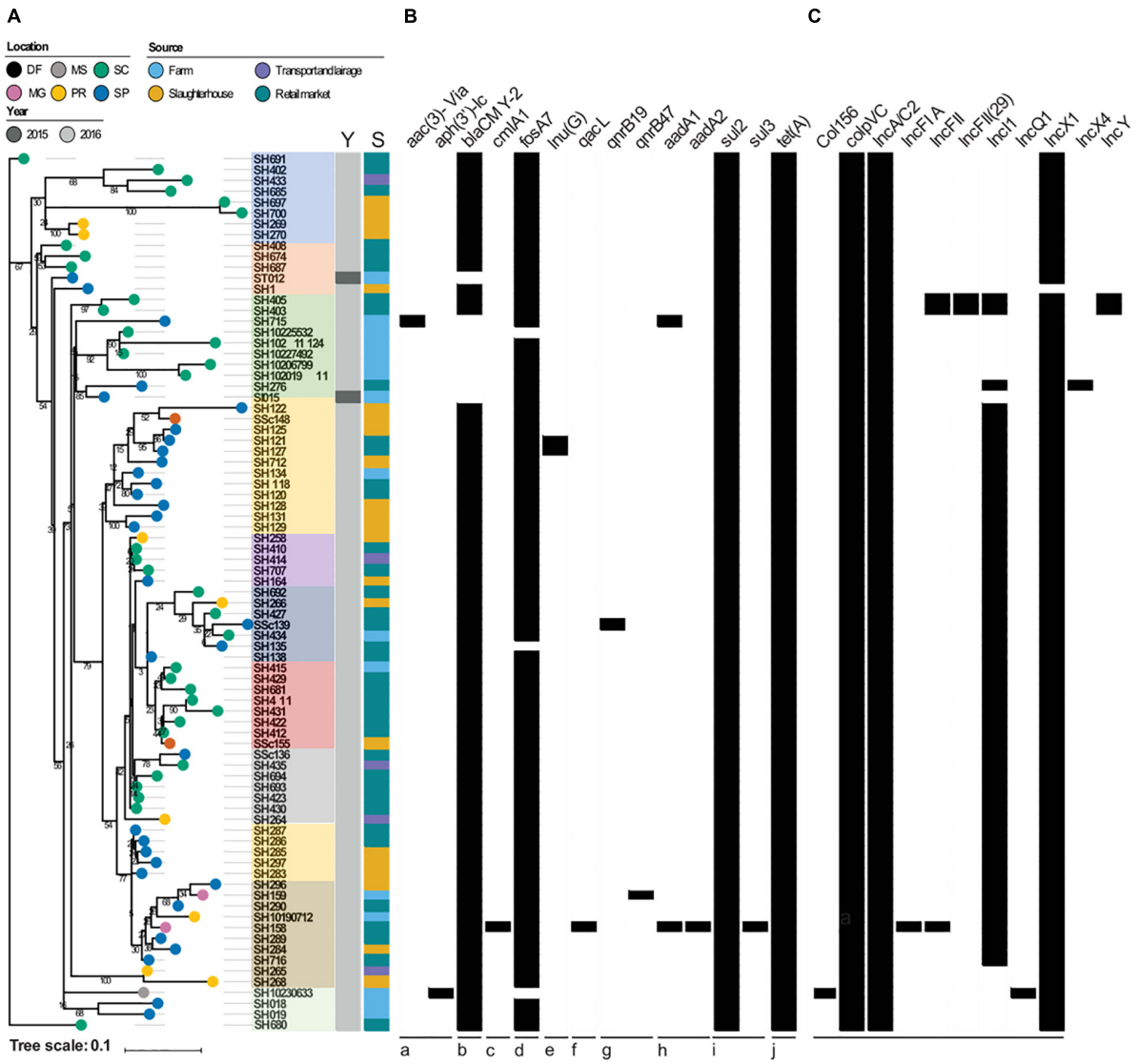


FIGURE 1 | (A) Reconstructed phylogeny based on the core genome (4,139 genes) of the 81 *S.* Heidelberg strains. The percentage of bootstrap samples in which nodes appeared is shown. The location of isolation of each strain is labeled on its respective branch. Color strips depict the year (Y) and source (S) of isolation, respectively. **(B)** The presence and absence of selected antimicrobial resistance genes are shown, with black indicating presence. The drug classes impacted by these genes are: (a) aminoglycosides, (b) beta-lactams, (c) chloramphenicol, (d) fosfomycin, (e) lincosamide, (f) quaternary ammonium compounds, (g) quinolones, (h) streptomycin, (i) sulfonamides, (j) tetracylines. **(C)** Presence and absence of plasmid incompatibility groups, with black indicating presence. Brazilian States: PR, Paraná; SC, Santa Catarina; SP, São Paulo; MG, Minas Gerais; DF, Distrito Federal; MS, Mato Grosso do Sul.

We further evaluated the multi-locus sequence typing by *in silico* prediction, which revealed that all *S.* Heidelberg strains matched the international sequence type (ST15) (**Table 1**).

Salmonella Heidelberg Strains Isolated From Brazil Are Genetically Related to South American, European, and Asian Isolates

While assessing the phylogenetics of *S.* Heidelberg strains (n = 81) sequenced in this study, we noticed that the core genome, calculated from WGS data, represented 74% of the pan-genome (4,139 out of 5,582 total genes). Little genomic variation was present among the core genome, as only 704 SNP sites were detected. Bootstrap values varied across the phylogeny, likely attributed to the small genomic variation among strains. *S.* Heidelberg strains did not cluster by year, source, or geographic location across the phylogeny suggesting the widespread distribution [regions (n = 6), sources (n = 4), years of isolation (n = 2)] and persistence of *Salmonella* strains in Brazil (**Figure 1**), which validate the previous surveys (Monte et al., 2019). Furthermore, 11 different clusters were identified as shown in **Figure 1**. Of these, 14 strains appear to be from independent lineages, given that they nested out of the main branches.

TABLE 3 | Clustered regularly interspaced short palindromic repeats (CRISPR) patterns obtained from 81 *Salmonella* Heidelberg strains.

CRISPR profile	Location*	Source	Year of isolation
P1	SC (*n* = 7), PR (*n* = 2), SP (*n* = 4)	Retail market (*n* = 5), transport and lairage (*n* = 1), slaughterhouse (*n* = 4), broiler farm (*n* = 3)	2015 (*n* = 1), 2016 (*n* = 12)
P2	SC (*n* = 2), MS (*n* = 1)	Retail market (*n* = 2), broiler farm (*n* = 1)	2016 (*n* = 3)
P3	SC (*n* = 1)	Slaughterhouse (*n* = 1)	2016 (*n* = 1)
P4	SC (*n* = 1), SP (*n* = 2)	Retail market (*n* = 2), slaughterhouse (*n* = 1)	2016 (*n* = 3)
P5	SC (*n* = 15), SP (*n* = 8), PR (*n* = 2), DF (*n* = 1)	Retail market (*n* = 13), transport and lairage (*n* = 3), slaughterhouse (*n* = 5), broiler farm (*n* = 5)	2016 (*n* = 26)
P6	SP (*n* = 1)	Broiler farm (*n* = 1)	2016 (*n* = 1)
P7	SC (*n* = 8), SP (*n* = 7), DF (*n* = 1)	Retail market (*n* = 9), slaughterhouse (*n* = 4), broiler farm (*n* = 3)	2015 (*n* = 1), 2016 (*n* = 15)
P8	SP (*n* = 1)	Retail market (*n* = 1)	2016 (*n* = 1)
P9	PR (*n* = 1)	Slaughterhouse (*n* = 1)	2016 (*n* = 1)
P10	SP (*n* = 6), MG (*n* = 1), PR (*n* = 1)	Retail market (*n* = 3), slaughterhouse (*n* = 3), broiler farm (*n* = 2)	2016 (*n* = 8)
P11	SP (*n* = 4), MG (*n* = 1)	Retail market (*n* = 3), slaughterhouse (*n* = 2)	2016 (*n* = 5)
P12	PR (*n* = 2)	Transport and lairage (*n* = 1), slaughterhouse (*n* = 1)	2016 (*n* = 2)
P13	SC (*n* = 1)	Broiler farm (*n* = 1)	2016 (*n* = 1)

**Brazilian States: PR, Paraná; SC, Santa Catarina; SP, São Paulo; MG, Minas Gerais; DF, Distrito Federal; MS, Mato Grosso do Sul.*

We also observed through SNP clustering (PDS000037185.127; *n* = 765 isolates; NCBI pathogen detection tool), cases of international clustering of *S.* Heidelberg from our collection (*n* = 77) with strains isolated from a variety of sources (food, human, and environment) from Brazil (*n* = 201), Chile (*n* = 9), the United Kingdom (*n* = 444), Germany (*n* = 1), the Netherlands (*n* = 2), South Korea (*n* = 1), and China (*n* = 1), which suggest a common ancestor origin (**Supplementary Figure 1**). Another two strains (SH265 and SH268) from this study nested in the SNP cluster (PDS000029160.10) with strains isolated from Brazil (*n* = 11), the United Kingdom (*n* = 4), and Chile (*n* = 1) (**Supplementary Figure 2**).

DISCUSSION

There has been a great interest in surveying the adaptation of *Salmonella* serovars to the poultry production chain because of their extensive persistence in the past, notably with *S.* Typhimurium and *S.* Enteritidis, which have caused significant economic losses to this sector. Furthermore, the prevalence of *S.* Heidelberg shown in this study is not the only issue, but the fact that highly drug-resistant and/or MDR isolates are being recovered in most steps of the poultry production chain, particularly in Brazil could be considered a public health threat, as there is a risk of it becoming globalized.

Based on AMR results, the β-lactam resistance was essentially driven by the presence of plasmid-mediated AmpC (pAmpC) bla_{CMY-2} gene, given the isolates which did not carry this gene were susceptible to FOX, while QRDR such as *gyrA* and *parC* genes drove quinolone resistance (**Table 1**). Indeed, the presence of strains harboring bla_{CMY-2} gene could have implications on a one health interface, since this plasmid is more likely to persist (Teunis et al., 2018). Besides that, all strains harbored chromosomal mutations in *gyrA* and *parC* genes promoting high-level resistance against quinolones that could have implications on human health as treatment options become limited. Disturbingly, this result corroborates the findings by van den Berg et al. (2019) that found 98.4% of the *S.* Heidelberg isolates resistant to fluoroquinolones. On the other hand, all *S.* Heidelberg strains from our collection, displayed susceptibility to azithromycin, which could be considered a promising agent against Salmonellae infections (Crump et al., 2015; Wen et al., 2017). Azithromycin has been used as an alternative treatment option for enteric fever even when the guidelines on susceptibility testing were not available. Like fluoroquinolones, azithromycin is an antimicrobial agent with efficient intracellular penetration (Crump et al., 2015; Wen et al., 2017).

The total AMR load also included encoding resistance genes for aminoglycoside [*aac(3)-VIa*, *aph(3′)-Ic, aadA1, aadA2*], chloramphenicol (*cmlA1*), macrolides [*Inu(G)*], trimethoprim (*dfrA12*), and ammonium quaternary compounds (*qacL*) (**Figure 1**). Interestingly, the unique strain (SSc139) that carried the *qacL* gene was isolated from the retail market, which makes large use of such compounds to disinfect surfaces. Moreover, this strain nested in the same cluster with five strains isolated from broiler farms and slaughterhouse sources, in different states such as São Paulo, Santa Catarina, and Parana, denoting the successful establishment of this lineage (ST15) in the Southern and South regions of Brazil. In this regard, we visualized the co-occurrence of AMR genes in all *S.* Heidelberg strains.

The presence of AMR genes and transmissible plasmids demonstrated little variation across the strains. The broad distribution and abundance of *S.* Heidelberg in broiler farms, slaughterhouses, transport, lairages, and retail markets suggest the high adaptability of this serovar in the poultry production chain in Brazil. Similarly, a study by Edirmanasinghe et al. (2017) examining FOX-resistant *S.* Heidelberg strains isolated

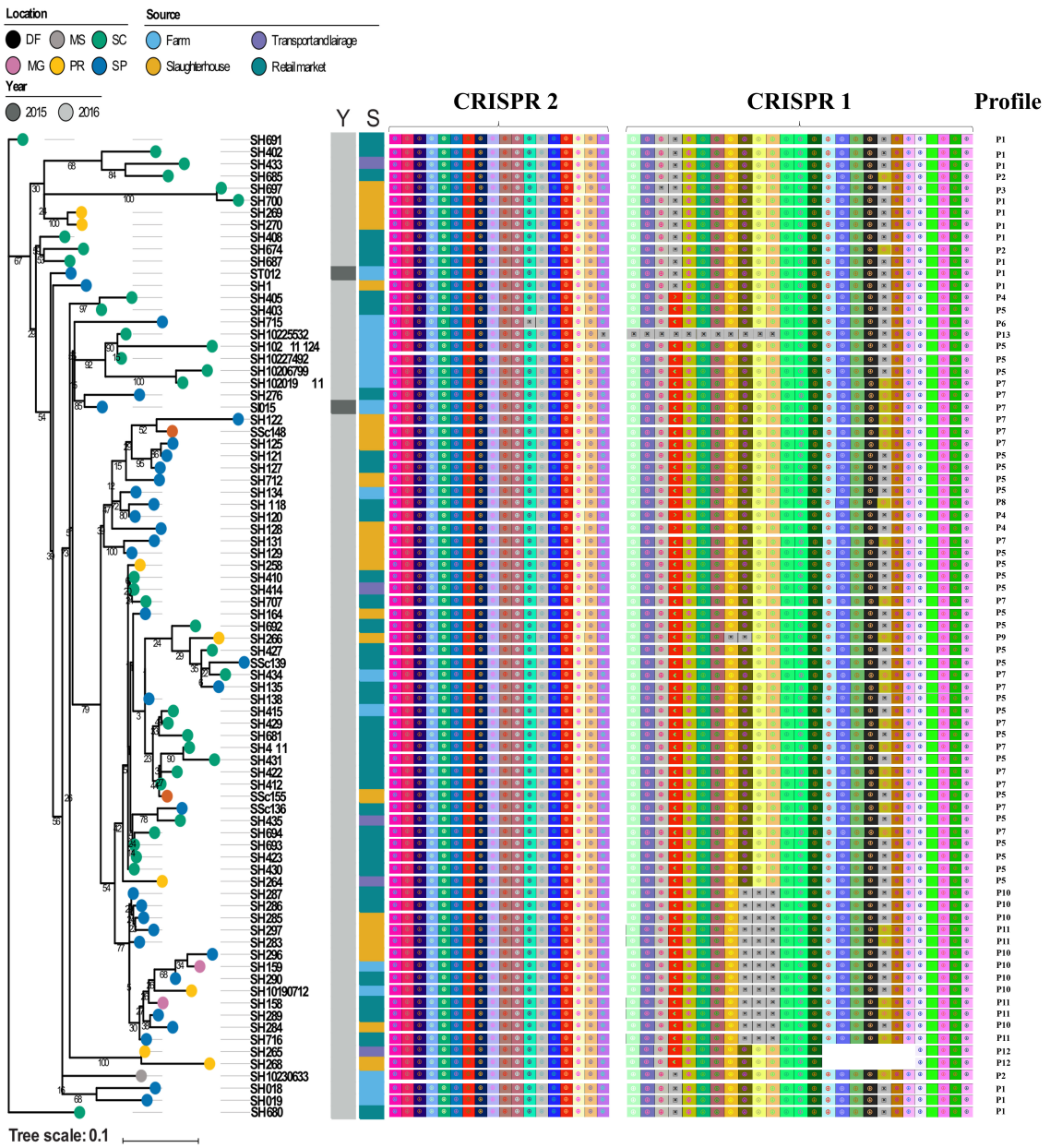


FIGURE 2 | Reconstructed phylogeny based on the core genome, distribution of spacers composition, clustered regularly interspaced short palindromic repeats (CRISPR) loci, and CRISPR profiles among *Salmonella* Heidelberg strains. The location of isolation of each strain is labeled on its respective branch. Color strips depict the year (Y) and source (S) of isolation, respectively. Brazilian States: PR, Paraná; SC, Santa Catarina; SP, São Paulo; MG, Minas Gerais; DF, Distrito Federal; MS, Mato Grosso do Sul.

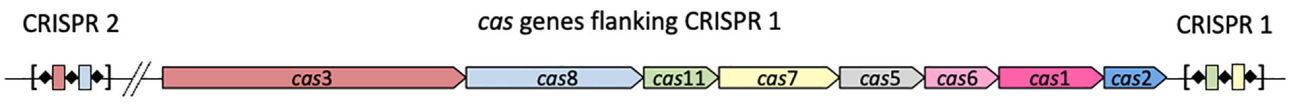


FIGURE 3 | *Salmonella enterica* subsp. *enterica* serovar Heidelberg type I-E CRISPR locus architecture. This system contains two distinct CRISPR arrays—one associated with the *cas* genes and one disparate locus upstream (5′) from the *cas* genes.

from human, abattoir poultry, and retail poultry sources in Canada revealed a potential common source among strains, which suggest the simultaneous dispersal of *S.* Heidelberg strains carrying CMY-2 gene in several sources and different geographical locations. In convergence with our results, another study revealed a high occurrence of *S.* Heidelberg in imported poultry meat in the Netherlands containing bla_{CMY-2} gene (van den Berg et al., 2019). Although we could not find colistin-resistant strains, it is worthwhile to note the presence of the IncX4 plasmid since it is a promiscuous plasmid with a high capacity of self-transmissibility that is commonly associated with the presence of plasmid-mediated colistin-resistance (*mcr-1*) in Brazil (Moreno et al., 2019).

Consistent with observations obtained in our study, recent surveys strongly support that *S.* Heidelberg may originate from a common ancestor, which circulates and persists in the Brazilian poultry production chain since at least 2004 (Kipper et al., 2021). More importantly, these strains are also nested with strains isolated from several sources and countries around the world supporting the hypotheses of intercontinental spread, which demonstrate that probably the common ancestor underwent diversification through genetic changes over time (**Supplementary Material**).

Protracted dissemination of *S.* Heidelberg *via* poultry might be a risk for a globalized food trade era. The introduction and clonal expansion of *Salmonella* strains across borders remain challenging due to the difficulties of identifying the origins of contamination. In light of this, the continued need for combined approaches between classical microbiology and high-resolution methods such as WGS and CRISPR genotyping truly illustrate to us what is hidden in plain sight.

For the purpose of discussion, other studies provide compelling validation data to support the usefulness of high-resolution methods for genotyping rare *Salmonella enterica* serovars (Monte et al., 2021) and/or to resolve *S.* Heidelberg isolates involved in foodborne outbreaks (Vincent et al., 2018). Of the latter, while assessing the CRISPR array of 145 *S.* Heidelberg isolates, Vincent et al. (2018) found 15 different CRISPR profiles endorsing our results.

This study further illustrates the potential of CRISPR for the tracking of variable genotypes in diverse *Salmonella* strains, as previously determined (DiMarzio et al., 2013; Shariat et al., 2013a,b, 2015; Monte et al., 2021), with noteworthy methodological convenience. Indeed, CRISPR-based analyses have proven relevant for subtyping of *Salmonella enterica* serovars Typhimurium and Heidelberg strains involved in outbreaks (Shariat et al., 2013b) and occasionally associated with antibiotic resistance (DiMarzio et al., 2013). Our findings underscore the potential role of *S.* Heidelberg as a key pathogen in the poultry production chain, particularly in Brazil.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

DM, RB, MN, PF-C, and ML designed the study. RB, PF-C, NL, and ML supervised the work. DM, MN, HB, SK, NL, PF-C, RB, and ML participated, coordinated, and analyzed the data. DM, MN, and RB wrote the original draft. All authors approved the final manuscript.

FUNDING

This study was granted by the North Carolina State University and Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP [Food Research Center (FoRC-2013/07914-8) and 2016/03044-7]. The project has been partially developed during DM's time as visiting scholar at the North Carolina State University under a fellowship grant from FAPESP (2017/15967-5).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.867278/full#supplementary-material

Supplementary Figures 1, 2 | SNP-based phylogram of S. Heidelberg isolates from various isolation sources and locations. Strain ID, isolate source, location, SNP cluster, and collection date were retrieved from Genbank. Red color indicates isolates from this study.

REFERENCES

Abdullah, K., Iwen, P. C., and Abdalhamid, B. (2021). Identification of an mcr-9-Carrying *Salmonella enterica* serotype heidelberg strain isolated from blood. *Microbiol. Resour. Announc.* 10:e00676-21. doi: 10.1128/MRA.00676-21

Andrysiak, A. K., Olson, A. B., Tracz, D. M., Dore, K., Irwin, R., Ng, L. K., et al. (2008). Genetic characterization of clinical and agri-food isolates of multi drug resistant *Salmonella enterica* serovar Heidelberg from Canada. *BMC Microbiol.* 8:89. doi: 10.1186/1471-2180-8-89

Anonymous (2007). *ISO 6579:2002 Amd. 1:2007 Microbiology of Food and Animal Feeding Stuffs–Horizontal Method for the Detection of Salmonella spp. – Annex D: Detection of Salmonella spp. in Animal Faeces and in Environmental Samples from the Primary Production Stage*. Geneva: International Organization for Standardization.

Anonymous (2017). *ISO 6579–1 Microbiology of the Food Chain–Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella–Part 1: Detection of Salmonella spp*. Geneva: International Organization for Standardization.

Antony, L., Behr, M., Sockett, D., Miskimins, D., Aulik, N., Christopher-Hennings, J., et al. (2018). Genome divergence and increased virulence of outbreak associated *Salmonella enterica* subspecies *enterica* serovar Heidelberg. *Gut Pathog.* 10:53. doi: 10.1186/s13099-018-0279-0

Barrangou, R., and Dudley, E. G. (2016). CRISPR-based typing and next-generation tracking technologies. *Annu. Rev. Food Sci. Technol.* 7, 395–411. doi: 10.1146/annurev-food-022814-015729

Cabral, C. C., Panzenhagen, P., Delgado, K. F., Silva, G., Rodrigues, D., Franco, R. M., et al. (2017). Contamination of carcasses and utensils in small swine slaughterhouses by *Salmonella* in the Northwestern Region of the State of Rio de Janeiro, Brazil. *J. Food Prot.* 80, 1128–1132. doi: 10.4315/0362-028X.JFP-16-387

Campos, J., Mourão, J., Silveira, L., Saraiva, M., Correia, C. B., Maçãs, A. P., et al. (2018). Imported poultry meat as a source of extended-spectrum cephalosporin-resistant CMY-2-producing *Salmonella* Heidelberg and *Salmonella* Minnesota in the European Union, 2014-2015. *Int. J. Antimicrob. Agents* 51, 151–154. doi: 10.1016/j.ijantimicag.2017.09.006

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., et al. (2014). *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother* 58, 3895–3903. doi: 10.1128/AAC.02412-14

Centers for Disease Control and Prevention (2014). *National Antimicrobial Resistance Monitoring System (NARMS): 2014 Human Isolates Surveillance Report*. Atlanta, GA: U.S. Department of Health and Human Services, 7.

Centers for Disease Control and Prevention [CDC] (2017). *Multistate Outbreak of Multidrug-Resistant Salmonella Heidelberg Infections Linked to Contact with Dairy Calves*. Atlanta, GA: Centers for Disease Control and Prevention.

Chittick, P., Sulka, A., Tauxe, R. V., and Fry, A. M. (2006). A summary of national reports of foodborne outbreaks of *Salmonella* Heidelberg infections in the United States: clues for disease prevention. *J. Food Prot.* 69, 1150–1153. doi: 10.4315/0362-028x-69.5.1150

Clinical and Laboratory Standards Institute [CLSI] (2021). *Performance Standards for Antimicrobial Susceptibility Testing, M100*, 31st Edn. Wayne, PA: Clinical and Laboratory Standards Institute.

Cox, G. W., Parmley, E. J., Avery, B. P., Irwin, R. J., Reid-Smith, R. J., Deckert, A. E., et al. (2021). A one-health genomic investigation of gentamicin resistance in *Salmonella* from Human and Chicken Sources in Canada, 2014 to 2017. *Antimicrob. Agents Chemother.* 65:e00966-21. doi: 10.1128/AAC.00966-21

Crump, J. A., Sjölund-Karlsson, M., Gordon, M. A., and Parry, C. M. (2015). Epidemiology, clinical presentation, laboratory diagnosis, antimicrobial resistance, and antimicrobial management of invasive *Salmonella* infections. *Clin. Microbiol. Rev.* 28, 901–937. doi: 10.1128/CMR.00002-15

Deblais, L., Lorentz, B., Scaria, J., Nagaraja, K. V., Nisar, M., Lauer, D., et al. (2018). Comparative genomic studies of *Salmonella* Heidelberg Isolated From Chicken- and Turkey-Associated Farm Environmental Samples. *Front. Microbiol.* 9:1841. doi: 10.3389/fmicb.2018.01841

DiMarzio, M., Shariat, N., Kariyawasam, S., Barrangou, R., and Dudley, E. G. (2013). Antibiotic Resistance in *Salmonella enterica* Serovar Typhimurium Associates with CRISPR Sequence Type. *Antimicrob. Agents Chemother.* 57, 4282–4289. doi: 10.1128/AAC.00913-13

Edirmanasinghe, R., Finley, R., Parmley, E. J., Avery, B. P., Carson, C., Bekal, S., et al. (2017). A whole-genome sequencing approach to study Cefoxitin-Resistant *Salmonella enterica* Serovar Heidelberg Isolates from Various Sources. *Antimicrob. Agents Chemother.* 61:e01919-16. doi: 10.1128/AAC.01919-16

Foley, S. L., Lynne, A. M., and Nayak, R. (2008). *Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. *J. Anim. Sci.* 86(Suppl. 14), E149–E162. doi: 10.2527/jas.2007-0464

Folster, J. P., Pecic, G., Rickert, R., Taylor, J., Zhao, S., Fedorka-Cray, P. J., et al. (2012). Characterization of multidrug-resistant *Salmonella enterica* serovar Heidelberg from a ground turkey-associated outbreak in the United States in 2011. *Antimicrob. Agents Chemother.* 56, 3465–3466. doi: 10.1128/AAC.00201-12

Grimont, P. A. D., and Weil, F. X. (2007). *Antigenic Formulae of the Salmonella serovars*, 9th Edn. (Paris: WHO Collaborating Centre for Reference and Research on Salmonella), 166.

Guibourdenche, M., Roggentin, P., Mikoleit, M., Fields, P. I., Bockemühl, J., Grimont, P. A., et al. (2010). Supplement 2003-2007 (No. 47) to the White-Kauffmann-Le Minor scheme. *Res. Microbiol.* 161, 26–29. doi: 10.1016/j.resmic.2009.10.002

Hadfield, J., Croucher, N. J., Goater, R. J., Abudahab, K., Aanensen, D. M., and Harris, S. R. (2018). Phandango: an interactive viewer for bacterial population genomics. *Bioinformatics* 34, 292–293. doi: 10.1093/bioinformatics/btx610

Hennessy, T. W., Cheng, L. H., Kassenborg, H., Ahuja, S. D., Mohle-Boetani, J., Marcus, R., et al. (2004). Egg consumption is the principal risk factor for sporadic *Salmonella* serotype Heidelberg infections: a case-control study in FoodNet sites. *Clin. Infect. Dis.* 38(Suppl. 3), S237–S243. doi: 10.1086/381593

Jackson, B. R., Griffin, P. M., Cole, D., Walsh, K. A., and Chai, S. J. (2013). Outbreak-associated *Salmonella enterica* serotypes and food Commodities, United States, 1998-2008. *Emerg. Infect. Dis.* 19, 1239–1244. doi: 10.3201/eid1908.121511

Kipper, D., Orsi, R. H., Carroll, L. M., Mascitti, A. K., Streck, A. F., Fonseca, A., et al. (2021). Recent evolution and genomic profile of *Salmonella enterica* Serovar Heidelberg Isolates from Poultry Flocks in Brazil. *Appl. Environ. Microbiol.* 87:e01036-21. doi: 10.1128/AEM.01036-21

Letunic, I., and Bork, P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res.* 44, W242–W245. doi: 10.1093/nar/gkw290

Liakopoulos, A., Olsen, B., Geurts, Y., Artursson, K., Berg, C., Mevius, D. J., et al. (2016a). Molecular Characterization of Extended-Spectrum-Cephalosporin-Resistant *Enterobacteriaceae* from Wild Kelp Gulls in South America. *Antimicrob. Agents Chemother.* 60, 6924–6927. doi: 10.1128/AAC.01120-16

Liakopoulos, A., Geurts, Y., Dierikx, C. M., Brouwer, M. S., Kant, A., Wit, B., et al. (2016b). Extended-Spectrum Cephalosporin-Resistant *Salmonella enterica* serovar Heidelberg Strains, the Netherlands(1). *Emerg. Infect. Dis.* 22, 1257–1261. doi: 10.3201/eid2207.151377

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Monte, D., Nethery, M. A., Barrangou, R., Landgraf, M., and Fedorka-Cray, P. J. (2021). Whole-genome sequencing analysis and CRISPR genotyping of rare antibiotic-resistant *Salmonella enterica* serovars isolated from food and related sources. *Food Microbiol.* 93:103601. doi: 10.1016/j.fm.2020.103601

Monte, D. F., Lincopan, N., Berman, H., Cerdeira, L., Keelara, S., Thakur, S., et al. (2019). Genomic Features of High-Priority *Salmonella enterica* Serovars Circulating in the Food Production Chain, Brazil, 2000-2016. *Sci. Rep.* 9:11058. doi: 10.1038/s41598-019-45838-0

Moreno, L. Z., Gomes, V., Moreira, J., de Oliveira, C. H., Peres, B. P., Silva, A., et al. (2019). First report of *mcr-1*-harboring *Salmonella enterica* serovar Schwarzengrund isolated from poultry meat in Brazil. *Diagn. Microbiol. Infect. Dis.* 93, 376–379. doi: 10.1016/j.diagmicrobio.2018.10.016

Nethery, M. A., and Barrangou, R. (2019). CRISPR Visualizer: rapid identification and visualization of CRISPR loci *via* an automated high-throughput processing pipeline. *RNA Biol.* 16, 577–584. doi: 10.1080/15476286.2018.1493332

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421

Page, A. J., Taylor, B., Delaney, A. J., Soares, J., Seemann, T., Keane, J. A., et al. (2016). SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. *Microb. Genom.* 2:e000056. doi: 10.1099/mgen.0.000056

Pattengale, N. D., Alipour, M., Bininda-Emonds, O. R., Moret, B. M., and Stamatakis, A. (2010). How many bootstrap replicates are necessary? *J Comput Biol.* 17, 337–354. doi: 10.1089/cmb.2009.0179

Public Health Agency of Canada, (2014). *National Enteric Surveillance Program (NESP) Annual Report*. http://publications.gc.ca/collections/collection_2016/aspc-phac/HP37-15-2014-eng.pdf (accessed April 2019).

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Shariat, N., DiMarzio, M. J., Yin, S., Dettinger, L., Sandt, C. H., Lute, J. R., et al. (2013a). The combination of CRISPR-MVLST and PFGE provides increased discriminatory power for differentiating human clinical isolates of *Salmonella enterica* subsp. *enterica* serovar Enteritidis. *Food Microbiol.* 34, 164–173. doi: 10.1016/j.fm.2012.11.012

Shariat, N., Sandt, C. H., DiMarzio, M. J., Barrangou, R., and Dudley, E. G. (2013b). CRISPR-MVLST subtyping of *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Heidelberg and application in identifying outbreak isolates. *BMC Microbiol.* 13:254. doi: 10.1186/1471-2180-13-254

Shariat, N., Timme, R. E., Pettengill, J. B., Barrangou, R., and Dudley, E. G. (2015). Characterization and evolution of *Salmonella* CRISPR-Cas systems. *Microbiology* 161, 374–386. doi: 10.1099/mic.0.000005

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Teunis, P., Evers, E. G., Hengeveld, P. D., Dierikx, C. M., Wielders, C., and van Duijkeren, E. (2018). Time to acquire and lose carriership of ESBL/pAmpC producing *E. coli* in humans in the Netherlands. *PLoS One* 13:e0193834. doi: 10.1371/journal.pone.0193834

Thompson, C. P., Doak, A. N., Amirani, N., Schroeder, E. A., Wright, J., Kariyawasam, S., et al. (2018). High-Resolution Identification of Multiple *Salmonella* Serovars in a Single Sample by Using CRISPR-SeroSeq. *Appl. Environ. Microbiol.* 84:e01859-18. doi: 10.1128/AEM.01859-18

US Food and Drug Administration [FDA] (2015). *The National Antimicrobial Resistance Monitoring System Manual of Laboratory Methods*. Silver Spring, MD: US Food and Drug Administration.

van den Berg, R. R., Dissel, S., Rapallini, M., van der Weijden, C. C., Wit, B., and Heymans, R. (2019). Characterization and whole genome sequencing of closely related multidrug-resistant *Salmonella enterica* serovar Heidelberg isolates from imported poultry meat in the Netherlands. *PLoS One* 14:e0219795. doi: 10.1371/journal.pone.0219795

Vincent, C., Usongo, V., Berry, C., Tremblay, D. M., Moineau, S., Yousfi, K., et al. (2018). Comparison of advanced whole genome sequence-based methods to distinguish strains of *Salmonella enterica* serovar Heidelberg involved in foodborne outbreaks in Québec. *Food Microbiol.* 73, 99–110. doi: 10.1016/j.fm.2018.01.004

Wen, S. C., Best, E., and Nourse, C. (2017). Non-typhoidal *Salmonella* infections in children: review of literature and recommendations for management. *J. Paediatr. Child Health* 53, 936–941. doi: 10.1111/jpc.13585

Wu, H., Xia, X., Cui, Y., Hu, Y., Xi, M., Wang, X., et al. (2013). Prevalence of extended-spectrum b-lactamase-producing *Salmonella* on retail chicken in six provinces and two national cities in the People's Republic of China. *J. Food Prot.* 76, 2040–2044. doi: 10.4315/0362-028X.JFP-13-224

Yousfi, K., Usongo, V., Berry, C., Khan, R. H., Tremblay, D. M., Moineau, S., et al. (2020). Source Tracking Based on Core Genome SNV and CRISPR Typing of *Salmonella enterica* Serovar Heidelberg Isolates Involved in Foodborne Outbreaks in Québec, 2012. *Front. Microbiol.* 11:1317. doi: 10.3389/fmicb.2020.01317

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 June 2022
doi: 10.3389/fmicb.2022.880043

Microbial Antagonism in Food-Enrichment Culture: Inhibition of Shiga Toxin-Producing *Escherichia coli* and *Shigella* Species

Tanis C. McMahon[1,2], Cesar Bin Kingombe[3†], Amit Mathews[4], Karine Seyer[5], Alex Wong[2], Burton W. Blais[1] and Catherine D. Carrillo[1]*

[1]Research and Development, Ottawa Laboratory (Carling), Ontario Laboratory Network, Canadian Food Inspection Agency, Ottawa, ON, Canada, [2]Department of Biology, Carleton University, Ottawa, ON, Canada, [3]Independent Researcher, Ontario, ON, Canada, [4]Microbiology, Greater Toronto Area Laboratory, Ontario Laboratory Network, Canadian Food Inspection Agency, Toronto, ON, Canada, [5]Microbiology (Food), St-Hyacinthe Laboratory, Eastern Laboratories Network, Canadian Food Inspection Agency, St-Hyacinthe, QC, Canada

OPEN ACCESS

Edited by:
Laurent Dufossé,
Université de la Réunion, France

Reviewed by:
Yujie Zhang,
United States Department of Agriculture, United States
Christian Menge,
Friedrich-Loeffler-Institute, Germany

***Correspondence:**
Catherine D. Carrillo
catherine.carrillo@inspection.gc.ca

[†]*Deceased*

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *20 February 2022*
Accepted: *18 May 2022*
Published: *23 June 2022*

Citation:
McMahon TC, Kingombe CB, Mathews A, Seyer K, Wong A, Blais BW and Carrillo CD (2022) Microbial Antagonism in Food-Enrichment Culture: Inhibition of Shiga Toxin-Producing Escherichia coli and Shigella Species. Front. Microbiol. 13:880043. doi: 10.3389/fmicb.2022.880043

Bacterial pathogens, such as Shiga toxin-producing *Escherichia coli* (STEC) and *Shigella* spp., are important causes of foodborne illness internationally. Recovery of these organisms from foods is critical for food safety investigations to support attribution of illnesses to specific food commodities; however, isolation of bacterial cultures can be challenging. Methods for the isolation of STEC and *Shigella* spp. from foods typically require enrichment to amplify target organisms to detectable levels. Yet, during enrichment, target organisms can be outcompeted by other bacteria in food matrices due to faster growth rates, or through production of antimicrobial agents such as bacteriocins or bacteriophages. The purpose of this study was to evaluate the occurrence of *Shigella* and STEC inhibitors produced by food microbiota. The production of antimicrobial compounds in cell-free extracts from 200 bacterial strains and 332 food-enrichment broths was assessed. Cell-free extracts produced by 23 (11.5%) of the strains tested inhibited growth of at least one of the five *Shigella* and seven STEC indicator strains used in this study. Of the 332 enrichment broths tested, cell-free extracts from 25 (7.5%) samples inhibited growth of at least one of the indicator strains tested. Inhibition was most commonly associated with *E. coli* recovered from meat products. Most of the inhibiting compounds were determined to be proteinaceous (34 of the 48 positive samples, 71%; including 17 strains, 17 foods) based on inactivation by proteolytic enzymes, indicating presence of bacteriocins. The cell-free extracts from 13 samples (27%, eight strains, five foods) were determined to contain bacteriophages based on the observation of plaques in diluted extracts and/or resistance to proteolytic enzymes. These results indicate that the production of inhibitors by food microbiota may be an important challenge for the recovery of foodborne pathogens, particularly for *Shigella sonnei*. The performance of enrichment media for recovery of *Shigella* and STEC could be improved by mitigating the impact of inhibitors produced by food microbiota during the enrichment process.

Keywords: bacteriocin, bacteriophage, Shiga toxin-producing *Escherichia coli*, *Shigella*, foodborne pathogen

INTRODUCTION

Foodborne illnesses due to Shiga toxin-producing *Escherichia coli* (STEC) and *Shigella* spp. are an important public health concern around the world (EFSA, 2013; Gould et al., 2013; Thomas et al., 2015; Adam and Pickings, 2016; The et al., 2016). In Canada, it is estimated that there are approximately 1,200 domestically-acquired cases of foodborne shigellosis and approximately 33,000 illnesses attributed to STEC per year (Thomas et al., 2013). Infections by both STEC and *Shigella* can result in serious illnesses. Complications such as hemolytic-uremic syndrome (HUS), hemorrhagic colitis and Reiter's syndrome can have long-term effects and are sometimes fatal (Donnenberg and Whittam, 2001; Perelle et al., 2004; Warren et al., 2006; Smith et al., 2014; The et al., 2016). Food contamination with *Shigella* is exclusively from human sources (Warren et al., 2006), whereas animals are important reservoirs associated with foodborne STEC (Kim et al., 2020).

Infection with STEC or *Shigella* spp. can occur due to ingestion of as little as 10–100 cells (Kothary and Babu, 2001; Thorpe, 2004). Therefore, methods used to test foods for these pathogens must be very sensitive, and must be able to identify a small number of target cells that are likely present as a miniscule portion of the food microbiota. Current methods used to detect foodborne STEC and *Shigella* in Canada, United States, and Europe involve an enrichment step followed by screening of enrichment cultures for characteristic virulence genes (Bin Kingombe et al., 2006; Warren et al., 2006; ISO, 2012; Blais et al., 2014; USDA-FSIS, 2019). Typically, an enrichment procedure is conducted with the intention of favoring the growth of the target bacteria while limiting the growth of non-target or background bacteria present in the sample. Nonetheless, the non-target bacteria can often outcompete the target pathogen under these conditions due to faster growth rates, or production of compounds that actively interfere with their growth (Uyttendaele et al., 2001; Blais et al., 2019). This can lead to false-negative results in cases where the pathogen dies, or is present at a proportionally lower level after enrichment, and cannot be detected or isolated in downstream analyses (Kozak et al., 2013). There are many examples of foodborne outbreaks associated with STEC or *Shigella* spp. in which pathogens were not successfully recovered from implicated foods despite strong epidemiological evidence supporting food attribution (Heier et al., 2009; Lewis et al., 2009; Marshall et al., 2020; Mikhail et al., 2021). Failure to recover target pathogens may result in delays in attribution of an outbreak to a food commodity.

A number of studies have investigated growth dynamics in food enrichment cultures to gain a better understanding of the growth of interfering organisms in these environments (Jarvis et al., 2015; Margot et al., 2016; Ottesen et al., 2016; Kang et al., 2021). The aim of many of these studies has largely been to catalog species in the food microbiome that can interfere with the successful recovery of target pathogens in enrichment cultures, and to evaluate the strengths and weaknesses of different methodological approaches in reducing the growth of non-target organisms. Few studies have looked specifically at the role of antimicrobial compounds produced by food microbiota in food enrichment culture. Although, one study of tomato enrichment culture microbiomes identified *Paenibacillus* as a potential *Salmonella*-inhibiting organism in this system, based on known activity of this organism (Ottesen et al., 2013).

Bacteria can produce a variety of antimicrobial compounds. Almost all bacteria encode bacteriocins, which are antimicrobial peptides (Kim et al., 2014; Yang et al., 2014; Simons et al., 2020). Bacteriocins produced by Gram-negative bacteria that target related species are classified as colicins (25–80 kDa) or microcins (10 kDa; Duquesne et al., 2007; Yang et al., 2014; Mader et al., 2015). These bacteriocins damage host cells through pore formation, DNA/RNA degradation, protein synthesis inhibition or DNA replication inhibition (Alonso et al., 2000; Duquesne et al., 2007; Yang et al., 2014). Another mechanism of microbial antagonism is through the production of bacteriophages that can infect and kill bacteria (Penadés et al., 2015; Shahin et al., 2019). Bacteriophages have two cycles of viral reproduction: the lysogenic and lytic cycles. In the lysogenic cycle, the bacteriophage genome is integrated into the bacterial host's genome as a prophage and is propagated through replication of the host's chromosome without damaging the host cell (Parasion et al., 2014; Penadés et al., 2015). Induction involves conversion of the lysogenic infection into a lytic infection, where the host's machinery is used to produce mature phages, which are released through lysis of the infected cells. Bacteriophages in the lysogenic cycle can be induced into the lytic cycle when the bacteria undergo stress or through induction of the SOS response. Most phages can only affect a subset of bacteria within a species, and specificity of the phage depends on the receptors to which it binds (Koskella and Meaden, 2013; Parasion et al., 2014). Finally, other antibiotic compounds (e.g., lipopeptides, aminoglycosides, tetracyclines, and aminocoumarins) may be produced by bacterial groups such as Actinomycetales, Bacillales, and Enterobacterales (Mandal et al., 2013; Challinor and Bode, 2015; Mohr, 2016).

The purpose of this study was to evaluate the occurrence of antimicrobial compounds produced by food microbiota in food enrichment cultures. *Shigella* spp. and STEC were selected for this study as they are related genera that have low infectious doses, and are among the most difficult pathogens to recover from foods. Cell-free extracts derived from food-associated bacterial strains and from food enrichments [modified Tryptone Soya Broth (mTSB) or *Shigella* broth (SB)] were tested for inhibitory activity against STEC and *Shigella*. Samples containing inhibitors were further characterized to determine the likely mechanisms of inhibition. Results of this study will be of great value in the development of improved methods for a more reliable recovery of STEC and *Shigella* spp. from foods.

MATERIALS AND METHODS

Growth and Maintenance of Bacterial Strains

A selection of 200 predominantly Enterobacteriaceae strains, most of which were previously isolated from food enrichment broths, were selected for this study (**Supplementary Table S1**).

Seven *E. coli* strains representing clinically important STEC serotypes and five *Shigella* strains were used as the indicator strains for testing sensitivity to inhibition from cell-free extracts (**Table 1**). All strains were stored at −80°C in 15% glycerol and were plated on Brain-Heart Infusion agar (BHI; OXOID, Nepean, ON, Canada) overnight (14–16 h) at 37°C prior to use.

Preparation of Cell-Free Extracts

Cell-free extracts were prepared from (1) overnight cultures of bacterial strains, and (2) food enrichment cultures from negative samples collected by the CFIA's food testing laboratories in Ottawa (ON), Toronto (ON), and Saint-Hyacinthe (QC).

Bacterial strains ($n = 200$; **Supplementary Table S1**) were grown overnight at 37°C in 10 ml of Nutrient broth (OXOID) and broths were filtered using a 0.22 µm Vacuum filter (EMD Millipore Steriflip™ Sterile Disposable Vacuum Filter Units; Thermo Fisher Scientific, Ottawa). The cell-free extracts were stored at 4°C for up to 3 months and at −20°C for longer storage (**Figure 1**).

Food products were sampled between the fall 2016 and winter 2017 and were representative of the types of foods tested in regulatory food testing programs (**Supplementary Table S2**). Extracts were prepared from 332 enrichment cultures derived from 235 food samples. Categories of food products tested included fruits [46 (14%)], salads and coleslaws [78 (23%)], meats [85 (26%)] and vegetables, cheese and other [123 (37%)]. Enrichment cultures were generated using methods described in the Canadian Compendium of Analytical Methods: either the method for detection of *Shigella* spp. in foods (MFLP-26; $n = 23$; Bin Kingombe et al., 2006) or the method for detection of STEC in foods (MFLP-30; $n = 115$; Microbiological Methods Committee, 2012) or both ($n = 97$). The meats and cheeses were only enriched in mTSB and the rest were enriched in SB or both broths. For the MFLP-26 method, samples are enriched in a 1:10 dilution of sample to *Shigella* broth (SB; SB base, OXOID) with Tween-80 (Sigma, Markham, ON, Canada) containing 0.5 µg/ml of novobiocin (Sigma-Aldrich, Oakville, ON). The SB is enriched for 20 h at 42°C in a CO_2 incubator or CO_2 jar system. The MFLP-30 method involves a 1:10 dilution of sample to modified Tryptone Soya Broth (mTSB, OXOID) containing 20 µg/ml of novobiocin, followed by aerobic incubation of the enrichment at 42°C for 18–24 h. Typically, 25 g of food are enriched in 225 ml of enrichment broth using both methods. Note that enrichments were negative for targeted pathogens and that bacterial growth was observed for all samples based on turbidity of broths.

TABLE 1 | STEC and *Shigella* strains used as indicator organisms.

Isolate	Genus	Species	Serotype	Description/Isolation source
OLC0024	*Shigella*	*sonnei*		ATCC29930/feces
OLC2340	*Shigella*	*sonnei*		Pasta salad outbreak/ human feces
OLC0603	*Shigella*	*flexneri*	1a	ATCC25929/human feces
OLC1597	*Shigella*	*flexneri*	1b	ATCC12022/missing
OLC0608	*Shigella*	*dysenteriae*		Human feces
OLC0455	*Escherichia*	*coli*	O111:H11	STEC *stx1a*, *eae*/ missing
OLC0464	*Escherichia*	*coli*	O26:H11	STEC *stx1a*, *eae*/ missing
OLC0675	*Escherichia*	*coli*	O145:NM	STEC *stx1a*, *eae*/ human feces
OLC0679	*Escherichia*	*coli*	O103:H2	STEC *stx1a*, *eae*/ human feces
OLC0710	*Escherichia*	*coli*	O121:H19	STEC *stx2a*, eae/ human feces
OLC0716	*Escherichia*	*coli*	O45:H2	STEC *stx1a*, *eae*/ human feces
OLC0797	*Escherichia*	*coli*	O157:H7	STEC *stx1a*, *sxt2a*, *eae*/human feces

Enrichment broths were stored at 4°C prior to use. Cell-free extracts were prepared from 25 to 50 ml of enrichment broth by centrifugation at 500 × *g* for 5 min, transferring the supernatant to a new tube, followed by centrifugation at 14000 × *g* for 10 min (**Figure 1**). The supernatant was then filtered as described above and stored at 4°C or −20°C. The pellets from the high-speed spin were resuspended in 30% glycerol and stored at −80°C.

Detection of Inhibitors in Cell-Free Extracts

The methods for evaluating the inhibitory activity of cell-free extracts were modified from methods developed by (Arici et al., 2004) and (Vijayakumar and Muriana, 2015; **Figure 1**). *Shigella* and STEC were grown overnight in 10 ml of Nutrient Broth (NB, OXOID) at 37°C to obtain a concentration of approximately 10^8 cells/ml. Overnight cultures of each of the indicator strains were enumerated to assess reproducibility of this approximation for initial experiments, but enumeration was not done routinely (data not shown). The overnight *Shigella* or STEC culture was added at a concentration of approximately 1×10^6 cells/ml into soft agar cooled to 40°C [NB (OXOID) containing 0.5% (w/v) bacteriological agar (Sigma-Aldrich)] and poured into a Petri dish. Once the plates were solidified, 3 µl spots of the cell-free extracts were added and left to absorb into the agar before incubating plates overnight at 37°C. To enable high-throughput analyses, multichannel pipets were used to generate 36 spots on square Petri plates. All extracts were tested in triplicate.

Isolation of Bacteria Producing Inhibitors From Food Enrichments

The method from Henning et al. (2015) was used to isolate the inhibitor-producing bacteria from a subset of 13 of the food enrichment broths that were active against at least one indicator organism. Dilutions of the enrichment broths were spread plated on nutrient agar (OXOID) then immediately overlaid with a thin nutrient agar sandwich layer. The plates were incubated at 37°C overnight before a soft agar containing ~1×10^6 cells/ml of the indicator species (*Shigella sonnei* OLC2340) was layered on top (see above). The triple layer agar was incubated overnight at 37°C. Note that only *S. sonnei* OLC2340

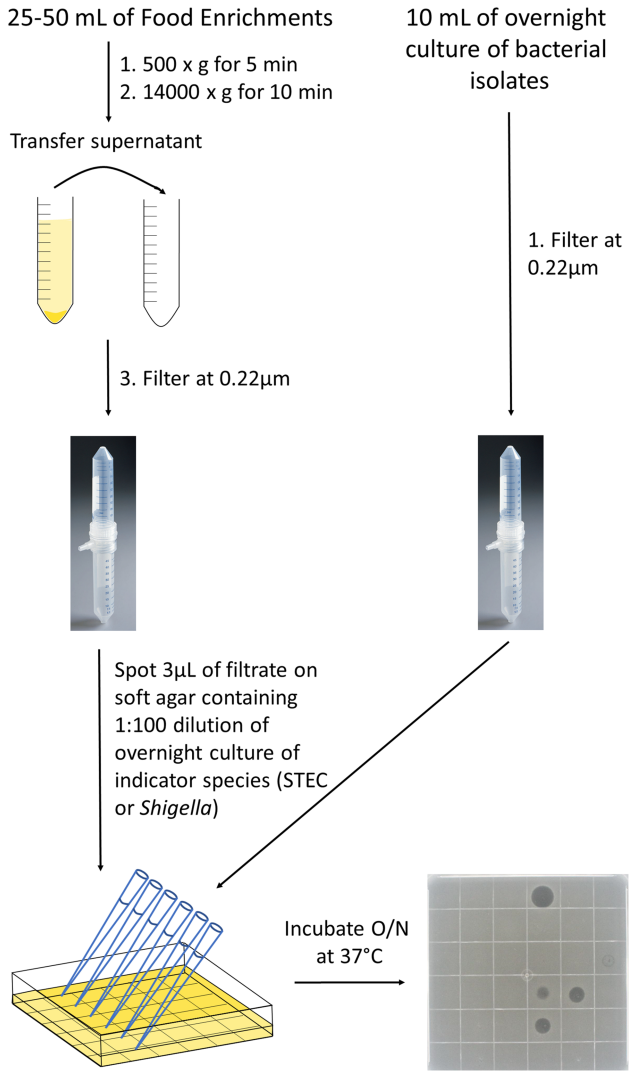


FIGURE 1 | Detection of inhibitors in cell-free extracts from food enrichments or bacterial strains. Bacterial strains were grown overnight in 10 ml of Nutrient broth at 37°C (right side) and food enrichments were incubated according to MLFP-26 or MLFP-30 methods. For food enrichments (left side), an aliquot of 25–50 ml was taken and centrifuged first at 500 × *g* to remove large food particles and then at 14,000 × *g* to remove bacterial cells and debris. Supernatants from cell enrichments or overnight bacterial cultures were then filtered through a 0.22 µm vacuum filter to remove remaining food particles and bacterial cells. Filtrates were spotted on soft agar containing indicator strains. Filtrate spots were absorbed into the agar, and plates were incubated overnight at 37°C. The following day, pictures were taken to observe the clearings and the diameter of the clearings were measured in millimeters. A representative plate with 35 different cell-free extracts and a negative control spotted in a 6 × 6 grid is shown.

was used in this experiment, due to the complexity of the method, and the susceptibility of this strain to most of the cell-free extracts.

Following incubation, the triple agar plates were inverted and placed onto their Petri dish covers, and colonies surrounded by zones of clearing were streaked onto a new nutrient agar plate. The streaked plates were incubated overnight at 37°C and isolated colonies were patched onto two nutrient agar plates. The two plates were incubated at 37°C for 4 h and then one of the duplicate plates was overlaid with soft agar containing ~1×10^6 cells/ml of *S. sonnei* OLC2340. The plates were again incubated at 37°C overnight. Isolates recovered from these plates were streaked for purity, then re-tested to confirm inhibitory activity. Isolates confirmed to cause inhibition were stored and maintained as described above.

Testing Cell-Free Extracts for Proteinaceous Properties, Bacteriophage, and pH

Proteolytic enzymes were used to assess the proteinaceous nature of the inhibitory cell-free extracts (Elayaraja et al., 2014). Proteinase K (Thermo Fisher Scientific) and trypsin (Sigma-Aldrich) were added to the extracts at a final concentration of 1 mg/ml or 1X, respectively. The extracts were incubated for 2 h at 30°C before spotting on soft agar containing target bacteria as described above, alongside an untreated control. The presence of bacteriophage was assessed using the dilution method from Hockett and Baltrus (2017). Cell-free extracts were serially diluted two times in nutrient broth (1:10 and 1:100). Dilutions were spotted on soft agar as described above. Bacteriophage presence was confirmed if individual plaques were visible in the diluted samples and/or the clearing was unaffected by proteolytic enzymes. The pH of the cell-free extracts were measured with pH indicator strips (Thermo Fisher Scientific).

RESULTS

Inhibition of Growth of *Shigella* and STEC by Cell-Free Extracts Derived From Bacterial Strains

Cell-free extracts from 200 bacterial strains, primarily recovered from food (**Supplementary Table S1**), were tested to detect inhibition of growth of five strains of *Shigella* and seven strains of STEC (**Table 1**). Twenty-three of the 200 strains tested (11.5%) inhibited the growth of at least one of the 12 indicator organisms (*Shigella* and EHEC) used in this study. Most of the strains tested were Enterobacteriaceae (194/200), except for six strains (Pseudomonadaceae and Aeromonadaceae; **Figure 2**; **Supplementary Table S1**). The three main genera evaluated were *Escherichia*, *Enterobacter* and *Hafnia* [118 (48.5%), 31(14.5%), and 20(10%), respectively]. Cell-free extracts produced by 21 *E. coli* (10.5% of all strains, 17.8% of *E. coli*) inhibited growth of at least one strain of *Shigella*, and cell-free extracts from two *Enterobacter* spp. strains (1% of all strains, 6.5% of *Enterobacter* spp.) inhibited growth of at least one strain of STEC (**Figure 2**). Most of the inhibitor-producing *E. coli* affected *S. sonnei* (18 of 21, 86%), with a smaller proportion ($n = 7$, 33%) affecting *Shigella flexneri*. *E. coli* classified as STEC were more likely to produce bacteriocins (10 of 19 strains,

52.6%), compared to other *E. coli* (11 of 99 strains, 11.1%). In most samples, both strains of *S. sonnei* (OLC0024 and OLC2340) were inhibited (16 out of 18) whereas only three out of the seven extracts affecting *S. flexneri* inhibited both of the strains used in this study (**Figure 3**). The inhibitor-producing *Enterobacter* spp. affected *E. coli* O45 (two extracts) and *E. coli* O103 (one extract; **Figure 3**). None of the strains evaluated in this study inhibited growth of *Shigella dysenteriae*.

The relative strength of inhibition varied among cell-free extracts and was assessed based on the diameter of the zone of inhibition and the opacity of the clearing (**Figure 3A**). Samples were designated as very weak (3 mm diameter) to very strong (11 mm diameter; **Figure 3**; **Supplementary Table S3**). The inhibitory activity of cell-free extracts from *E. coli* on *S. sonnei* was generally categorized as strong and very strong, whereas inhibitory activity on *S. flexneri* was largely determined to be medium or weak. Similarly, the cell-free extracts from *Enterobacter* spp. that inhibited STEC produced medium or weak inhibition of growth.

Inhibition of *Shigella* and STEC From Cell-Free Extracts From Food Enrichments

Cell-free extracts from 332 food-enrichment broths derived from 235 food products (**Figure 4**; **Supplementary Table S2**) were evaluated to determine prevalence of inhibitors to *Shigella* spp. or STEC in food enrichments. Of the 332 enrichment broths tested, cell-free extracts from 25 (7.55%) samples inhibited growth of at least one of the 12 *Shigella* or STEC strains used in this study (**Figure 5**). Twenty-one samples (6%) inhibited growth of *Shigella* spp. and seven (2%) inhibited growth of STEC (**Figure 5**). Among the 25 cell-free extracts containing inhibitors, 21 (84%) affected *S. sonnei*, six (24%) affected *S. flexneri* and seven (28%) affected STEC growth (**Figure 5**). One of the extracts (GTA-1452) inhibited growth of all STEC strains tested in this study and three extracts inhibited both *Shigella* and STEC strains. None of the extracts affected growth of *S. dysenteriae*.

As with the extracts from bacterial strains, the relative strength of inhibition was assessed based on the diameter of

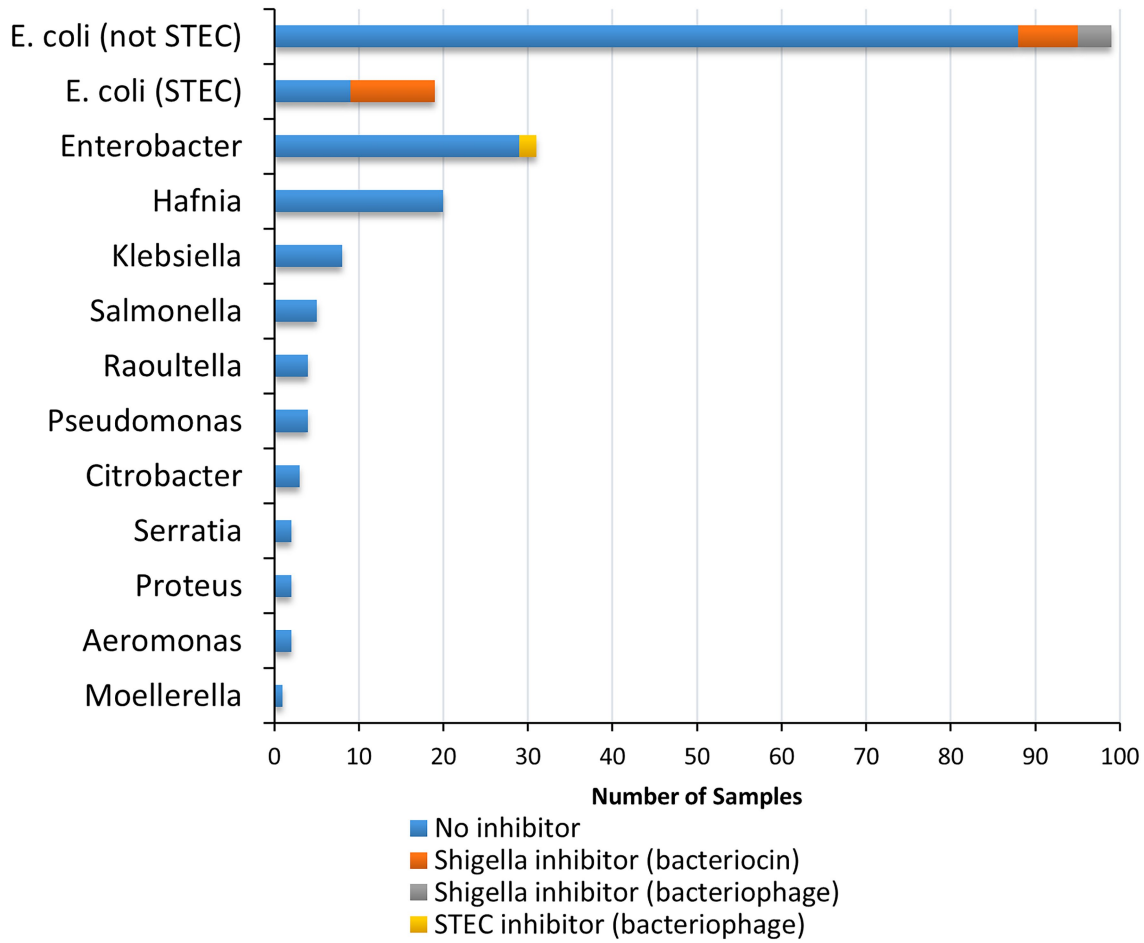


FIGURE 2 | Species and inhibitory activity of foodborne bacterial strains tested against *Shigella* and STEC. In total cell-free extracts from 200 bacterial strains were tested on five *Shigella* spp. strains (two *Shigella sonnei*, two *Shigella* flexneri, and one *Shigella dysenteriae*) and seven STEC (Serotypes O26, O45, O103, O111, O121, O145, and O157) samples. Relative proportion of strains producing inhibitors are indicated according to genus impacted (*Shigella* spp. vs. STEC), and predicted inhibitor (bacteriocin vs. bacteriophage; see legend).

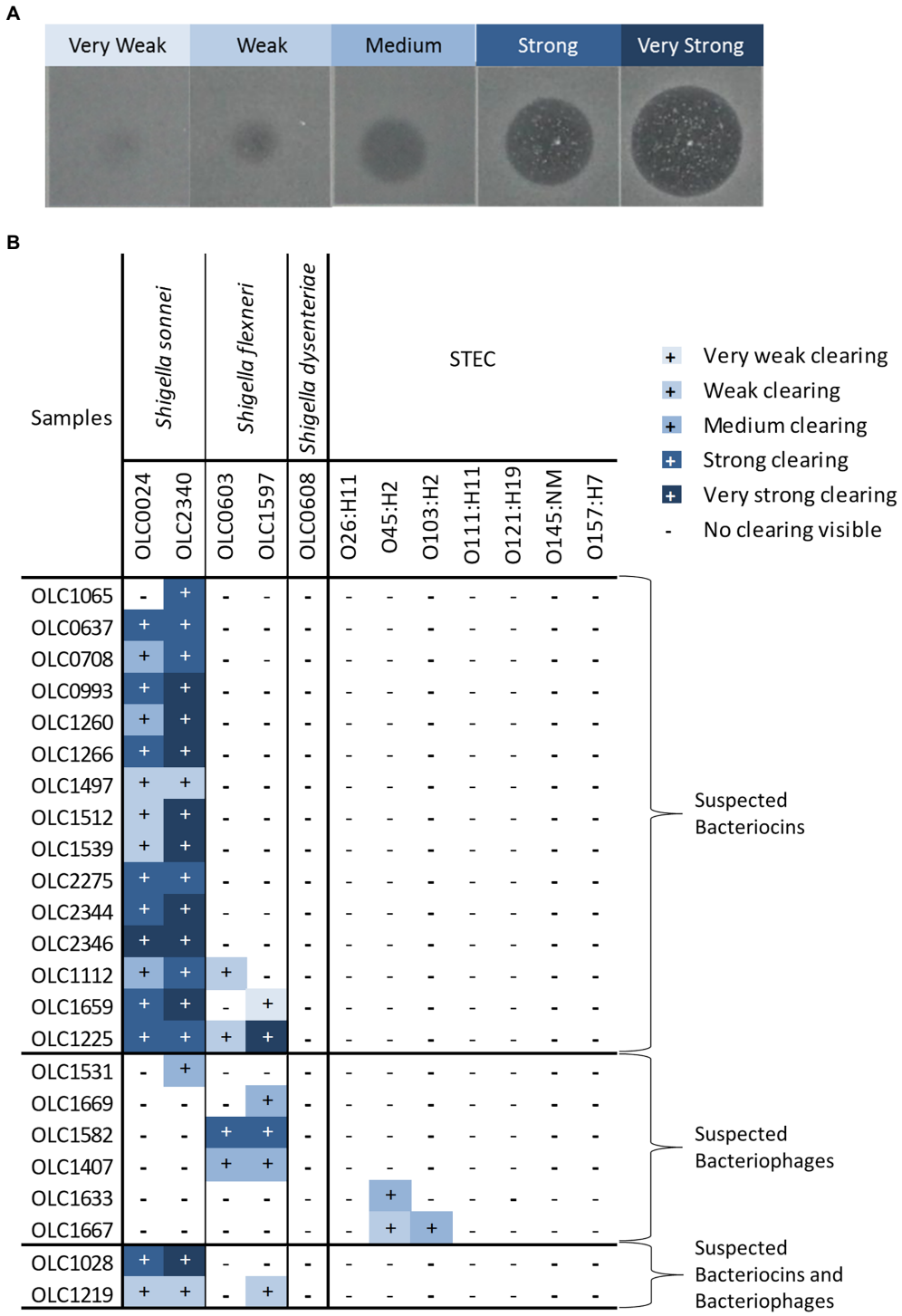


FIGURE 3 | Strengths of inhibitory activity of bacterial strains. **(A)** Representative image of the different strengths of the inhibitory activity observed in the strains used in this study. Level of activity (e.g., very weak to very strong) was classified based on diameter of the zone of clearing and opacity of the spot. **(B)** Relative strength of inhibitory activity of cell-free extracts from bacterial strains on *Shigella* spp. and STEC is indicated. The strains are grouped based on predicted inhibitor present in the sample (bacteriocins, bacteriophage, or both).

the zone of inhibition and the opacity of the clearing (**Figure 3A**). Strength of inhibition varied among the extracts and among indicator organisms tested (**Figure 5**; **Supplementary Table S3**). Inhibitory activity against *S. sonnei* tended to be strong, whereas inhibitory activity against *S. flexneri* and STEC was weaker (**Figure 5**). *Shigella* inhibitors were found in two (1.7%) of

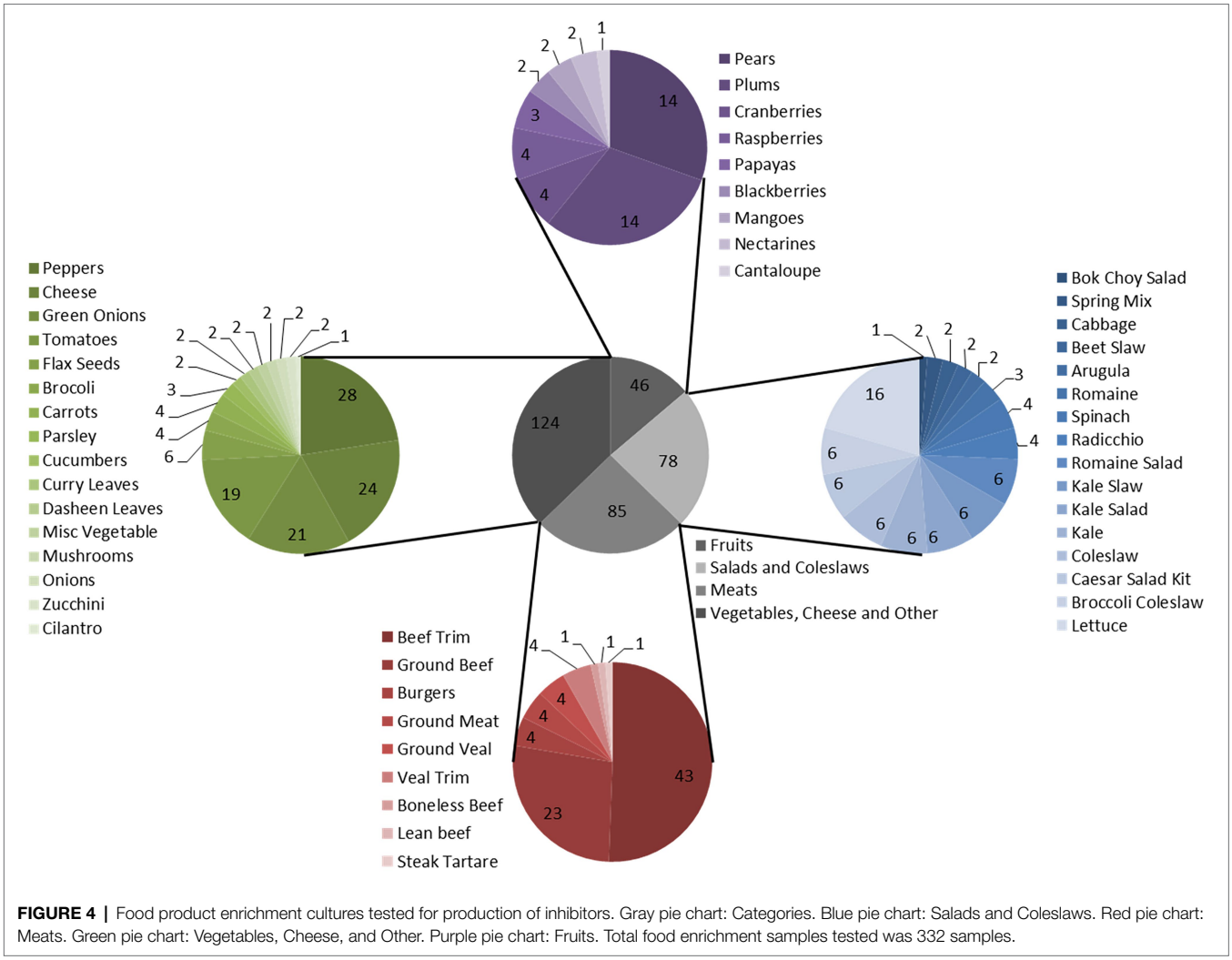


FIGURE 4 | Food product enrichment cultures tested for production of inhibitors. Gray pie chart: Categories. Blue pie chart: Salads and Coleslaws. Red pie chart: Meats. Green pie chart: Vegetables, Cheese, and Other. Purple pie chart: Fruits. Total food enrichment samples tested was 332 samples.

the 120 SB extracts (curry leaves and broccoli slaw) and STEC inhibitors were present in seven (3.2%) of 213 mTSB extracts tested. In contrast, mTSB extracts examined in this study were more likely to contain *Shigella* spp. inhibitors (8.9%), whereas none of the SB extracts contained STEC inhibitors. All the food enrichments had relatively neutral pH values between 6.0 and 7.5.

Cell-free extracts derived from the 85 meat mTSB extracts were most likely to contain inhibitors to either *Shigella* and/or STEC (19 extracts, 22% of meat enrichments; **Figure 6**; **Supplementary Table S2**). These extracts inhibited growth of *Shigella* spp. (14 extracts), STEC (two extracts) or both (three extracts). Two of the 23 cell-free extracts derived from cheese mTSB enrichments inhibited growth of either *Shigella* spp. or STEC. In contrast, only four of the 224 extracts derived from plant products (flax seeds, fruit, and vegetables) enriched in mTSB ($n = 104$) or SB ($n = 120$) contained inhibitors. In the 97 samples where plant products were enriched in both mTSB and SB, inhibition was only observed in one of the broths. For two of these samples (curry leaves and broccoli slaw), inhibitory compounds were detected in the SB extracts, but not the mTSB extracts. For two samples (both leafy greens) inhibitory compounds were detected the mTSB extracts, but not in the SB extracts.

Characterization of Inhibitory Properties of Cell-Free Extracts

All the cell-free extracts causing inhibition in at least one of the indicator *Shigella* or STEC strains were treated with proteolytic enzymes (proteinase-K and trypsin) to determine if inhibition was eliminated by the removal of the protein components of the extracts indicating that inhibitor was likely to be a bacteriocin (**Figure 7A**). The inhibitory activity of the cell-free extracts from most of the food strains [17 (74%)] and the food enrichments [15 (60%)] were affected by at least one proteolytic enzyme (suspected bacteriocins in **Figures 3**, **5**). For the food enrichment broths, there were two samples that were indeterminate for all inhibited strains and one sample indeterminate for *S. flexneri* due to lack of inhibition in the untreated control. Inconclusive results were likely associated with prolonged storage of these extracts as inhibitory activity

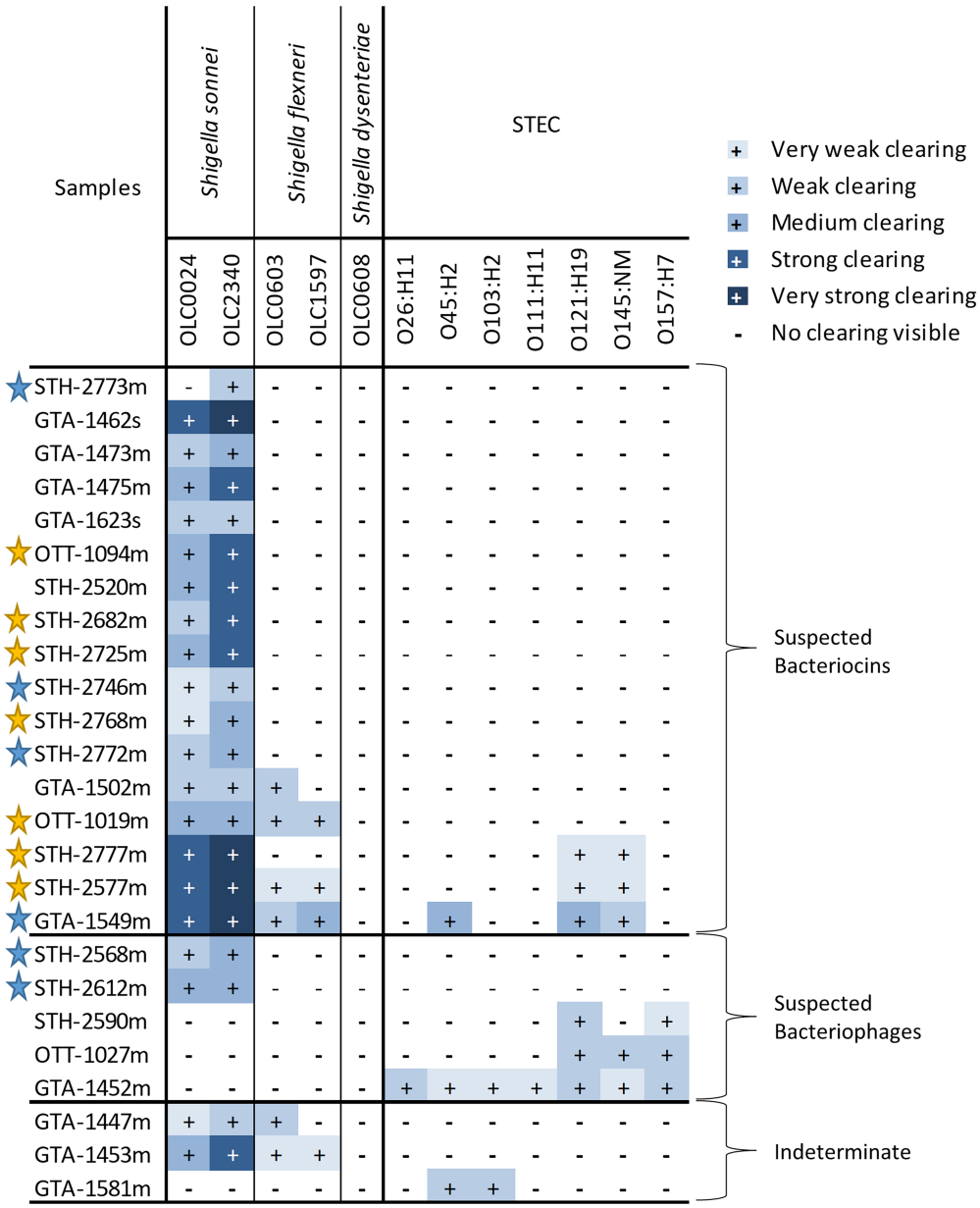


FIGURE 5 | Strengths of inhibitory activity of food enrichments on *Shigella* and STEC. Relative strength of inhibitory activity of extracts from mTSB ("m" in sample name) and SB enrichment ("s" in sample name) on *Shigella* spp. and STEC is indicated (see **Figure 3A** for representative images of different strengths of inhibitory activity). The enrichments are grouped based on predicted inhibitor present in the sample (bacteriocins, bacteriophage, or both). Samples were deemed to be indeterminate if observed inhibition of untreated samples was weak and impacts of proteolytic enzymes and dilutions could not be observed. Stars indicate samples for which attempts were made to recover isolates of the bacteria responsible for inhibition. Yellow stars indicate that an isolate producing an inhibitor was recovered, blue stars indicate attempts to recover isolates from samples were unsuccessful.

was found to generally decrease over time during storage at 4°C (data not shown). For the cell-free extract from OLC1219, the proteinase treatment reduced inhibition for *S. sonnei* strains but not for *S. flexneri* (**Figure 3**) indicating the presence of different inhibition mechanisms for these two species. Most of the cell-free extracts that inhibited *Shigella* (particularly *S. sonnei*) were affected by proteolytic enzymes (suspected bacteriocins in **Figures 3**, **5**). Cell-free extracts that inhibited STEC were generally not affected by proteolytic enzymes (**Figures 3**, **5**).

To identify bacteriophages, the inhibitory cell-free extracts from the food strains and food enrichments that were resistant to proteolytic digestion were also diluted to assess the presence of plaques (**Figure 7A**). Plaques were observed in dilutions derived from eight of the cell-free extracts from bacterial strains (suspected bacteriophage in **Figures 3**, **7B**). For strain OLC1219

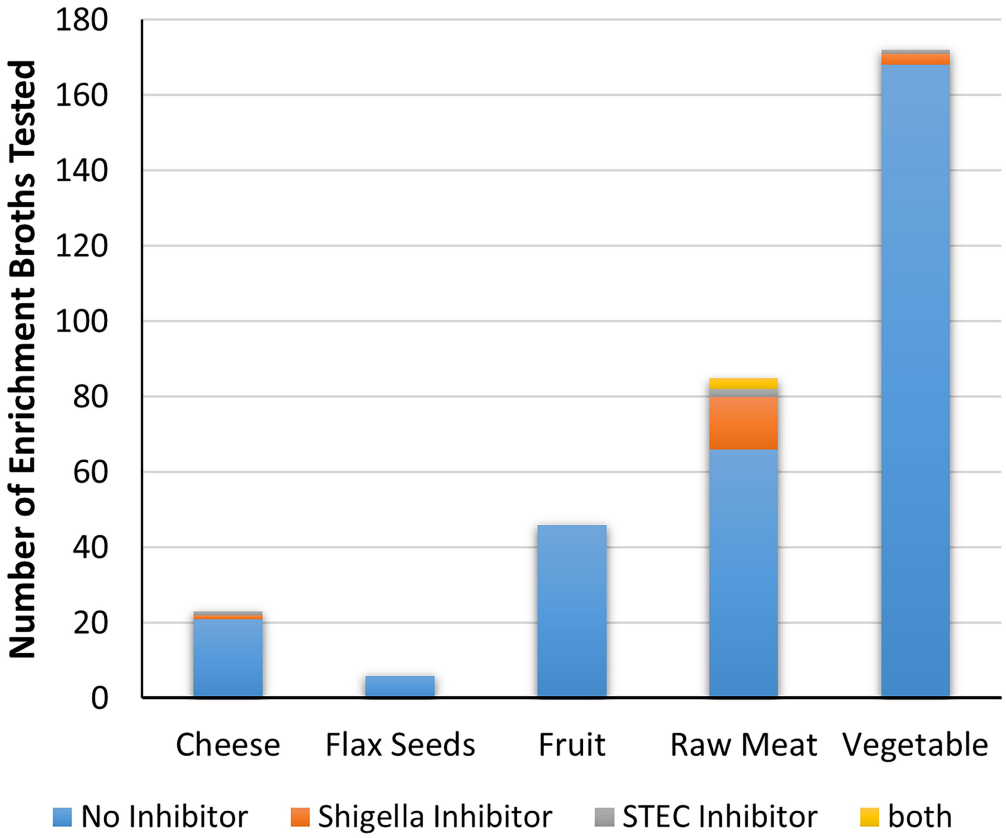


FIGURE 6 | Inhibitory activity of food enrichments on *Shigella* and STEC. The number of enrichment broths from each food category that did not produce inhibitors, or are inhibitory to either *Shigella*, STEC or both are indicated.

plaques were observed with *S. flexneri* but not *S. sonnei* indicating that inhibition of *S. flexneri* growth was likely due to bacteriophage, and inhibition against *S. sonnei* growth was likely due to production of bacteriocins (see above). Extracts from the OLC1028 strain were not completely affected by proteolytic enzymes and plaques were observed following treatment with proteolytic enzymes indicating production of both bacteriocin and bacteriophage inhibitors of *S. sonnei*. Plaques were observed in extracts from five of the food enrichment broths (bacteriophage in **Figure 5**, **7C**).

Recovery of Bacteria Producing Inhibitory Compounds From Food Enrichment Broths

Attempts were made to recover isolates of bacteria producing inhibitors from 13 of the mTSB enrichment broths producing inhibitory compounds (**Figure 5**, stars) using the triple overlay method (Henning et al., 2015). Inhibiting organisms were successfully recovered from seven of these samples (**Figure 5**, yellow stars and **Table 2**). All recovered isolates were determined to be *E. coli* based on whole-genome sequence analysis (data not shown). Cell-free extracts from isolates generally exhibited similar inhibitory properties to the cell-free extracts from enrichment cultures (**Table 2**; **Figure 5**, yellow stars). For example, all seven isolates inhibited growth of both strains of *S. sonnei*. Activity of the extracts from six of the *E. coli* isolates was affected by proteolytic enzymes indicating that inhibition was likely due to the production of bacteriocins (**Table 2**). The isolate recovered from STH-2768 m produced an inhibitor that affected both *S. sonnei* and *S. flexneri*, and was determined to produce bacteriophage as the inhibition was not impacted by proteolytic enzymes.

DISCUSSION

Recovery of the *Shigella* spp. and STEC from foods is important to support food safety investigations and to ensure the timely recall of implicated foods. Yet, this can be extremely challenging, in part due to problems culturing pathogens to detectable levels relative to non-target organisms. Studies conducted using current microbiological methods may underestimate the prevalence of *Shigella* spp. as this organism is easily outcompeted by other Enterobacteriaceae (Fishbein et al., 1971; Pollock and Dahlgren, 1974; Uyttendaele et al., 2001). Similarly, detection of STEC using existing methodology can be difficult due to lack of selective enrichment media, and competition with non-target organisms during food enrichment culture (Duffy et al., 1999; Catarame et al., 2003; Vimont et al., 2006;

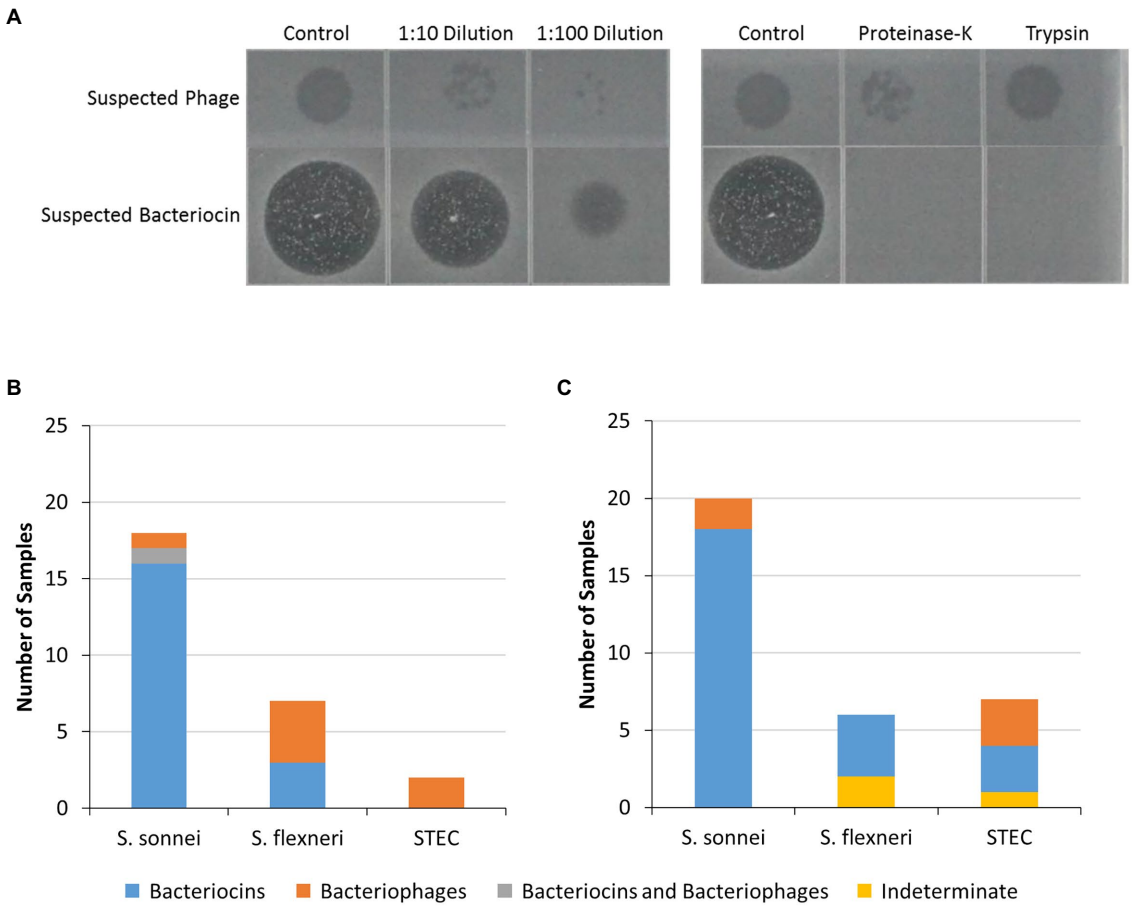


FIGURE 7 | Characterization of inhibitors in isolate and food-enrichment cell-free extracts. Cell-free extracts from food strains and enrichments which inhibited growth of at least one of the indicator strains tested were further evaluated to characterize inhibitors present. **(A)** The cell-free extracts were treated with two proteolytic enzymes (proteinase-K or trypsin) or were diluted (1:10 and 1:100) then spotted on soft agar containing indicator strains. Loss of activity following protein digestion indicated that growth inhibition was likely due to presence of bacteriocins (suspected bacteriocins bottom row), and resilience to protein digestion along with presence of plaques in diluted extracts indicated bacteriophage (suspected bacteriophage, top row). **(B,C)** Inhibitory activity of bacterial isolate extracts **(B)** or food enrichment extracts **(C)** against indicator organisms. Predicted bacteriocins are indicated by blue bars, bacteriophages (orange bars) or both (gray bars). Indeterminate samples could not be assessed due to weak zones of clearing (yellow bars).

TABLE 2 | Inhibitor-producing organisms recovered from food enrichment cultures.

Strain	Enrichment culture	Serotype	Activity	Species inhibited
OLC3028	STH-2577	O174:H8	Bacteriocin	*Shigella sonnei*
OLC3029	STH-2682	O23:H9	Bacteriocin	*Shigella sonnei*
OLC3030	STH-2725	O123:H16	Bacteriocin	*Shigella sonnei*
OLC3032	STH-2768	O105:H7	Bacteriophage	*Shigella sonnei*
				Shigella flexneri
OLC3035	STH-2777	O153/ O178:H11	Bacteriocin	*Shigella sonnei*
OLC3094	OTT-1094	O81:H7	Bacteriocin	*Shigella sonnei*
OLC3095	OTT-1019	O51:H10	Bacteriocin	*Shigella sonnei*

Knowles et al., 2016; Verhaegen et al., 2016; Blais et al., 2019). Very few studies have examined the impact of antimicrobial compounds produced by food microbiota on pathogen detection. This study was performed to determine whether antimicrobial compounds impacting STEC, or *Shigella* spp. were commonly produced by the microbiota associated with various foods.

Inhibition of Target Foodborne STEC and *Shigella* spp. by Food Microbiota

A collection of 200 food-associated bacterial strains (**Supplementary Table S1**) was tested for the production of inhibitory activity against a panel of seven STEC and five *Shigella* strains. The panel of indicator strains was selected to represent clinically important species and strains of pathogens that are targeted in food testing programs. The Gram-negative foodborne bacteria included in this study would typically be highly represented in food-enrichment cultures aimed at recovery of *Shigella*/STEC. Most methods integrate antibiotics such as novobiocin to reduce growth of Gram-positive bacteria, but generally do not include selective agents that reduce growth of Gram-negative bacteria (Vimont et al., 2007). Only two of

the 36 *Enterobacter* strains tested inhibited growth of STEC (**Figure 3B**; OLC1633 and OLC1667). In previous investigations organisms such as *Clostridium* spp., *E. coli*, *Hafnia alvei*, *Brochothrix thermosphacta*, and *Pediococcus acidilactici* present in meats were shown to outcompete STEC; however, the mechanism for this was not identified (Duffy et al., 1999; Kang et al., 2021). Similarly, Paquette et al. (2018) identified *E. coli* strains capable of inhibiting STEC due to production of diffusible antimicrobial compounds (Paquette et al., 2018). While *E. coli* with this activity was not found in the present study, analysis of a larger number of strains or food samples may have led to the identification of strains with similar properties. In contrast, extracts from 20 of the 118 *E. coli* strains tested were found to inhibit growth of *Shigella* spp., particularly *S. sonnei*, indicating that this species is highly susceptible to antimicrobial compounds produced by food microbiota. The inhibition of *Shigella* spp. by antibiotics produced by *E. coli* recovered from human sources was first described over 70 years ago (Halbert, 1948; Halbert and Gravatt, 1949), and *S. sonnei* strains have been used as indicator organisms for bacteriocins detection due to known susceptibility to a variety of colicins (Šmarda and Obdržálek, 2001; Micenková et al., 2014). It is interesting to note that STEC strains were more likely to produce inhibitors impacting growth of *Shigella* spp. compared to non-pathogenic strains. The association of bacteriocins and virulence factors in *E. coli* has been previously reported (Bradley et al., 1991; Micenková et al., 2014). Both of these traits may provide a competitive advantage in certain environments.

Analysis of cell-free extracts derived from a variety of food enrichments was done to evaluate the production of inhibitors by more complex communities of representative food microbiota. While *Shigella* broth would not be used for detection of STEC, and mTSB would not be used for detection of *Shigella*, similar analyses conducted with both types of enrichment broths enabled comparison of inhibitor production by organisms growing in the two media. Inhibition of at least one of the 12 indicator organisms tested was observed for 7.5% of the enrichment cultures tested, making it a relatively common occurrence overall. Inhibitors were more commonly associated with raw meat products enriched in mTSB (22% of raw meat enrichments) relative to plant products (1.8%). Similarly, a recent study found pathogen-killing bacteriophage to be more prevalent in raw beef and chicken than in vegetables and seafood (Premarathne et al., 2017). The relatively high prevalence of *Shigella* inhibition in the mTSB enrichments may be due to the species of bacteria present in the food matrix rather than differences in the enrichment broths. For example, *E. coli* is known to be associated with animal products (Barco et al., 2015). *Shigella*-inhibiting *E. coli* has long been known to be associated with human clinical samples (Halbert, 1948; Halbert and Gravatt, 1949; Levine and Tanimoto, 1954), but to confirm animal association, samples of animal fecal matter should be further examined. The association of inhibition to mTSB is partly due to the fact that meat and cheese products were not enriched in *Shigella* broth in this study. Paired enrichments of raw meat products in both broths could be evaluated to confirm the association of inhibitor-production with raw meats rather than enrichment broths. In the 97 plant products enriched with both broths, no association of the inhibiting organisms to the enrichment broths was observed; however, only four of the 224 broths examined produced inhibitors.

Similar to what was observed with the bacterial strains, *S. sonnei* was the indicator species that was the most sensitive to inhibition (21 out of 25 extracts), followed by *S. flexneri* and STEC (**Figure 5**). The observation of similar trends between the bacterial strains and enrichments indicates that the bacterial strains used in this study are representative of the bacteria in food enrichments. Most enrichments affected only one species (e.g., *S. sonnei* or STEC for 19 of 25 extracts) however there were some samples that showed a broader activity. For instance, cell-free extracts from one enrichment culture inhibited all seven STEC strains (GTA-1452, **Figure 5**). There were also three enrichment broths that inhibited both STEC and *Shigella* strains (GTA-1549, STH-2577 and STH-2777). This is likely indicative of an inhibitor that has a larger spectrum of activity, or potentially more than one strain with this activity in the samples (see below). Bacteriocins and bacteriophages can have broad or narrow spectrums of activity (Penadés et al., 2015; Simons et al., 2020). Note that the approach used to evaluate cell-free extracts was only semi-quantitative, as interpretation could be somewhat subjective, particularly for "weak" and "very weak" spots. Size of spots was also influenced by length of storage, with reduced activity over time (data not shown). Nonetheless, general trends could be determined with the screening approach used in this study.

Recovery of inhibiting organisms from enrichment cultures was challenging. Out of 13 enrichment cultures, seven *E. coli* isolates were recovered from cultures derived from raw meat products using a triple agar overlay method (Henning et al., 2015; **Table 2**). Recovery of inhibitor-producing *E. coli* is consistent with observations for the food-associated bacterial strains, and further supports the finding that *E. coli* may be an important source of inhibition, particularly for growth of *S. sonnei*. Results may be biased by the use of only one of the indicator strains (*S. sonnei*, OLC2340) for the recovery of inhibitory isolates, which were all derived from meat enrichments. *E. coli* is an indicator of fecal matter contamination which could occur during the slaughter process, potentially explaining the association of these organisms with raw meat products (Tallon et al., 2005; Odonkor and Ampofo, 2013; Barco et al., 2015). Failure to recover isolates in six samples may be because the method required recovery of a bacterial strain producing the inhibition. This approach would not work for the recovery of bacteriophage that may have been present in the foods, but not associated with a bacterial host. Cell-free extracts from the isolated *E. coli* strains had similar inhibitory properties to the extracts from the food samples from which they were isolated (**Table 2**; **Supplementary Table S3**), particularly to *S. sonnei*, which was the organism used for the recovery of these isolates. However, differences in activity against other indicator strains were observed. For example, OLC3032 was active against *S. flexneri*, unlike in the original enrichment culture where only *S. sonnei* was

affected and conversely OLC3028 did not inhibit growth of *S. flexneri*, unlike the original food enrichment culture. This indicates that multiple strains may have been responsible for inhibitory activity in the more complex enrichment cultures. Use of multiple indicator strains, and characterization of additional bacterial isolates may have led to recovery of additional strains.

S. sonnei and *S. flexneri* were included as indicator organisms in this study as they are the *Shigella* species most commonly associated with outbreaks in North America (Thompson et al., 2015; Centers for Disease Control and Prevention (CDC), 2016; The et al., 2016). *Shigella sonnei* was most commonly affected by antimicrobial compounds, followed by *S. flexneri* (**Figures 3**, **5**). Previous studies have that shown that *S. flexneri* was more highly affected by organic acids (e.g., by-products from food and food microbiota) than *S. sonnei* (Hentges, 1969; Uyttendaele et al., 2001; Zhang et al., 2011). *S. dysenteriae* was not affected by any of the cell-free extracts, so it is possible this species has a tolerance or resistance to inhibitors such as bacteriocins due to differences in targeted receptors in this species (Alonso et al., 2000). The acidity of the cultures was not likely to be an important factor in the observed growth inhibition. The pH range of *Shigella* growth is between 4.8–9.3 and the range of STEC growth is 4.0–10.0 (Food and Drug Administration, 2011). The pH of the bacterial isolate cell-free extracts (6.5–7.5) and food enrichment extracts (6.0–7.5) was well within these ranges.

Foodborne *Shigella* outbreaks are often associated with plant products (Kozak et al., 2013; Nygren et al., 2013). While enrichment broths derived from plant products were less likely to contain bacteriocins than those derived from meats in this study, this does not mean that recovery of *Shigella* spp. from plant products would not be problematic. *Shigella* contamination of foods is exclusively from human sources, and such contamination would likely also include *E. coli*. Given that human isolates of *E. coli* commonly inhibit *Shigella* spp. (Halbert, 1948; Levine and Tanimoto, 1954), the combination of organisms could impact recovering *Shigella* from the implicated foods. In fact, in a foodborne outbreak associated with baby corn in 2007, Shigella was not recovered from implicated samples despite strong epidemiological evidence, however, samples had high levels of *E. coli* (100–350 cfu/g) indicating potential issues with food hygiene (Lewis et al., 2009).

Identification of Predicted Bacteriocins and Bacteriophage in Cell-Free Extracts

To gain a better understanding of the mechanism of the inhibition, cell-free extracts were digested with proteolytic enzymes to determine if the inhibitor was impacted, as would be expected for bacteriocins which are proteinaceous compounds (Elayaraja et al., 2014), and extracts were diluted to identify the plaques that would be attributed to bacteriophages (Hockett and Baltrus, 2017; **Figure 7**). Most of the inhibitors, particularly those affecting *S. sonnei*, were affected by proteolytic enzymes and are likely to be bacteriocins (**Figures 3**, **5**, **7**). Notably, there were two *E. coli* strains (OLC1028 and OLC1219) that were predicted to produce both bacteriocins and bacteriophages with antimicrobial activity against *Shigella* spp. (**Figure 3**). In contrast, STEC inhibition was more commonly associated with bacteriophages (**Figures 3**, **5**). The *Enterobacter* strains that inhibited growth of the O45 and O103 were both predicted to produce bacteriophages (**Figure 3**). Similarly, the broad spectrum inhibition of STEC observed for sample GTA-1452 was also determined to be associated with bacteriophages. This inhibition could be due to the presence of a bacteriophage that has a wide spectrum of activity, or multiple bacteriophages targeting a few strains (Penadés et al., 2015; Simons et al., 2020). For example, STEC-killing phage can have broad or narrow host ranges (Litt et al., 2018; Mangieri et al., 2020). Interestingly, the inhibition produced by the isolate recovered from the STH-2768 m (OLC3032) was determined to be due to bacteriophages (**Table 2**) rather than the bacteriocins observed in the original culture (**Supplementary Table S3**), indicating that there was likely at least one bacteriocin-producing organism in the original culture that was not recovered. The identification of multiple inhibiting organisms within a single enrichment culture is further evidence that these strains may be an important factor in the success of competitive enrichment for food microbiology methods.

Bacteriophages active against STEC have been isolated from numerous zoonotic sources and may be relatively common indicating potential importance for enrichment cultures (Litt et al., 2018; Mangieri et al., 2020; Pinto et al., 2021). Presence of bacteriophages active against target species in enrichment cultures has been shown to reduce relative proportions of target *Salmonella* and *S. sonnei* by over a log indicating their critical impact on food microbiological methods (Muniesa et al., 2005). Bacteriocins would be expected to have similar inhibitory activity in food enrichment cultures. The ability to produce bacteriocins impacting closely related species is a common feature of *E. coli* enabling their survival in competitive environments (Micenková et al., 2016; Cameron et al., 2019). *E. coli*, particularly human isolates, frequently produce colicins, some of which have been shown to specifically target *Shigella* spp. (Halbert and Gravatt, 1949; Levine and Tanimoto, 1954). Only two colicins (colicin Z and J_s) that specifically target Enteroinvasive *E. coli* (EIEC) and *Shigella* species have been characterized (Micenková et al., 2019). Further analysis of the *E. coli* recovered in this study will be conducted to determine if the strains recovered encode related colicins.

In this study, *S. sonnei* were more susceptible to bacteriocins than *S. flexneri* and STEC. Due to practical considerations, the number of indicator strains used in this study was necessarily limited. It is possible that some of the indicator organisms used had higher resilience to phage and bacteriocins due to biological factors such as presence of immunity proteins, or other defense mechanisms (Rendueles et al., 2022). Use of a larger panel of *Shigella* strains, particularly strains associated with food outbreaks, will be necessary to establish whether *S. sonnei* strains are more susceptible than others. An understanding of the impact of bacteriocins on enrichment dynamics could lead to approaches to mitigating their impact. For example, fermentable sugars have been shown to reduce colicin production by *S. sonnei* (Lavoie et al., 1973). Future

studies will target enrichment dynamics associated with bacteriocins in food matrices.

While outside of the scope of the current study, there is currently significant interest in alternatives to antibiotics for the prevention of food contamination with these *Shigella* spp. and STEC, and for the development of therapeutics (Yang et al., 2014; Mangieri et al., 2020; Telhig et al., 2020; Jiang et al., 2022). The bacteria recovered in this study are highly active against pathogenic strains of *Shigella* spp. and STEC, with potential for future use in the production of alternatives to antibiotics.

CONCLUSION

Isolation of a foodborne bacterial pathogen is an important component of a food safety investigation that is sometimes difficult to achieve. This study provides evidence that production of antimicrobial compounds such as bacteriocins and bacteriophages by food microbiota is a relatively common occurrence which could impede growth of *Shigella* and STEC in enrichment cultures, reducing effectiveness of microbiological methods aimed at recovering these organisms. Presence of bacteriocin-producing *E. coli* may be an important challenge for the recovery of *Shigella* spp. and presence of bacteriophage may be of greater concern for STEC. Development of new methods should mitigate the potential interference due to inhibitor production by food microbiota. Organisms recovered in this study could be included as interfering organisms in future method validations, leading to a more rigorous assessment of methods aimed at recovery of *Shigella* and STEC from foods.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, and further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CK originally identified bacteriocins as an important inhibitor for *Shigella* spp. TM, CC, AW, and BB conceived and designed the experiments and contributed to writing of the manuscript. TM performed laboratory experiments. TM and CC analyzed the data and wrote the first draft of the manuscript. TM, CC, AM, KS, and BB contributed reagents, materials, and analysis tools. All authors contributed to the article and approved the submitted version.

FUNDING

This study has received funding from the Canadian Food Inspection Agency and the Government of Canada interdepartmental Genomic Research Development Initiative (GRDI).

ACKNOWLEDGMENTS

The authors gratefully acknowledge staff at the CFIA food microbiology laboratories in Ottawa (Austin Markell), Scarborough (Katherine Han), and Saint-Hyacinthe (José Riva, Julie Plamondon, Lyne Laflamme) for providing food enrichment cultures. The authors also appreciate technical assistance from Bridgette Kelly, Susan Van Zanten, Hilda Hoo, Paul Manninger, Martine Dixon, and Mylène Deschênes. The authors would also like to thank Sam Mohajer, Chloe Anastasiadis and Lang Yao for critical review of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.880043/full#supplementary-material

REFERENCES

Adam, P. R., and Pickings, W. D. (2016). "*Shigella* and shigellosis," in *Shigella: Molecular and Cell Biology*. eds. W. D. Pickings and W. L. Pickings (UK: Caister Academic Press), 7–26.

Alonso, G., Vílchez, G., and Rodríguez Lemoine, V. (2000). How bacteria protect themselves against channel-forming colicins. *Int. Microbiol.* 3, 81–88.

Arici, M., Bilgin, B., Sagdic, O., and Ozdemir, C. (2004). Some characteristics of *Lactobacillus* isolates from infant faeces. *Food Microbiol.* 21, 19–24. doi: 10.1016/S0740-0020(03)00044-3

Barco, L., Belluco, S., Roccato, A., and Ricci, A. (2015). A systematic review of studies on *Escherichia coli* and Enterobacteriaceae on beef carcasses at the slaughterhouse. *Int. J. Food Microbiol.* 207, 30–39. doi: 10.1016/j.ijfoodmicro.2015.04.027

Bin Kingombe, C., Cerqueira-Campos, M. L., Trottier, Y. L., and Houle, J. (2006). "Isolation and identification of *Shigella* (VTEC) spp. from foods (MFLP-25)," in *Compendium of Analytical Methods* (Ottawa, Canada: Health Canada).

Blais, B., Martinez, A., Gill, A., McIlwham, S., Mohajer, S., Gauthier, M., et al. (2014). "Isolation and identification of priority verotoxigenic *Escherichia coli* (VTEC) in meat and vegetable products (MFLP-52)," in *Laboratory Procedures of Microbiological Analysis of Foods* (Ottawa, Canada: Health Canada (Compendium of Analytical Methods)).

Blais, B. W., Tapp, K., Dixon, M., and Carrillo, C. D. (2019). Genomically informed strain-specific recovery of Shiga toxin-producing *Escherichia coli* during foodborne illness outbreak investigations. *J. Food Prot.* 82, 39–44. doi: 10.4315/0362-028X.JFP-18-340

Bradley, D. E., Howard, S. P., and Lior, H. (1991). Colicinogeny of O157:H7 enterohemorrhagic *Escherichia coli* and the shielding of colicin and phage receptors by their O-antigenic side chains. *Can. J. Microbiol.* 37, 97–104. doi: 10.1139/m91-014

Cameron, A., Zaheer, R., Adator, E. H., Barbieri, R., Reuter, T., and McAllister, T. A. (2019). Bacteriocin occurrence and activity in *Escherichia coli* isolated from bovines and wastewater. *Toxins* 11:475. doi: 10.3390/toxins11080475

Catarame, T. M. G., O'Hanlon, K. A., Duffy, G., Sheridan, J. J., Blair, I. S., McDowell, D. A., et al. (2003). Optimization of enrichment and plating procedures for the recovery of *Escherichia coli* O111 and O26 from minced beef. *J. Appl. Microbiol.* 95, 949–957. doi: 10.1046/j.1365-2672.2003.02065.x

Centers for Disease Control and Prevention (CDC) (2016) *National Shigella Surveillance Annual Report, 2013*. Atlanta, Georgia: US Department of Health and Human Services, CDC.

Challinor, V. L., and Bode, H. B. (2015). Bioactive natural products from novel microbial sources. *Ann. N. Y. Acad. Sci.* 1354, 82–97. doi: 10.1111/nyas.12954

Donnenberg, M. S., and Whittam, T. S. (2001). Pathogenesis and evolution of virulence in enteropathogenic and enterohemorrhagic *Escherichia coli*. *J. Clin. Investig.* 107, 539–548. doi: 10.1172/JCI12404

Duffy, G., Whiting, R. C., and Sheridan, J. J. (1999). The effect of a competitive microflora, pH and temperature on the growth kinetics of *Escherichia coli* O157:H7. *Food Microbiol.* 16, 299–307. doi: 10.1006/fmic.1998.0242

Duquesne, S., Destoumieux-Garzón, D., Peduzzi, J., and Rebuffat, S. (2007). Microcins, gene-encoded antibacterial peptides from enterobacteria. *Nat. Prod. Rep.* 24, 708–734. doi: 10.1039/B516237H

EFSA (2013). Scientific opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment. *EFSA J.* 11:3138. doi: 10.2903/j.efsa.2013.3138

Elayaraja, S., Annamalai, N., Mayavu, P., and Balasubramanian, T. (2014). Production, purification and characterization of bacteriocin from *Lactobacillus murinus* AU06 and its broad antibacterial spectrum. *Asian Pac. J. Trop. Biomed.* 4(Suppl 1), S305–S311. doi: 10.12980/APJTB.4.2014C537

Fishbein, M., Mehlman, I. J., and Wentz, B. (1971). Isolation of Shigella from foods. *J. Assoc. Off. Anal. Chem.* 54, 109–111. doi: 10.1093/jaoac/54.1.109

Food and Drug Administration (2011). "Appendix 4: Bacterial Pathogen Growth and Inactivation," in *Fish and Fishery Products Hazards and Controls Guidance. 4th Edn.*

Gould, L. H., Mody, R. K., Ong, K. L., Clogher, P., Cronquist, A. B., Garman, K. N., et al. (2013). Increased recognition of non-O157 Shiga toxin–producing *Escherichia coli* infections in the United States during 2000–2010: epidemiologic features and comparison with *E. coli* O157 infections. *Foodborne Pathog. Dis.* 10, 453–460. doi: 10.1089/fpd.2012.1401

Halbert, S. P. (1948). The antagonism of coliform bacteria against shigellae. *J. Immunol.* 58, 153–167. PMID: 18905191

Halbert, S. P., and Gravatt, M. (1949). The relation of *Shigella* type-specificity and susceptibility to antibiotic-producing strains of *Escherichia coli*. *J. Immunol.* 61, 271–282. PMID: 18117864

Heier, B. T., Nygård, K., Kapperud, G., Lindstedt, B. A., Johannessen, G. S., and Blekkan, H. (2009). *Shigella sonnei* infections in Norway associated with sugar peas, May–June 2009. *Eur. Secur.* 14:19243. doi: 10.2807/ese.14.24.19243-en

Henning, C., Vijayakumar, P., Adhikari, R., Jagannathan, B., Gautam, D., and Muriana, P. (2015). Isolation and taxonomic identity of bacteriocin-producing lactic acid bacteria from retail foods and animal sources. *Microorganisms* 3, 80–93. doi: 10.3390/microorganisms3010080

Hentges, D. J. (1969). Inhibition of *Shigella flexneri* by the normal intestinal flora II. Mechanisms of inhibition by coliform organisms. *J. Bacteriol.* 97, 513–517. doi: 10.1128/jb.97.2.513-517.1969

Hockett, K. L., and Baltrus, D. A. (2017). Use of the soft-agar overlay technique to screen for bacterially produced inhibitory compounds. *J Vis Exp.* 119. doi: 10.3791/55064

ISO (2012) ISO/TS 13136:2012, Microbiology of food and animal feed -- Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups. International Organization for Standardization. Available at: http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=53328 (Accessed: 24 July 2016).

Jarvis, K. G., White, J. R., Grim, C. J., Ewing, L., Ottesen, A. R., Beaubrun, J. J. G., et al. (2015). Cilantro microbiome before and after nonselective pre-enrichment for Salmonella using 16S rRNA and metagenomic sequencing. *BMC Microbiol.* 15:160. doi: 10.1186/s12866-015-0497-2

Jiang, Y.-H., Xin, W. G., Zhang, Q. L., Lin, L. B., and Deng, X. Y. (2022). A novel bacteriocin against *Shigella flexneri* from *Lactiplantibacillus plantarum* isolated From Tilapia intestine: purification, antibacterial properties and antibiofilm activity. *Front. Microbiol.* 12:779315. doi: 10.3389/fmicb.2021.779315

Kang, S., Ravensdale, J. T., Coorey, R., Dykes, G. A., and Barlow, R. S. (2021). Changes in STEC and bacterial communities during enrichment of manufacturing beef in selective and non-selective media. *Food Microbiol.* 96:103711. doi: 10.1016/j.fm.2020.103711

Kim, J.-S., Lee, M.-S., and Kim, J. H. (2020). Recent updates on outbreaks of Shiga toxin-producing *Escherichia coli* and its potential reservoirs. *Front. Cell Infect. Microbiol.* 10:273. doi: 10.3389/fcimb.2020.00273

Kim, Y. C., Tarr, A. W., and Penfold, C. N. (2014). Colicin import into *E. coli* cells: a model system for insights into the import mechanisms of bacteriocins. *Biochim. Biophys. Acta* 1843, 1717–1731. doi: 10.1016/j.bbamcr.2014.04.010

Knowles, M., Stinson, S., Lambert, D., Carrillo, C., Koziol, A., Gauthier, M., et al. (2016). Genomic tools for customized recovery and detection of foodborne Shiga toxigenic *Escherichia coli*. *J. Food Prot.* 79, 2066–2077. doi: 10.4315/0362-028X.JFP-16-220

Koskella, B., and Meaden, S. (2013). Understanding bacteriophage specificity in natural microbial communities. *Viruses* 5, 806–823. doi: 10.3390/v5030806

Kothary, M. H., and Babu, U. S. (2001). Infective dose of foodborne pathogens in volunteers: a review. *J. Food Saf.* 21, 49–68. doi: 10.1111/j.1745-4565.2001.tb00307.x

Kozak, G. K., MacDonald, D., Landry, L., and Farber, J. M. (2013). Foodborne outbreaks in Canada linked to produce: 2001 through 2009. *J. Food Prot.* 76, 173–183. doi: 10.4315/0362-028X.JFP-12-126

Lavoie, M., Mathieu, L. G., and Charron-Allie, L. (1973). Inhibition of colicin production by fermentable sugars. *Can. J. Microbiol.* 20, 269–272. doi: 10.1139/m74-044

Levine, M., and Tanimoto, R. H. (1954). Antagonisms among enteric pathogens and coliform bacteria. *J. Bacteriol.* 67, 537–541. doi: 10.1128/jb.67.5.537-541.1954

Lewis, H. C., Ethelberg, S., Olsen, K. E. P., Nielsen, E. M., Lisby, M., Madsen, S. B., et al. (2009). Outbreaks of *Shigella sonnei* infections in Denmark and Australia linked to consumption of imported raw baby corn. *Epidemiol. Infect.* 137, 326–334. doi: 10.1017/S0950268808001829

Litt, P. K., Saha, J., and Jaroni, D. (2018). Characterization of bacteriophages targeting non-O157 Shiga toxigenic *Escherichia coli*. *J. Food Prot.* 81, 785–794. doi: 10.4315/0362-028X.JFP-17-460

Mader, A., von Bronk, B., Ewald, B., Kesel, S., Schnetz, K., Frey, E., et al. (2015). Amount of colicin release in *Escherichia coli* is regulated by lysis gene expression of the colicin E2 operon. *PLoS One* 10:e0119124. doi: 10.1371/journal.pone.0119124

Mandal, S. M., Sharma, S., Pinnaka, A. K., Kumari, A., and Korpole, S. (2013). Isolation and characterization of diverse antimicrobial lipopeptides produced by Citrobacter and Enterobacter. *BMC Microbiol.* 13:152. doi: 10.1186/1471-2180-13-152

Mangieri, N., Picozzi, C., Cocuzzi, R., and Foschino, R. (2020). Evaluation of a potential bacteriophage cocktail for the control of Shiga-toxin producing *Escherichia coli* in food. *Front. Microbiol.* 11:1801. doi: 10.3389/fmicb.2020.01801

Margot, H., Tasara, T., Zwietering, M. H., Joosten, H., and Stephan, R. (2016). Effects of different media on the enrichment of low numbers of Shiga toxin-producing *Escherichia coli* in mung bean sprouts and on the development of the sprout microbiome. *Int. J. Food Microbiol.* 232, 26–34. doi: 10.1016/j.ijfoodmicro.2016.05.005

Marshall, K. E., Hexemer, A., Seelman, S. L., Fatica, M. K., Blessington, T., Hajmeer, M., et al. (2020). Lessons learned from a decade of investigations of Shiga toxin–producing *Escherichia coli* outbreaks linked to leafy greens, United States and Canada. *Emerg. Infect. Dis.* 26, 2319–2328. doi: 10.3201/eid2610.191418

Micenková, L., Bosák, J., Kucera, J., Hrala, M., Dolejšová, T., Šedo, O., et al. (2019). Colicin Z, a structurally and functionally novel colicin type that selectively kills enteroinvasive *Escherichia coli* and *Shigella* strains. *Sci. Rep.* 9:11127. doi: 10.1038/s41598-019-47488-8

Micenková, L., Bosák, J., Vrba, M., Ševčíková, A., and Šmajs, D. (2016). Human extraintestinal pathogenic *Escherichia coli* strains differ in prevalence of virulence factors, phylogroups, and bacteriocin determinants. *BMC Microbiol.* 16:218. doi: 10.1186/s12866-016-0835-z

Micenková, L., Štaudová, B., Bosák, J., Mikalová, L., Littnerová, S., Vrba, M., et al. (2014). Bacteriocin-encoding genes and ExPEC virulence determinants are associated in human fecal *Escherichia coli* strains. *BMC Microbiol.* 14:109. doi: 10.1186/1471-2180-14-109

Microbiological Methods Committee (2012) 'MFLP-30: Detection of *Escherichia coli* O157:H7 in select foods using the BAX® System *E. coli* O157:H7 MP', in *Compendium of Analytical Methods*. Ottawa: Health Canada.

Mikhail, A. F. W., Pereboom, M., Utsi, L., Hawker, J., Lighthill, J., Aird, H., et al. (2021). Utility of whole-genome sequencing during an investigation of multiple foodborne outbreaks of *Shigella sonnei*. *Epidemiol. Infect.* 149:e71. doi: 10.1017/S0950268821000509

Mohr, K. I. (2016). History of antibiotics research. *Curr. Top. Microbiol. Immunol.* 398, 237–272. doi: 10.1007/82_2016_499

Muniesa, M., Blanch, A. R., Lucena, F., and Jofre, J. (2005). Bacteriophages may bias outcome of bacterial enrichment cultures. *Appl. Environ. Microbiol.* 71, 4269–4275. doi: 10.1128/AEM.71.8.4269-4275.2005

Nygren, B. L., Schilling, K. A., Blanton, E. M., Silk, B. J., Cole, D. J., Mintz, E. D., et al. (2013). Foodborne outbreaks of shigellosis in the USA, 1998–2008. *Epidemiol. Infect.* 141, 233–241. doi: 10.1017/S0950268812000222

Odonkor, S. T., and Ampofo, J. K. (2013). *Escherichia coli* as an indicator of bacteriological quality of water: An overview. *Microbiol. Res.* 4:e2. doi: 10.4081/mr.2013.e2

Ottesen, A. R., Gonzalez, A., Bell, R., Arce, C., Rideout, S., Allard, M., et al. (2013). Co-enriching microflora associated with culture based methods to detect *Salmonella* from tomato Phyllosphere. *PLoS One* 8:e73079. doi: 10.1371/journal.pone.0073079

Ottesen, A., Ramachandran, P., Reed, E., White, J. R., Hasan, N., Subramanian, P., et al. (2016). Enrichment dynamics of *listeria monocytogenes* and the associated microbiome from naturally contaminated ice cream linked to a listeriosis outbreak. *BMC Microbiol.* 16:275. doi: 10.1186/s12866-016-0894-1

Paquette, S.-J., Zaheer, R., Stanford, K., Thomas, J., and Reuter, T. (2018). Competition among *Escherichia coli* strains for space and resources. *Vet. Sci.* 5:93. doi: 10.3390/vetsci5040093

Parasion, S., Kwiatek, M., Gryko, R., Mizak, L., and Malm, A. (2014). Bacteriophages as an alternative strategy for fighting biofilm development. *Pol. J. Microbiol.* 63, 137–145. doi: 10.33073/pjm-2014-019

Penadés, J. R., Chen, J., Quiles-Puchalt, N., Carpena, N., and Novick, R. P. (2015). Bacteriophage-mediated spread of bacterial virulence genes. *Curr. Opin. Microbiol.* 23, 171–178. doi: 10.1016/j.mib.2014.11.019

Perelle, S., Dilasser, F., Grout, J., and Fach, P. (2004). Detection by 5′-nuclease PCR of Shiga-toxin producing *Escherichia coli* O26, O55, O91, O103, O111, O113, O145 and O157:H7, associated with the world's most frequent clinical cases. *Mol. Cell. Probes* 18, 185–192. doi: 10.1016/j.mcp.2003.12.004

Pinto, G., Minnich, S. A., Hovde, C. J., Oliveira, H., Smidt, H., Almeida, C., et al. (2021). The interactions of bacteriophage ace and Shiga toxin-producing *Escherichia coli* during biocontrol. *FEMS Microbiol. Ecol.* 97:p. fiab105. doi: 10.1093/femsec/fiab105

Pollock, H. M., and Dahlgren, B. J. (1974). Clinical evaluation of enteric media in the primary isolation of *Salmonella* and *Shigella*. *Appl. Microbiol.* 27, 197–201. doi: 10.1128/am.27.1.197-201.1974

Premarathne, J. M. K. J. K., Thung, T. Y., New, C. Y., Huat, J. T. Y., Basri, D. F., Rukayadi, Y. et al. (2017). Distribution of bacteriophages in food and environment samples. *Int. Food Res. J.* 24, 888–896.

Rendueles, C., Duarte, A. C., Escobedo, S., Fernández, L., Rodríguez, A., García, P., et al. (2022). Combined use of bacteriocins and bacteriophages as food biopreservatives. A review. *Int. J. Food Microbiol.* 368:109611. doi: 10.1016/j.ijfoodmicro.2022.109611

Shahin, K., Bouzari, M., Wang, R., and Yazdi, M. (2019). Prevalence and molecular characterization of multidrug-resistant *Shigella* species of food origins and their inactivation by specific lytic bacteriophages. *Int. J. Food Microbiol.* 305:108252. doi: 10.1016/j.ijfoodmicro.2019.108252

Simons, A., Alhanout, K., and Duval, R. E. (2020). Bacteriocins, antimicrobial peptides from bacterial origin: overview of their biology and their impact against multidrug-resistant bacteria. *Microorganisms* 8:639. doi: 10.3390/microorganisms8050639

Šmarda, J., and Obdržálek, V. (2001). Incidence of colicinogenic strains among human *Escherichia coli*. *J. Basic Microbiol.* 41, 367–374. doi: 10.1002/1521-4028(200112)41:6<367::AID-JOBM367>3.0.CO;2-X

Smith, J. L., Fratamico, P. M., and Gunther, N. W. (2014). Shiga toxin-producing *Escherichia coli*. *Adv. Appl. Microbiol.* 86, 145–197. doi: 10.1016/B978-0-12-800262-9.00003-2

Tallon, P., Magajna, B., Lofranco, C., and Leung, K. T. (2005). Microbial indicators of faecal contamination in water: a current perspective. *Water Air Soil Pollut.* 166, 139–166. doi: 10.1007/s11270-005-7905-4

Telhig, S., Ben Said, L., Zirah, S., Fliss, I., and Rebuffat, S. (2020). Bacteriocins to thwart bacterial resistance in Gram negative bacteria. *Front. Microbiol.* 11:586433. doi: 10.3389/fmicb.2020.586433

The, H. C., Thanh, D. P., Holt, K. E., Thomson, N. R., and Baker, S. (2016). The genomic signatures of *Shigella* evolution, adaptation and geographical spread. *Nat. Rev. Microbiol.* 14, 235–250. doi: 10.1038/nrmicro.2016.10

Thomas, M. K., Murray, R., Flockhart, L., Pintar, K., Fazil, A., Nesbitt, A., et al. (2015). Estimates of foodborne illness-related hospitalizations and deaths in Canada for 30 specified pathogens and unspecified agents. *Foodborne Pathog. Dis.* 12, 820–827. doi: 10.1089/fpd.2015.1966

Thomas, M. K., Murray, R., Flockhart, L., Pintar, K., Pollari, F., Fazil, A., et al. (2013). Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, circa 2006. *Foodborne Pathog. Dis.* 10, 639–648. doi: 10.1089/fpd.2012.1389

Thompson, C. N., Duy, P. T., and Baker, S. (2015). The rising dominance of *Shigella sonnei*: An intercontinental shift in the etiology of bacillary dysentery. *PLoS Negl. Trop. Dis.* 9:e0003708. doi: 10.1371/journal.pntd.0003708

Thorpe, C. M. (2004). Shiga toxin-producing *Escherichia coli* infection. *Clin. Infect. Dis.* 38, 1298–1303. doi: 10.1086/383473

USDA-FSIS (2019) Detection, isolation and identification of top seven Shiga toxin-producing *Escherichia coli* (STECs) from meat products and carcass and environmental sponges. Athens GA: United States Department of Agriculture. Available at: https://www.fsis.usda.gov/wps/wcm/connect/fsis-content/internet/main/topics/science/laboratories-and-procedures/guidebooks-and-methods/microbiology-laboratory-guidebook/microbiology-laboratory-guidebook (Accessed: 18 April 2019).

Uyttendaele, M., Bagamboula, C. F., de Smet, E., van Wilder, S., and Debevere, J. (2001). Evaluation of culture media for enrichment and isolation of *Shigella sonnei* and *S. flexneri*. *Int. J. Food Microbiol.* 70, 255–265. doi: 10.1016/s0168-1605(01)00549-9

Verhaegen, B., van Damme, I., Heyndrickx, M., Botteldoorn, N., Elhadidy, M., Verstraete, K., et al. (2016). Evaluation of detection methods for non-O157 Shiga toxin-producing *Escherichia coli* from food. *Int. J. Food Microbiol.* 219, 64–70. doi: 10.1016/j.ijfoodmicro.2015.12.006

Vijayakumar, P. P., and Muriana, P. M. (2015). A microplate growth inhibition assay for screening bacteriocins against *Listeria monocytogenes* to differentiate their mode-of-action. *Biomol. Ther.* 5, 1178–1194. doi: 10.3390/biom5021178

Vimont, A., Delignette-Muller, M.-L., and Vernozy-Rozand, C. (2007). Supplementation of enrichment broths by novobiocin for detecting Shiga toxin-producing *Escherichia coli* from food: a controversial use. *Lett. Appl. Microbiol.* 44, 326–331. doi: 10.1111/j.1472-765X.2006.02059.x

Vimont, A., Vernozy-Rozand, C., Montet, M. P., Lazizzera, C., Bavai, C., and Delignette-Muller, M. L. (2006). Modeling and predicting the simultaneous growth of *Escherichia coli* O157:H7 and ground beef background microflora for various enrichment protocols. *Appl. Environ. Microbiol.* 72, 261–268. doi: 10.1128/AEM.72.1.261-268.2006

Warren, B. R., Parish, M. E., and Schneider, K. R. (2006). *Shigella* as a foodborne pathogen and current methods for detection in food. *Crit. Rev. Food Sci. Nutr.* 46, 551–567. doi: 10.1080/10408390500295458

Yang, S.-C., Lin, C. H., Sung, C. T., and Fang, J. Y. (2014). Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Front. Microbiol.* 5:241. doi: 10.3389/fmicb.2014.00241

Zhang, Y., Zhang, L., du, M., Yi, H., Guo, C., Tuo, Y., et al. (2011). Antimicrobial activity against *Shigella sonnei* and probiotic properties of wild lactobacilli from fermented food. *Microbiol. Res.* 167, 27–31. doi: 10.1016/j.micres.2011.02.006

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership