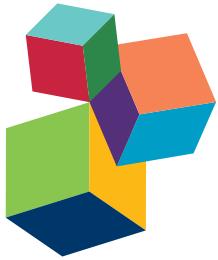


MICROBIAL FOOD SAFETY ALONG THE DAIRY CHAIN

EDITED BY: Edward M. Fox, Kieran Jordan, Séamus Fanning and Aldo Corsetti
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MICROBIAL FOOD SAFETY ALONG THE DAIRY CHAIN

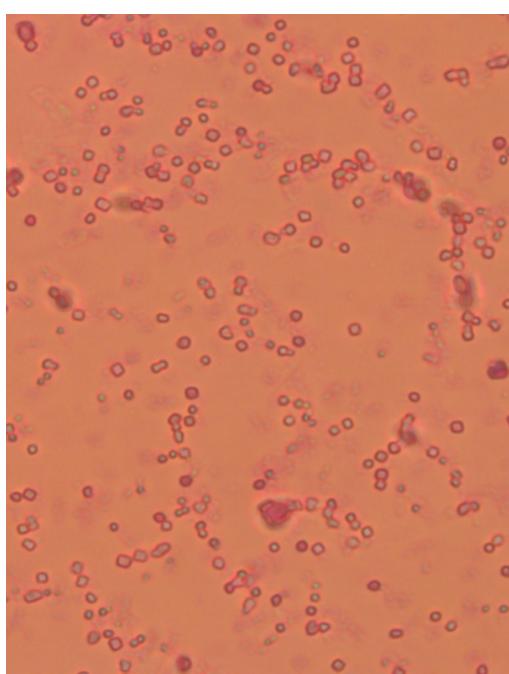
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Fuchsin-stained sporulated *Bacillus thuringiensis* isolated from a dairy farm, with characteristic bipyramidal toxin crystals. Image: Paul Drean of CSIRO, Australia.

Cover image: ggw/Shutterstock.com

The dairy chain is an integral part of global food supply, with dairy food products a staple component of recommended healthy diets. The dairy food chain from production through to the consumer is complex, with various opportunities for microbial contamination of ingredients or food product, and as such interventions are key to preventing or controlling such contamination. Dairy foods often include a microbial control step in their production such as

pasteurization, but in some cases may not, as with raw milk cheeses. Microbial contamination may lead to a deterioration in food quality due to spoilage organisms, or may become a health risk to consumers should the contaminant be a pathogenic microorganism. As such, food safety and food production are intrinsically linked.

This Research Topic eBook includes publications on issues relating to the microbiological integrity of the dairy food chain, such as the ecology of pathogenic and spoilage organisms through the dairy farm to fork paradigm, their significance to dairy foods and health, and genomic analysis of these microorganisms.

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Editorial: Microbial Food Safety along the Dairy Chain

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Editorial on the Research Topic

Microbial Food Safety along the Dairy Chain

Milk is susceptible to contamination with pathogenic and spoilage organisms and, therefore, **Microbial food safety along the dairy chain** is an important topic, from public health and industry perspectives. The dairy chain is an integral part of global food supply, with dairy food products a staple component of recommended healthy diets. The dairy food chain from production through to the consumer is complex, with various opportunities for microbial contamination of ingredients or food products, and as such interventions are key to preventing or controlling such contamination. Dairy foods often include a microbial control step in their production such as pasteurization, but in some cases may not, as with raw milk products. Microbial contamination may lead to a deterioration in food quality due to spoilage organisms, or may become a health risk to consumers should the contaminant be a pathogenic microorganism. As such food safety and food production are intrinsically linked.

This e-Book brings together a series of articles related to the microbiological integrity of the dairy food chain, with regard to pathogenic and spoilage organisms, genomic and other analyses of these contaminants, and alternative methods for their study.

The first two papers concern sporeforming bacteria, which are a concern both from safety and processing perspectives. The paper McHugh et al. the specifications for spores in milk powders can be low and so traditional detection methods used in industry to enumerate them have limitations in terms of time, efficiency, accuracy, and sensitivity. The review provides an insight into recent advances in methodology for detection of spores, highlighting the advantages and limitations with respect to the application of such methodologies for dairy food. Optimisation and application of these methods can ensure safety and quality standards.

The paper Doll et al. is more specific. It addresses the issue of the spoilage of extended shelf life milk and concluded that the shelf-life was not influenced to a large extent by raw-milk-associated factors, but by recontamination with spores, particularly from the *B. cereus* complex. To enhance milk quality throughout the entire shelf life, improved plant sanitation and disinfection that target the elimination of spores are necessary.

Machado et al. is a review of the growth potential of psychrotrophic bacteria, the heat resistant enzymes they produce and the consequences for dairy products with a long shelf life. Due to their ability to produce extracellular heat resistant enzymes such as peptidases and lipases, psychrotrophic bacteria, such as species of *Pseudomonas*, can contribute to spoilage of ultra-high temperature (UHT) treated and sterilized milk and other dairy products with a long shelf life. This problem is of increasing importance because of the large worldwide trade in fluid milk and milk powder.

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For many years, coliforms have been used as indicators of contamination of dairy products, and continue to be used for this purpose. Martin et al. highlights that recent discoveries regarding this diverse group of bacteria indicates that only some are of fecal in origin, while the majority are environmental contaminants, raising questions regarding the validity of coliforms as indicators of unhygienic conditions for dairy products. The role that coliforms play in raw and finished dairy products, their sources and the future of this diverse group as indicator organisms in dairy products is discussed.

The next six papers deal specifically with foodborne pathogens; three with *S. aureus*, and one with each of the pathogens *Salmonella*, pathogenic *E. coli* and *L. monocytogenes*.

The paper Merz et al. determined that *S. aureus* isolates from sheep and goats were similar, but were different from bovine isolates. Sixty-seven percent of the *S. aureus* strains detected exhibited at least one enterotoxin gene, indicating that many caprine, or ovine raw milk products may be contaminated with low levels of enterotoxigenic *S. aureus*, stressing the importance of strict maintenance of the cold chain.

The paper Kümmel et al. showed that dairy cattle represent an important, yet underreported, entrance point of *S. aureus* into the dairy chain. It was shown that certain *S. aureus* subtypes were present in primary production as well as in the cheese processing at the dairy plant and although a considerable diversity of *S. aureus* subtypes was observed, only certain *S. aureus* subtypes were able to enter and persist in the cheese manufacturing at the dairy plant, and could be isolated from cheese up to day 14 of ripening.

The paper Yu et al. concerns the control of *S. aureus* infection in cows. They demonstrated that the *in vivo* bacterial killing activity elevated when dosage increased or when dosing intervals were shortened, and recommended a regimen of three infusions of 75 mg per quarter every 12 h to achieve a 76.7% cure rate in clinical treatment of bovine mastitis caused by *S. aureus* infection.

The paper Gunn et al. used molecular characterization to relate two historical outbreaks associated with *Salmonella* serovars Anatum and Ealing. Pulsed-field gel electrophoresis (PFGE) revealed the clonal nature of the two outbreaks and whole genome sequencing (WGS) of representative isolates, one from each serovar, focused on the *Salmonella* pathogenicity islands. The results suggested a high level of genetic diversity that may have contributed to survival and virulence of isolates from these outbreaks.

Shiga toxin-producing *E. coli* (STEC) are significant pathogens in the dairy chain. The paper Murphy et al. examined the issue of super-shedding (shedding of >10,000 CFU/g of feces) of *E. coli* O157 and O26 by lactating cows and its

impact (if any) on raw milk. The results showed that of the 529 samples taken from 40 animals, 4 animals were deemed as super-shedders, one shedding *E. coli* O157 and 3 shedding *E. coli* O26. No STEC O157 or O26 were recovered from any of the raw milk, milk filter, or water samples. The results show that vigilance is required with regard to super-shedding of non-O157 STEC.

Another important pathogen in the dairy chain is *L. monocytogenes*. The paper Casey et al. contributes to understanding the pathogenicity of *L. monocytogenes*. Two serotype 1/2b strains of *L. monocytogenes* with differing infection abilities were subjected to comparative genomic analysis. The results showed the importance of accessory genes (genes that are not part of the conserved core genome) in *L. monocytogenes* pathogenesis and suggested that the emergence of an apparently non-pathogenic isolate of *L. monocytogenes* may result from a cumulative loss of functionality rather than by a single isolated genetic event.

Biofilm formation is an important factor in the community life of bacteria in the environment, providing protection against inactivation processes for pathogenic bacteria. The paper Miljkovic et al. demonstrated that deletion of the collagen binding repeats II, III, and IV, necessary for auto-aggregation, resulted in a loss of the strong auto-aggregation, collagen and fibronectin binding abilities whereas the biofilm forming capability was increased.

In the final paper, Anvarian et al. a combination of flow cytometry (FCM) and 16S rDNA sequencing was used to investigate the microbiome in a powdered infant formula (PIF) production facility. The greatest diversity in the microbiome was observed in the low care area. The genera present in low, medium and high care were mostly associated with soil, water, and humans, respectively. The integration of FCM and metagenomic data provided information on the density of different species in the facility.

AUTHOR CONTRIBUTIONS

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

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Detection and Enumeration of Spore-Forming Bacteria in Powdered Dairy Products

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With the abolition of milk quotas in the European Union in 2015, several member states including Ireland, Luxembourg, and Belgium have seen year on year bi-monthly milk deliveries to dairies increase by up to 35%. Milk production has also increased outside of Europe in the past number of years. Unsurprisingly, there has been a corresponding increased focus on the production of dried milk products for improved shelf life. These powders are used in a wide variety of products, including confectionery, infant formula, sports dietary supplements and supplements for health recovery. To ensure quality and safety standards in the dairy sector, strict controls are in place with respect to the acceptable quantity and species of microorganisms present in these products. A particular emphasis on spore-forming bacteria is necessary due to their inherent ability to survive extreme processing conditions. Traditional microbiological detection methods used in industry have limitations in terms of time, efficiency, accuracy, and sensitivity. The following review will explore the common spore-forming bacterial contaminants of milk powders, will review the guidelines with respect to the acceptable limits of these microorganisms and will provide an insight into recent advances in methods for detecting these microbes. The various advantages and limitations with respect to the application of these diagnostics approaches for dairy food will be provided. It is anticipated that the optimization and application of these methods in appropriate ways can ensure that the enhanced pressures associated with increased production will not result in any lessening of safety and quality standards.

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INTRODUCTION

The European Union's removal of milk quotas in April, 2015 led to a 2% increase in milk deliveries to dairies in the EU for 2015. Some countries are taking full advantage of the new limitless system in the EU, with Ireland, Luxembourg, and Belgium increasing bi-monthly milk deliveries to dairies by in excess of 20% (Eurostat, 2016). Although the production rate has slowed in some other major dairy exporters, including New Zealand and Australia, the US has seen continued increases in production (Dairy Australia, 2015; DCANZ, 2016; USDA, 2016). The surplus milk produced can be processed into a wide variety of dairy products, including yogurt, butter, cheeses, and dairy powders. Dairy powders are a popular commodity due to their long shelf life, ease of storage and versatile nature. A wide variety of dairy powders can be produced, each with

individual properties. These include whole milk powder (WMP), skimmed milk powder (SMP), whey protein concentrate (WPC), whey protein isolate (WPI), milk protein concentrate (MPC), milk protein isolate (MPI), casein and caseinates (Lagrange et al., 2015). Dairy powders can be used in fortification of other dairy products (Karam et al., 2013), as well as an ingredient in a wide array of foods including soups and sauces, confectionary (Sharma et al., 2012), infant formula, sports dietary supplements and in foods for health recovery (Gill et al., 2001; Lagrange et al., 2015). However, the increased production of dairy powders may create safety and economic risks to the dairy sector, specifically when controlling microbial loads in these products. Several key steps are involved in producing dairy powders including pasteurization, separation, evaporation, and spray drying (**Figure 1**). These thermal and mechanical processes can reduce the microbes present in the milk. However, spore forming bacteria may survive. It has been shown that the spore-forming bacterial composition of raw milk differs considerably from their associated dairy powders (Miller et al., 2015), highlighting that the processing of milk into powder changes the composition of the specific spore-formers present. Post-production, powders can be stored for extended periods and in the absence of water, bacterial metabolic activity and growth is limited (Deng et al., 2012), thus preventing spoilage and product defects. However, under these conditions, bacterial spores can remain dormant until more favorable conditions are encountered, when germination and outgrowth can proceed (Setlow, 2003, 2014).

BACTERIAL CONTAMINANTS OF DAIRY POWDERS

Sources of Bacterial Contamination of Dairy Powders

Spore-forming bacteria can contaminate dairy powders through a variety of means. Bacteria can originate from the soil (Heyndrickx, 2011), feces, bedding, feed, or milking equipment (Gleeson et al., 2013), or can enter the raw milk *via* contaminated teats, milking cups and bulk tanks. Additionally, contamination can occur during transport from the farm to the processing plant (Pantoja et al., 2011), and also within the processing facility itself from poor handling and contaminated equipment (Burgess et al., 2010; Faille et al., 2014). The formation of homogeneous or heterogeneous multicellular bacterial communities on the surface of processing equipment in the form of biofilms is a particular concern for the dairy processing sector and, when present, can lead to recurring problems of microbial contamination. The biofilms, which are themselves resistant to cleaning, can serve as a reservoir for bacterial spores which can slough off and contaminate dairy powders (Branda et al., 2001; Faille et al., 2014).

Common Bacterial Contaminants

Common contaminants identified in dairy powders include species of the class Bacilli (**Table 1**), many of which are capable of forming endospores (Checinska et al., 2015). Taxa

other than Bacilli have also been found to contaminate powdered dairy products with species reported including *Clostridium halophilum*, *Klebsiella oxytoca* (Buehner et al., 2015), *C. perfringens*, *C. septicum*, *C. novyi/haemolyticum*, *C. sporogenes* (Barash et al., 2010), *Staphylococcus aureus* (Zhang et al., 2015), and *Cronobacter sakazakii* (Minami et al., 2012). Bacteria of the genus *Clostridium*, as well as many of the contaminants of the class Bacilli (**Table 1**), including *Bacillus*, *Anoxybacillus*, *Geobacillus*, *Lysinibacillus*, *Brevibacillus*, and *Paenibacillus*, have a considerable advantage due to being capable of forming stress-resistant endospores. These genera, and their associated species, vary considerably with respect to the range of temperatures in which they can grow, and include some psychrophilic (Ivy et al., 2012) and thermophilic (Burgess et al., 2010; Watterson et al., 2014) species. Dairy product contaminating spore-formers can also differ by virtue of preferring anaerobic (Doyle et al., 2015) or aerobic (Gopal et al., 2015) conditions. Although many spore-formers are not pathogenic and are seen primarily as indicators of poor hygiene during milk collection and or processing (Burgess et al., 2010), some can cause disease (Andersson et al., 1995). Of the spore-formers identified in powders, specific representatives of *Clostridium* spp. and *Bacillus* spp. are the most worrying from a food safety point of view. *Clostridium* are anaerobic spore-formers, of which *C. botulinum* is the most notorious due to its highly potent botulinum toxin. There are many types of botulism including foodborne botulism, wound botulism, infant botulism and adult intestinal botulism. Infant botulism is the most common form (Sobel, 2005). Strains of *C. botulinum* isolated clinically have been identified in containers of opened milk powder from the home of patients with infant botulism (Brett et al., 2005; Johnson et al., 2005). Despite this, and although many species of *Clostridium* have been identified in dairy powders (Barash et al., 2010; Buehner et al., 2015), dairy powders have never been found to be responsible for a case of infant botulism (Brett et al., 2005; Johnson et al., 2005; Doyle et al., 2015). However, it is worth noting that anaerobic spore-forming bacteria, like *C. botulinum*, are less common than aerobic spore-formers in dairy powders. This may be due to the high degree of aeration involved in dairy powder processing or that testing criteria for spore-formers has been optimized to identify aerobic spore-formers except in the case of phenotype based assays for specific groups of anaerobic species. The ability of certain *Clostridium* species to reduce sulphite to sulfide under anaerobic conditions resulting in black colonies on specific media has been widely utilized. The accuracy of these qualitative and quantitative approaches has previously been discussed (Doyle et al., 2015). Of the aerobic spore-formers identified, the majority have been of the genus *Bacillus* (**Table 1**). Many species of this genus are generally regarded as safe and some are even used as probiotics (Hong et al., 2005); e.g., Bactisubtil, Biovicerin and Biosubtyl containing *B. cereus*, Bidisubtilis containing *B. subtilis*, Biosporin and Primal Defense containing *B. subtilis* and *B. licheniformis*, Biosubtyl containing *B. pumilus*, Enterogermina containing *B. clausii* and Lactospore containing *B. coagulans* (Hong et al., 2005). Other species of *Bacillus* have been used in the production of animal feed-stuffs; e.g., *B. subtilis* has been utilized for

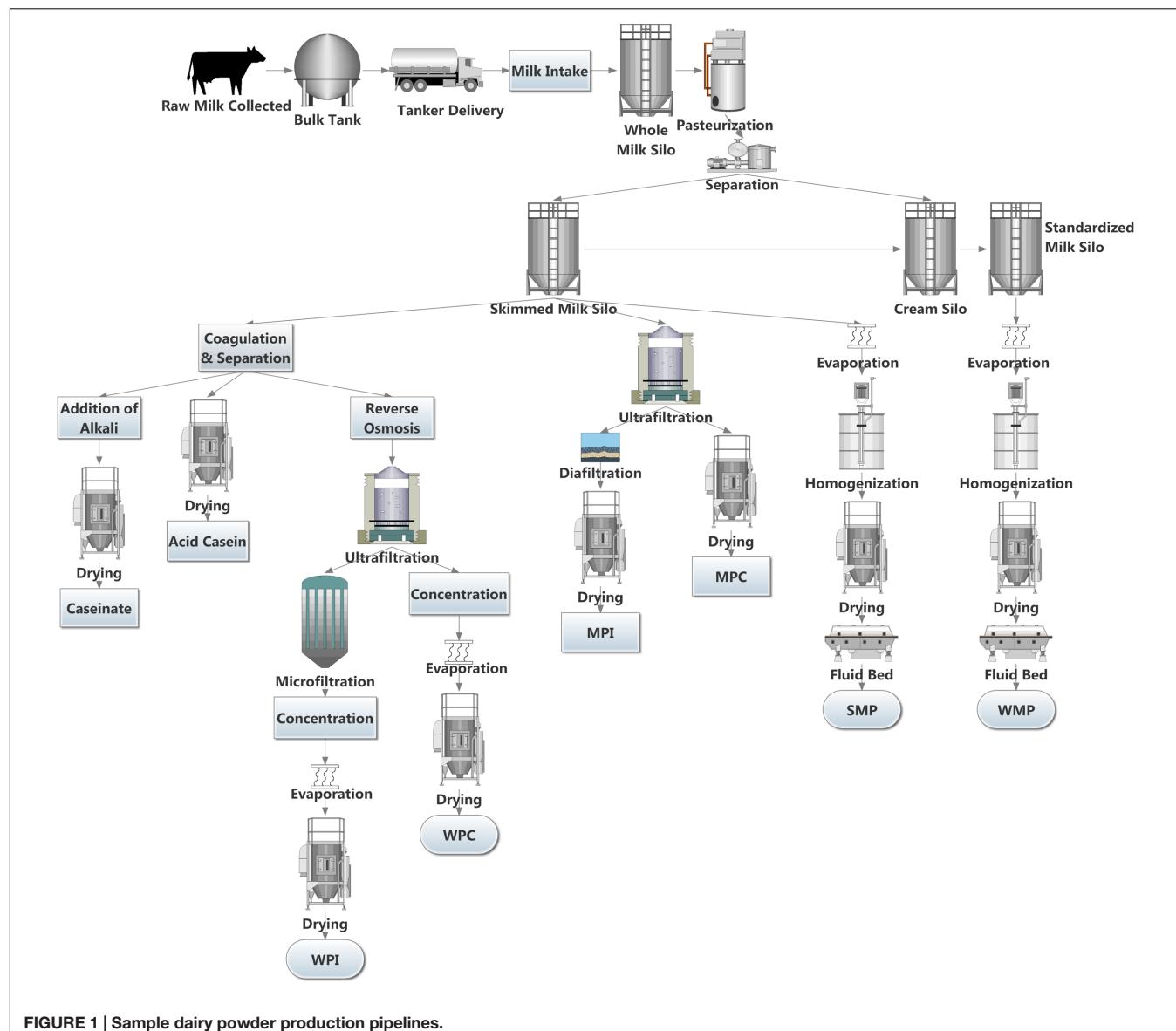


FIGURE 1 | Sample dairy powder production pipelines.

the fermentation of indigestible by-products of soya bean oil production to yield a suitable food source for monogastric animals (Wongputtisin et al., 2014). *B. cereus* sensu lato is the most important group of species identified from a pathogenic perspective (Bottone, 2010). This group, containing up to 11 individual, highly related species (Okstad and Kolsto, 2011; Liu et al., 2015), includes species that are regarded as non-pathogenic (Okstad and Kolsto, 2011). Other species include *B. thuringiensis* which is used as pesticides (Schnepp et al., 1998; Bravo et al., 2013); *B. cereus*, a class 2 pathogen capable of food poisoning which gave this species group its name (Bottone, 2010) and even a class 3 human pathogenic species, *B. anthracis* (Rasko et al., 2005). All of these are notoriously difficult to classify and differentiate from each other (Helgason et al., 2000; Radnedge et al., 2003; Rasko et al., 2005; Liu et al., 2015). *B. cereus* is the main cause of food poisoning

from within this group. *B. cereus* strains can contain many enterotoxins which are associated with diarrheal food poisoning including non-hemolytic enterotoxin (Nhe; Lund and Granum, 1996; Lindblad et al., 2004), hemolysin BL (Hbl; Beecher and Wong, 1997), and cytotoxin K (CytK; Lund et al., 2000). It should be noted that the description of CytK as a viable enterotoxin has been called into question as, in isolation, the presence of the corresponding gene has not been linked to virulence in diarrheal pathogenesis (Castiaux et al., 2015). Other molecules previously thought to be enterotoxins associated with food poisoning but which have since been reclassified include EntFM (Tran et al., 2010) and BcET (Choma and Granum, 2002). Some strains of *B. cereus* also produce an emetic toxin, cereulide (Ces), a product of non-ribosomal peptide synthesis, which can cause emetic food poisoning (Horwood et al., 2004; Toh et al., 2004).

TABLE 1 | Contaminants of the class Bacilli identified in powdered dairy products.

Bacilli contaminants	Reference
<i>Bacillus licheniformis</i>	Ronimus et al., 2003; Ruckert et al., 2004; Rueckert et al., 2005; Reginensi et al., 2011; Buehner et al., 2015; Miller et al., 2015; Sadiq et al., 2016; VanderKelen et al., 2016
<i>Bacillus subtilis</i> sensu lato	Ronimus et al., 2003; Ruckert et al., 2004; Rueckert et al., 2005; Reginensi et al., 2011; Miller et al., 2015; Sadiq et al., 2016
<i>Bacillus pumilus</i>	Ruckert et al., 2004; Reginensi et al., 2011; Buehner et al., 2015; Miller et al., 2015; Sadiq et al., 2016; VanderKelen et al., 2016
<i>Bacillus circulans</i>	Ruckert et al., 2004; Sadiq et al., 2016
<i>Bacillus coagulans</i>	Ruckert et al., 2004; Sadiq et al., 2016
<i>Bacillus cereus</i> sensu lato	Reyes et al., 2007; Buehner et al., 2015; Miller et al., 2015; Sadiq et al., 2016; Zhang et al., 2016
<i>Bacillus megaterium</i>	Reginensi et al., 2011; Buehner et al., 2015
<i>Bacillus sonorensis</i>	Buehner et al., 2015; Sadiq et al., 2016
<i>Bacillus altitudinis</i>	Buehner et al., 2015
<i>Oceanobacillus</i> spp.	Buehner et al., 2015
<i>Bacillus clausii</i>	Miller et al., 2015; Sadiq et al., 2016
<i>Bacillus thermoamylorovans</i>	Miller et al., 2015; Sadiq et al., 2016
<i>Anoxybacillus</i> spp.	Miller et al., 2015; Trmcic et al., 2015; Sadiq et al., 2016
<i>Anoxybacillus flavithermus</i>	Ronimus et al., 2003; Ruckert et al., 2004; Rueckert et al., 2005; Reginensi et al., 2011; Sadiq et al., 2016; VanderKelen et al., 2016
<i>Geobacillus</i> spp.	Miller et al., 2015; Trmcic et al., 2015
<i>Geobacillus stearothermophilus</i>	Ronimus et al., 2003; Ruckert et al., 2004; Rueckert et al., 2005; Buehner et al., 2015; Sadiq et al., 2016
<i>Geobacillus thermolevorans</i> group	Sadiq et al., 2016; VanderKelen et al., 2016
<i>Ureibacillus</i> spp.	Miller et al., 2015
<i>Ureibacillus thermosphaericus</i>	Ruckert et al., 2004
<i>Aeribacillus pallidus</i>	Miller et al., 2015; Sadiq et al., 2016
<i>Lysinibacillus</i> spp.	Miller et al., 2015
<i>Lysinibacillus sphaericus</i>	Sadiq et al., 2016
<i>Paenibacillus</i> spp.	Miller et al., 2015
<i>Paenibacillus cookii</i>	Sadiq et al., 2016
<i>Paenibacillus macerans</i>	Sadiq et al., 2016
<i>Bacillus aerophilus</i> sensu lato	Sadiq et al., 2016
<i>Brevibacillus brevis</i>	Sadiq et al., 2016
<i>Brevibacillus parabrevis</i>	Sadiq et al., 2016
<i>Virgibacillus puumi</i>	Sadiq et al., 2016
<i>Bacillus shackletonii</i>	Sadiq et al., 2016
<i>Sporosarcina contaminans</i>	Sadiq et al., 2016
<i>Laceyella sacchari</i>	Sadiq et al., 2016
<i>Bacillus amyloliquefaciens</i>	VanderKelen et al., 2016

Spore Formation

Endospores are formed in *Bacillus* and *Clostridium* species in response to environmental stress, by the activation of the master transcriptional regulator Spo0A (Hoch, 1993) following

a cascade of phosphorylation including five autokinases and two phosphorelay proteins (Molle et al., 2003). Spo0A binds to DNA and influences the expression of over 500 genes (Molle et al., 2003). It does so directly, for example it can control efficient replication of a single chromosome for both the mother cell and fore spore by binding to the origin of replication in the mother cell (Boonstra et al., 2013). But it can also work indirectly, through regulation of other transcription factors (Molle et al., 2003). There are over 100 genes known to be required for spore formation, with more being identified as research in the field develops (Meeske et al., 2016). Steps involved in spore formation include segregation of DNA, formation of a septum, engulfment and formation of a fore spore, formation of spore protein layers, cortex, membranes and spore coat and maturation of the spore before lysing the mother cell and being released. This process has previously been comprehensively reviewed elsewhere (Sella et al., 2014; Pompeo et al., 2016). Following its formation, an endospore can remain dormant and can persist in unfavorable environmental conditions without moisture or nutrients due to the protective structure and properties of the endospore.

Spore Structure

Endospores contain several thick layers. The outer coat, or exosporium, is a thick layer only found in some species, usually those of *B. cereus* sensu lato (Matz et al., 1970; Lai et al., 2003). The exosporium contains two layers, a basal layer surrounded by an external layer with hair like projections consisting mainly of the glycoprotein *Bacillus* collagen-like protein A (BclA; Sylvestre et al., 2002; Stewart, 2015). The exosporium, and especially BclA, contributes to hydrophobicity and aids the binding of spores to their substrates, including food preparation surfaces and stainless steel. This, along with its ability to assist spores in their avoidance of innate immune cells (Stewart, 2015), and also aids the spores' survival, spread and pathogenicity potential in the food chain. The exosporium, if present, surrounds the spore coat. The spore coat is a complex, semipermeable, proteinaceous layer found on all endospores. It is the outermost layer of *B. subtilis* spores (Setlow, 2006) and gives resistance to chemicals and enzymes, as well as structurally holding the spore together. It excludes large molecules, while allowing nutrients pass through and interact with germination receptors deeper in the spore structure (Driks, 2002; Lai et al., 2003). The spore coat surrounds an outer membrane, which encapsulates the cortex. The cortex is made of specific peptidoglycan (Popham, 2002) that is assembled into rod shaped structures, located perpendicularly to the spore surface (Li et al., 2016). It confers resistance to wet heat and is essential in the dormancy of the spore as well as reducing the water content of the core (Setlow, 2006). The cortex surrounds the germ cell wall, which becomes the bacterial cell wall following germination (Setlow, 2006; Wells-Bennik et al., 2016). The germ cell wall surrounds an inner membrane. This too protects the bacterial spore against chemicals, and contains the proteins required for germination back to active cells (Setlow, 2003). Proteins including transporters (some of which are associated with efflux processes and unique to the spore inner membrane), proteases (essential for sporulation and germination), DNA repair and replication enzymes (including

nucleotide excision repair enzymes, spore specific lyases and endonucleases), heat shock proteins and proteins involved in control of cellular processes in response to stress (including, but not limited to UV and oxidative stress) have all been identified in the spore inner membrane (Zheng et al., 2016). These all contribute to the resistance and persistence of spores in unfavorable conditions. Inside the inner membrane is the core of the endospore, which is severely dehydrated and compacted. This dehydration allows immobilization of proteins, preventing their coagulation following heat denaturation (Sunde et al., 2009). The core also contains high levels (up to 15–25% of the spores dry weight) of dipicolinic acid (DPA), most of which is chelated by divalent ions, allowing protection of spore DNA from external stressors as well as synthesis of new DNA in response to UV radiation (Setlow, 2006, 2007; Sunde et al., 2009). Also found in the spore core of *Bacillus* species is a group of small, acid-soluble spore proteins (SASP) of the α/β -type. These bind DNA in the spore core and alter its structure, thus aiding its resistance to heat, chemicals, UV radiation, and osmotic pressure (Setlow, 2006, 2007).

Survival of Spore-Forming Bacteria in Processing Environments

Spores can survive processing to which vegetative cells would normally succumb. Such processing-related stressed include desiccation, dry and wet heat, UV radiation, mechanical agitation, γ -radiation, chemical exposure and hydrostatic and osmotic pressure (Nicholson et al., 2000; Setlow, 2006). Indeed, while the temperatures and drying conditions used in the processing of milk to powders kills most vegetative bacterial cells, it also inadvertently selects for these spore-formers. Once powders are rehydrated, the spores may germinate by activation of germination receptors, either in response to nutrients called germinants (Setlow, 2003) or by heat activation (Luu et al., 2015). Germination independent of these receptors may also be triggered by calcium chelated dipicolinic acid (CaDPA), dodecylamine, or peptidoglycan fragments, although these mechanisms may not be applicable to the food industry (Setlow, 2014). Germination initiated by high pressure, either by activation of germination receptors or independent of them, can also occur (Setlow, 2014). Following germination, these spore-formers can proliferate in the absence of competition from other bacteria that were eradicated during processing (Brown, 2000).

LEGISLATION GOVERNING BACTERIAL CONTAMINATION IN DAIRY POWDERS

Guidelines governing the levels and types of bacteria permitted in dairy powders are not very comprehensive, except in the case of infant formula. There are many different governing bodies that have set testing parameters; including the U.S. Food and Drug Administration (FDA), Food Standards Australia New Zealand (FSANZ), and The European Commission (EC). In Ireland, the Food Safety Authority of Ireland (FSAI) implements limits based on the Commission Regulation (EC) No 2073/2005 (European Commission, 2005). FSAI state that aerobic colony counts

in dairy powders should ideally be $<10^4$ cfu/g (FSAI, 2014). However, this is not a legal obligation, and does not mean that the food is unsafe as characterization of the species isolated would need to be performed in order to determine product safety. The U.S. Department of Agriculture (USDA) implements the following microbial limits in US extra grade dairy powders using the standard plate count; dry buttermilk $<20,000$ cfu/g (USDA, 2001a), dry whey $<30,000$ cfu/g (USDA, 2000), dry whole milk $<10,000$ cfu/g (USDA, 2001b), dry casein (acid) $<30,000$ cfu/g (USDA, 1968), instant non-fat dry milk $<10,000$ cfu/g (USDA, 2013), non-fat dry milk (roller dried) $<50,000$ cfu/g (USDA, 1984), and non-fat dry milk (spray process) $<10,000$ cfu/g (USDA, 2001c). The US Dairy Export Council (USDEC) implements limits for US dairy powders destined for international customers with limits on aerobic spore-formers set to between <500 cfu/g and <1000 cfu/g for thermophilic and mesophilic spores, respectively, in SMP, non-fat dry milk and WMP destined for infant powder, and <500 cfu/g and <2000 cfu/g, respectively, in SMP and WMP (Watterson et al., 2014).

In Australia and New Zealand, state agencies enforce limits set by FSANZ. *B. cereus* must be <100 cfu/g in 4/5 samples, and $<1,000$ cfu/g in 1/5 samples in dried milk powder and powdered infant formula products with added lactic acid producing cultures, and must be absent in five samples of 1 g in powdered infant formula. The EC regulation, as amended (European Commission, 2005) sets similar legal microbiological criteria including a limit of <50 cfu/g presumptive *B. cereus* in 4/5 samples and <500 cfu/g in 1/5 analyzed is set in accordance to EN/ISO 7932 (Standards, 2004).

Due to the competitive market for dairy ingredients, individual purchasers often set their own microbiological limits to ensure high standards. In many cases dairy powders will not receive any further treatments before incorporation into other products. For example, powdered infant formula manufacturers often have close relationships with the dairy powder supplier to ensure high microbiological standard are met, and set strict criteria (Kent et al., 2015).

DETECTION OF SPORE-FORMING BACTERIA

Apart from dairy powder that is due for export from the US, no legislation thoroughly covers the enumeration or identification of all spore-formers in dairy powder. This is in spite of recent research highlighting the need for accurate spore quantification and identification (Burgess et al., 2010). Identification and enumeration of all spore-formers present in dairy powders allows identification of potential problematic species whether from a hygiene, quality or pathogenic perspective. This information would allow manufacturers implement more comprehensive and/or directed preventative measures (Pennacchia et al., 2014) resulting in continued economic and safety confidence in the sector. Understanding composition of total spore-formers within a product contributes to a clearer understanding of the source of potential quality or safety issues should they arise and allows

faster implementation of control measures (Burgess et al., 2010; Pennacchia et al., 2014). Indeed, efforts have continued to be made in recent years to improve the detection and identification of spore-forming bacteria present in dairy powders (Watterson et al., 2014; Miller et al., 2015; Sadiq et al., 2016).

Culture Based Methods

Spore Count Methods

Typical spore count tests involve the heating of a reconstituted powder sample to 80°C for 12 min before cooling, culturing and enumerating colonies (Frank and Yousef, 2004; Watterson et al., 2014). Highly thermo-resistant spores are selected by heating to 100°C for 30 min before cooling and culturing while numbers of especially thermo-resistant spores are quantified by heating to 106°C for 30 min, cooling and culturing. Media is incubated in the presence or absence of oxygen to select for aerobic or anaerobic spore-forming species, respectively. Incubation can also be at different temperatures. Incubation at 6°C will select for psychrophilic spore-formers, incubation at 30–35°C will select for mesophilic spore-formers and incubation at 55°C will select for thermophilic spore-formers (Watterson et al., 2014; Kent et al., 2016). Further analysis of isolated colonies is required in order to determine the species present, and the options available for this analysis are discussed at a later stage in this review (see Culture-Based Identification of Spore-Forming Species and Post-Culture DNA-Based Classification Methods). Total bacterial counts and spore counts, although informative, are not without their limitations. Almost a century ago it was highlighted that different media will result in different bacterial counts (Ayers and Mudge, 1920) and that more than just quantitative data is needed with respect to contamination of dairy products, in order to determine the significance of the contamination (Ayers and Mudge, 1920). The use of various heating methods is somewhat redundant in terms of identification of different species (Miller et al., 2015). However, the actual abundance of these spore-forming bacteria does differ depending on the test method used (Kent et al., 2016). In order to get a clear picture of the total spore-former composition present in a powder sample through culture-based approaches, a variety of incubation conditions, temperatures, agars and, possibly, heat treatments would be needed. This highlights the need for stronger/more robust test methods to determine the abundance of (spore-forming) bacteria in dairy powders.

Culture-Based Identification of Spore-Forming Species

Numerous culture-based tests have been developed in order to help identify spore-forming bacteria. These involve the use of selective media and, in some cases, additional tests to provide further information regarding the identity of the species present. Both Bacara and Mannitol Egg Yolk Polymyxin (MYP) agars have been developed for the isolation of *B. cereus*. The testing used for presumptive *B. cereus* in Europe (Standards, 2004) involves the use of MYP agar and the hemolysis test. However, MYP has been shown to be not as selective as Bacara agar for *B. cereus* (Tallent et al., 2012), potentially leading to false positives. Some *Clostridium* species, the sulphite reducing Clostridia (SRCs),

have the ability to reduce sulphite to sulfide under anaerobic conditions. A number of sulphite containing agars have been developed for their selection (Wilson and Blair, 1924; Gibbs and Freame, 1965; Weenk et al., 1995). SRCs are identified by a black color change, however, other bacteria capable of reducing sulphite and can also grow on these media, these are referred to as sulphite reducing bacteria (SRBs) (Doyle et al., 2015). Other tests can involve analyzing phenotypes by visualizing morphological properties and performing biochemical tests to narrow down the possible species (Janda and Abbott, 2002; Reyes et al., 2007).

Limitations of Culture-Dependent Analysis

A common limitation with all of the aforementioned methods is a requirement that the bacteria first be cultured. This can result in important difficult-to-culture species being overlooked due to inappropriate culturing conditions, temperature, aeration, and/or media type. Furthermore, colony selection may favor the selection of the largest/most plentiful colonies above the smaller/less plentiful types. Although these methods allow isolation and enumeration of culturable species, accurate identification of each species present is difficult, very time-consuming, labor intensive and can be biased. The aforementioned isolation methods can be coupled with the following, more recently developed, protein- and DNA-based methods, to provide more robust identification.

Protein-Based Methods

Enzyme Immunoassays

A sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) has been developed for the detection of whole cells of *B. cereus*, by recognizing surface antigens specifically associated with *B. cereus* cells. This assay was developed by multiple location immunization of animal models with whole cell immunogen to develop hybridomas and subtractive screen was used to eliminate cross reactivity with closely related species (Zhu L. et al., 2016). The subtractive screen ensured the mAbs are highly specific against *B. cereus* and the assay has a lower detection limit of 0.9×10^3 cells/ml in phosphate buffered saline. This assay has been tested using food samples spiked with various pathogens without the need for culturing. It was highly effective at identifying *B. cereus* cells in mixed samples, without interference by the food matrix or influence by other related species. Although this ELISA for detection of surface antigens is specific for *B. cereus*, it is not clear if it can recognize spores as well as vegetative bacteria, or if it can distinguish between live and dead *B. cereus* (Zhu L. et al., 2016). Failure to detect spores could lead to a false negative result, whereas detection of free floating antigens from dead *B. cereus* cells could lead to false positive results. Additional culturing may be needed to detect cell numbers below the lower detection limit, and thus eliminate these concerns. Enzyme immunoassays have also been developed for the detection of *B. cereus* toxins (Wehrle et al., 2009; Cui et al., 2016). Specific conditions are needed to ensure efficient protein production. Casein hydrolysate-glucose-yeast with 1% glucose is used for the production of enterotoxins in *B. cereus*, and 10% skim milk medium is used for cereulide production in *B. cereus* (Cui et al., 2016). A negative result from a proteomic based assay

would not imply that the bacteria is not present, rather protein synthesis might not be currently active.

Limitations of Protein Based Methods

The requirement for correct expression conditions in order to identify proteins of interest is a hugely limiting step in protein based method for species identification. This is particularly true for spore-forming bacteria, whose presence is of concern but are currently in a dormant state during sample testing. Such requirements for specific growth conditions increase the analysis time and complexity, which may not be possible for large scale analysis of many possible toxin producers in laboratory situations. Furthermore, it is expected that the proteinaceous nature of dairy samples would greatly impeded the sensitivity of any protein analysis performed without initial culturing, even if expression was occurring.

DNA-Based Methods

Post-Culture DNA-Based Classification Methods

Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR)

Random amplified polymorphic DNA polymerase chain reaction uses short random primers to amplify multiple random DNA segments which, once visualized on an agarose gel, give unique patterns (Williams et al., 1990). Analysis of these fingerprints allows differentiation of species and strains by comparing profiles of various known strains (Ronimus et al., 1997, 2003). This method has been applied to colonies obtained from dairy powders in New Zealand to identify *Geobacillus stearothermophilus*, *Anoxybacillus flavithermus*, *Bacillus licheniformis*, and *B. subtilis* as the main contaminants of WMPs and SMPs, as well as buttermilk and goat milk powders (Ronimus et al., 2003). It has also been applied to whole and SMPs in Uruguay, correctly identifying the presence of *B. licheniformis*, *B. megaterium*, *B. pumilus*, *A. flavithermus*, and *B. subtilis* (Reginensi et al., 2011). Indeed, using this approach, *G. stearothermophilus*, *A. flavithermus*, and *B. licheniformis* have been identified as the dominant species in whole and SMPs from multiple countries including; Poland, Germany, Switzerland, France, Portugal, Netherlands, Great Britain, Ireland, Canada, USA, Mexico, Chile, Brazil, South Africa, Thailand, Australia, and New Zealand. *B. subtilis*, *B. circulans*, *Ureibacillus thermosphaericus*, *B. coagulans*, and *B. pumilus* have also being identified, albeit in lower quantities (Ruckert et al., 2004). A common feature of the RAPD-PCR approach is the highlighting of the 3–4 most dominant species. However, species of lower abundance might be the most interesting in terms of food security and spoilage. One exceptional study described the use of RAPD-PCR, and revealed a more in depth array of species, in Chinese dairy powders (Table 1) (Sadiq et al., 2016). Apart from identifying previously unreported species, other details worth noting are that *B. licheniformis*, *G. stearothermophilus*, and *A. flavithermus* were again established as being present in high abundance while, importantly, *B. cereus* group species were also identified. This observation obviously has implications for food safety (Sadiq et al., 2016). Although informative, analysis of the gel bands in RAPD PCR is very subjective allowing errors

in classification and bias. Furthermore, the method requires time-consuming and laborious preparation of reference strains and there may also be variability between gels with the same samples, thus large-scale analysis would be difficult.

Sequencing housekeeping genes

Housekeeping genes are genes that are essential for the functions of the cell and viability of the organism, and thus typically contain highly conserved regions (Gil et al., 2004; Eisenberg and Levanon, 2013). Genes that contain such highly conserved regions at either end of a more variable region are particularly useful for strain identification purposes as the conserved regions can be targeted using degenerate primers to facilitate PCR amplification and sequencing of the variable region (Case et al., 2007). Identification of genera present is facilitated by comparison with databases of corresponding variable region sequences of known origin (Case et al., 2007). Many genes have been utilized for classification of species in fluid milk in the form of molecular typing (Durak et al., 2006). Other typing methods have been described for milk powder isolates of *Geobacillus* spp. and *B. licheniformis* based on variable number tandem repeat analysis (Seale et al., 2012; Dhakal et al., 2013). The 16S rRNA gene is ubiquitous among bacteria, and contains multiple conserved and variable regions making it extremely useful, in general, for taxonomic classification. However, 16S rRNA gene sequencing cannot differentiate between closely related species or subtypes and other housekeeping genes such as *gyrB* or *rpoB* have been utilized to do so (Durak et al., 2006; Case et al., 2007). Recently, both the *rpoB* and 16S rRNA genes have been used to characterize the contaminating psychrophilic, mesophilic, and thermophilic spore populations isolated from sweet whey, WPC, non-fat dry milk and acid whey powders. At least 14 different species were identified, with *B. licheniformis*, *Geobacillus* spp., and *Anoxybacillus* spp. being the most abundant (Miller et al., 2015). These methods have the potential to allow identification and monitoring of persistent species and subtypes throughout dairy powder processing plants (Seale et al., 2012; Dhakal et al., 2013). Although not currently employed in sequencing dairy powder isolates, *cpn60* (Durak et al., 2006; Schellenberg et al., 2011), *pycA*, *ccpA* (Liu et al., 2015), and *groEL* (Chang et al., 2003) have all been used to varying success in the sequencing of isolates from fluid milk (Durak et al., 2006), vaginal (Schellenberg et al., 2016) and marine (Liu et al., 2013) populations and remain as potential targets for future application to study dairy powder-associated microbes.

Pyroprinting

Pyroprinting utilizes sequencing by synthesis on multiple copy polymorphic loci simultaneously. The sequence reads are digitalized and can be compared using Pearson's correlation distance matrix to identify strains (Black et al., 2014). This method has been developed and utilized for source tracking, i.e., tracing sources of microbial contamination in end products or, more specifically, of endospore-forming bacilli in raw milk through to dairy powders. Presumptive species identified in powder included *G. thermoleovorans*, *A. flavithermus*, *B. licheniformis*, *B. pumilus*, and *B. amyloliquefaciens*.

(VanderKelen et al., 2016). These results correlate well with previous studies on raw milk and powders using the Sanger sequencing approach (Durak et al., 2006; Miller et al., 2015).

Limitations

All of the above tests allow identification of the most abundant culturable species identified in dairy powders. However, they are limited by an initial requirement for culturing and, unless these methods are modified for identification of species directly from dairy powders, they are not suitable for the identification of non-culturable species or species of lower abundance which can be out competed when culturing, unless selective media is employed. Ultimately, while promising, these methods when compared to culture-independent sequencing (see Next Generation Sequencing for the Identification of Dairy Powder Contaminants) are labor intensive and time consuming.

Targeted DNA Based Approach

A more targeted approach can be taken in the food sector to detect specific pathogens or groups of interest. These assays allow detection of toxin genes, possible pathogenic groups or members of a species of interest. Most of these have been adapted to allow amplification directly from mixed DNA extracted from foodstuffs and thus avoid the limiting step of culturing. Many also allow quantification of the species/toxin gene containing group. Of particular relevance to this review is the fact that a great deal of research has been performed with respect to such assays and the *B. cereus* sensu lato.

PCR assays

Polymerase Chain Reaction (PCR)-based assays have been developed for the detection of *B. cereus* toxin genes. Taqman quantitative PCR (qPCR) assay of a single component of the hemolysin toxin gene in *B. cereus* has been developed (Cattani et al., 2016), amplifying the sequence corresponding to one component of one tripartite toxin. It has been reported that the Taqman probe is specific for *B. cereus* strains that contain this gene, however, not all *B. cereus* strains contain the hemolysin gene (Cui et al., 2016). This assay reportedly does not give false positives with related species, such as other members of the *B. cereus* sensu lato including *B. thuringiensis* and *B. mycoides*. However, this assay could lead to false negatives. The assay may fail to detect other species that have the toxin genes, or other strains of *B. cereus* that do not have this particular toxin, but may be pathogenic due to the presence of other toxins. This assay also gives accurate quantification of viable *B. cereus* by comparison to standard curves. Multiplex endpoint PCR of toxin genes has also been performed to identify *B. cereus* in dairy samples. These assays included primers to amplify single components of *B. cereus* enterotoxin genes, i.e., those encoding Nhe, CytK, and Hbl (Zhang et al., 2016) as well as enterotoxin FM (EntFM) and emetic toxin Ces (Forghani et al., 2015). However, the specificity of these assays was only tested using *B. cereus* and non-*Bacillus* species. Multiplex PCR of multiple components of *B. cereus* toxin genes has also been performed on single bacterial colonies isolated from dairy products and environments (Wehrle et al., 2009). This approach allows detection of all components needed to produce viable

enterotoxins, and thus lessening the chance of false readings compared to other assays that only identify one toxin gene component. Multiplex endpoint PCR assays have also been developed for hygiene indicator species, *G. stearothermophilus* and *A. flavithermus* isolated from dairy powders. These assays rely in the species specific conserved regions of ITS 16S-23S rRNA region and the *rpoB* gene (Pennacchia et al., 2014). Further validation of these assays could lead to their use on DNA isolated directly from dairy powders. Finally, droplet digital PCR (ddPCR) allows precise, absolute quantification of a target DNA sequence. The DNA is encapsulated into many water in oil emulsion droplets and a PCR performed on each (Pinheiro et al., 2012). This culture-independent method has recently been used to detect *B. cereus* in fluid milk and can provide absolute quantification without need for comparison to standard curves. In this instance ddPCR was implemented using primers that target the *gyrB* gene of *B. cereus* sensu lato and the assay was found to have a lower detection limit than traditional qPCR (Porcellato et al., 2016), which is ideal for dairy powders that have low levels of contamination.

Biosensors

The assays described above also have the potential to be employed in the form of biosensors. Indeed, biosensors are already being developed for detection of a toxin gene found in *B. cereus* in milk and powder (Izadi et al., 2016). These biosensors are DNA based pencil graphite electrode (PGE) biosensors, in which a *nhe* toxin gene primer is immobilized on gold nanoparticles. Positive results are measured by an increase in charge resistance on the biosensor from the hybridization of the target DNA to *nhe* toxin sequence.

Limitations of targeted DNA assays

Although these methods do not give a complete view of the microbial composition in a dairy powder, they are useful as a test for key spoilage and pathogenic bacteria, including producers of harmful toxins. It is important to note that *B. cereus* sensu lato toxin genes are not specific to any one species of the group, nor is one toxin found in all *B. cereus* (Liu et al., 2015; Cui et al., 2016; Zhu K. et al., 2016). However, targeting toxins allows detection of all possible pathogenic species. Singleplex assays that target one component of one toxin may be prone to false negatives (Cui et al., 2016), i.e., producers of other toxin types being overlooked, thus underestimating the number of pathogenic *B. cereus* cells in a sample. Multiplex assays targeting many toxins, are more robust and can be beneficial for the food industry as they are a good indicator of potential food pathogens. Targeting all components of a toxin system may be required to confirm if there is a true potential for toxin production. Furthermore, while the genes for toxins may be present, it is unclear from these assays whether any active proteins are functionally expressed. The alternative use of a non-toxin gene for identification of *B. cereus* (*gyrB*) does not distinguish between members of *B. cereus* sensu lato, nor does it identify if the species identified are capable of being pathogenic. Overall the detection of toxin and species specific genes are a good indicator of potential pathogenic and other species of interest being present.

Although issues remain, future improvement and development should result in the full potential of these approaches being realized.

Culture-Independent, Non-targeted DNA Analysis

As outlined, there are limitations associated with the aforementioned culture-dependent and targeted assays. Culture-independent DNA-based analysis should be considered when striving to obtain an overview of all (i.e., culturable and non-culturable) spore-forming species present in dairy powders. This involves a shift away from testing for and identifying only specific known spore-forming bacteria in order to eliminate the possibility of currently unknown or underappreciated microbiology-related food security threats.

Next generation sequencing for the identification of dairy powder contaminants

In the last decade, considerable advances have meant that next generation DNA sequencing platforms have surpassed traditional Sanger sequencing platforms in terms of speed and potential applications. Their initially extremely short sequencing read lengths are less of a concern as sequencing lengths of Illumina and Ion platforms have increased (Quail et al., 2012) and new, even longer read, platforms have been developed by PacBio and Oxford Nanopore (Quail et al., 2012; Madoui et al., 2015). The advantages and disadvantages of the various sequencing platforms have been previously reviewed elsewhere (Goodwin et al., 2016). Regardless, research laboratories now have a much greater choice when determining which sequencing technology to use, though it should be noted that results generated using different methods, technologies or bioinformatics pipelines are not always consistent (Cloonan et al., 2016). Whole genome shotgun sequencing is the process by where the whole genome of a single colony is sequenced. The DNA is extracted and sheared it into small pieces, before sequencing of these pieces and the use of computer software to assemble these sequences reads back together. This process can be applied to metagenomics, the term used to denote all of the genomic information from an entire community of different cells, for example the contaminants in dairy powders (Sharpton, 2014). The application of metagenomic techniques to the analysis of dairy products presents exciting opportunities. Metagenomic sequencing eliminates the need to culture, thus reducing bias, and allows the identification of species that are difficult to, or cannot be, cultured in the laboratory. Metagenomic sequencing has been applied to single gene products, such as the aforementioned 16S rRNA gene that can differentiate between all bacteria present to the genus level, while the *spo0A* gene has been targeted to specifically identify spore-forming Firmicutes in mixed populations. A whole metagenome 'shotgun,' i.e., untargeted, approach has also been attempted and comparison of 16S amplicon sequencing, *spo0A* amplicon sequencing and metagenomic shotgun sequencing performed for the identification of Firmicutes in metagenomic samples (Filippidou et al., 2015). Each method has advantages and disadvantages. Amplicon sequencing is more cost effective, high throughput and rapid but often only gives accurate classification to genus level, and may over-estimate microbial

diversity in the sample (Acinas et al., 2004; Poretsky et al., 2014). In contrast, shotgun sequencing is more expensive, less samples can be analyzed at one time, but it gives the opportunity to accurately classify to species level provided there are accurate reference databases to compare sequence reads to Sharpton (2014). Shotgun sequencing also reduces the bias of amplicon sequencing that can arise due to need for an initial PCR amplification and, where relevant, variable gene copy numbers (Sharpton, 2014; Brooks et al., 2015). The other advantage of shotgun metagenomic approaches is that additional information regarding other genes of interest within the microbial community can be generated. Such genes include toxin genes (Steffen et al., 2012; Leonard et al., 2015), sporulation genes (Filippidou et al., 2015), non-ribosomal peptide synthase (NRPS) gene clusters (Schirmer et al., 2005), antibiotic resistance genes (Bengtsson-Palme et al., 2014), and phage genes (Dutilh et al., 2014), all of which may be interesting from a food safety point of view. The sequencing reads from this approach can be difficult to analyze as they can be biased toward genomes of higher abundance. This is a particular issue when studying samples from specific human and animal microbiomes where there is a considerable amount of DNA from host cells present (Feehery et al., 2013). It is important to note that, due to the high sensitivity of shotgun metagenomic sequencing, care needs to be taken to ensure the absence of contaminating cells or DNA from other environments (Salter et al., 2014; Glassing et al., 2016).

Regardless of the sequencing approach taken, bioinformatic expertise is needed to analyze sequencing data and compare sequence reads to databases. Databases and bioinformatics software are updated continuously and newer, more accessible programs are constantly being developed (Vincent and Charette, 2015), including more targeted programs and databases specifically for food microbes (Vangay et al., 2013; Parente et al., 2016).

Limitations

Both amplicon and shotgun metagenomic sequencing reveal the relative abundance of bacteria in a sample. Furthermore, the quantification of total bacterial load can be achieved by coupling these techniques with qPCR or ddPCR analysis, (Porcellato et al., 2016).

While the benefits of next generation sequencing in determining the safety and quality of dairy powders provide cause for optimism, there are several hurdles. Culture-independent DNA analyses rely on one's ability to extract all genomic DNA directly from the substrate for analysis. Extracting DNA from dairy powder can be difficult, especially from spore-forming bacteria. Although, many studies have endeavored to optimize methods for the extraction of DNA from spores that have been spiked into food, success has been varied (Wielinga et al., 2011; Mertens et al., 2014). Furthermore, the bacterial load is likely to be lower in dried dairy powders than other environmental samples in which this sort of analysis has been previously performed, such as the gut (Gill et al., 2006), soil (Fierer et al., 2012), and fermented food (Jung et al., 2011). Low DNA concentration can be overcome through use of whole genome

amplification kits (Yokouchi et al., 2006; Binga et al., 2008). Although expensive, these provide for culture independent non-targeted analysis of all bacteria present in dairy powders even if present at low cell numbers. However, these kits are notoriously susceptible to contamination (de Bourcy et al., 2014) and, ideally, ultra clean laboratory environments are needed for their use (Weinmaier et al., 2015).

Isolation of DNA solely from spore-formers. There may be instances where there is a specific desire to specifically focus on the sequencing of DNA from the spore-forming community within a powder sample. Isolation of DNA solely from spores/spore-forming bacteria is a challenge. One possible method would be to perform standard spore pasteurization at 80°C for 12 min (see Spore Count Methods) (Frank and Yousef, 2004; Watterson et al., 2014) or other forms of targeted vegetative cell lysis (Wunderlin et al., 2016). However, free DNA could still be present in the samples from the lysed vegetative cells. Elimination of this signal could be performed using an intercalating dye (described below). Post-heat treatment, subsequent culture-based enrichment could be employed prior to DNA extraction (Frank and Yousef, 2004; Watterson et al., 2014) but, as described with respect to the culture-based approaches, this has the potential to lead to bias.

Sequencing-based approaches can also be adapted to specifically focus on spore-formers by, for example targeting of the *spo0A* gene for amplicon sequencing, or through focusing specifically on this gene from within shotgun sequence data. However, yet again, the need to ensure optimal DNA extraction and the removal of DNA from dead cells is a key consideration. A less conventional way of overcoming such challenges could involve the isolation of spores from dairy powder using density gradient centrifugation (Tamir and Gilvarg, 1966).

As noted above, free DNA from lysed vegetative cells can be present in samples following heat-treatments. Elimination of this signal could be performed using an intercalating dye. The use of intercalating dyes is especially relevant in the case of amplicon metagenomic sequencing where PCR amplification is performed (Rudi et al., 2005). This has been performed utilizing the dyes propidium monoazide (PMA) or ethidium monoazide bromide (EMA) to bind free DNA in the samples (Rudi et al., 2005; Forghani et al., 2015; Cattani et al., 2016; Zhang et al., 2016). Further testing and optimization would be needed to determine if its results are as promising for dairy powder samples with mixed populations. There are contradicting studies with regard to whether EMA or PMA is best for particular applications (Seinige et al., 2014; Wu et al., 2015). Very few studies have compared EMA and PMA in mixed populations, though EMA was reported to be favorable at penetrating heat damaged bacterial cells in fish fillets (Lee and Levin, 2009). EMA has been known to penetrate some live bacteria (Nocker et al., 2006; Seinige et al., 2014) whereas PMA has been seen not to penetrate all dead cells (Cattani et al., 2016). The concentrations of EMA used

has seen a decrease in recent years (possibly to circumvent the penetration of live cells) and, so, while early studies used 100 µg/ml (Rudi et al., 2005; Nocker and Camper, 2006), more recent studies used 8–10 µg/ml (Seinige et al., 2014; Wu et al., 2015). Alternatives, including the use of platinum (Soejima et al., 2016) to bind extracellular DNA, appear promising as they have been reported to be more selective at differentiating live/dead *E. coli* and *C. sakazakii* than PMA in water and milk. Ultimately, optimization needs to take place to develop the system that is best suited to the low microbial load of mixed populations present in powdered dairy products. It should also be noted that these approaches are not effective when performing metagenomic shotgun sequencing, as there is no amplification step to eliminate the dye-bound DNA.

Outlook

Currently culture-independent, population-based, analysis is relatively expensive and, thus, further developments are needed to increase its relevance to the food industry. It is, however, becoming more accessible as a test method for companies to strategically analyze processing pipelines and end products, allowing development of targeted treatments and intervention strategies against persistent or troublesome microorganisms. To provide thorough and reproducible analysis of dairy powders in this fashion, it will be particularly important to arrive at a consensus regarding the standardized sample preparation, use of specific sequencing platforms and analysis methodologies to facilitate comparison across multiple investigations (Clooney et al., 2016).

CONCLUSION

Newer technologies have paved the way for an overhaul in the approaches taken to detect and enumerate of spore-forming bacteria in dairy powders. This can lead to a more accurate, high throughput system. Although the newer technologies themselves are not without their limitations, they are continuously improving. Optimization of these newer technologies could lead to their routine use, allowing development of improved targeted treatments and preventative measures in the powder processing industry.

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Spoilage of Microfiltered and Pasteurized Extended Shelf Life Milk Is Mainly Induced by Psychrotolerant Spore-Forming Bacteria that often Originate from Recontamination

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Premature spoilage and varying product quality due to microbial contamination still constitute major problems in the production of microfiltered and pasteurized extended shelf life (ESL) milk. Spoilage-associated bacteria may enter the product either as part of the raw milk microbiota or as recontaminants in the dairy plant. To identify spoilage-inducing bacteria and their routes of entry, we analyzed end products for their predominant microbiota as well as the prevalence and biodiversity of psychrotolerant spores in bulk tank milk. Process analyses were performed to determine the removal of psychrotolerant spores at each production step. To detect transmission and recontamination events, strain typing was conducted with isolates obtained from all process stages. Microbial counts in 287 ESL milk packages at the end of shelf life were highly diverse ranging from <1 to 7.9 log cfu/mL. In total, 15% of samples were spoiled. High G+C Gram-positive bacteria were the most abundant taxonomic group, but were responsible for only 31% of spoilage. In contrast, psychrotolerant spores were isolated from 55% of spoiled packages. In 90% of samples with pure cultures of *Bacillus cereus* sensu lato and *Paenibacillus* spp., counts exceeded 6 log cfu/mL. In bulk tank milk, the concentration of psychrotolerant spores was low, accounting for merely 0.5 ± 0.8 MPN/mL. *Paenibacillus amylolyticus/xylanexedens* was by far the most dominant species in bulk tank milk (48% of all isolates), but was never detected in ESL milk, pointing to efficient removal during manufacturing. Six large-scale process analyses confirmed a high removal rate for psychrotolerant spores (reduction by nearly 4 log-units). *B. cereus* sensu lato, on the contrary, was frequently found in spoiled end products, but was rarely detected in bulk tank milk. Due to low counts in bulk tank samples and efficient spore removal during production, we suggest that shelf life is influenced only to a minor extent by raw-milk-associated factors. In contrast, recontamination with spores, particularly from the *B. cereus* complex, seems to occur. To enhance milk quality throughout the entire shelf life, improved plant sanitation and disinfection that target the elimination of spores are necessary.

Keywords: microfiltration, extended shelf life (ESL) milk, microbiota, spoilage, psychrotolerant spore-forming bacteria

INTRODUCTION

Extended shelf life (ESL) products have largely replaced conventionally pasteurized milk due to the growing demand for fluid milk products with prolonged shelf life. Technologies for producing ESL milk include high-heat treatment, high hydrostatic pressure (Datta and Deeth, 1999; Chawla et al., 2011), pulsed electric fields (Walkling-Ribeiro et al., 2011), bactofugation (Te Giffel and Van Der Horst, 2004), or microfiltration (Fernandez Garcia et al., 2013). The most commonly used technique in Germany is high-heat treatment, followed by microfiltration. To the authors' knowledge, high hydrostatic pressure and pulsed electric fields are currently not used in commercial production of ESL milk.

Combined microfiltration (MF) and pasteurization enables ESL milk production with only minor thermal treatment compared to that of ultra-high temperature (UHT) processed milk. Organoleptic properties similar to conventionally pasteurized milk are therefore maintained and promote broad consumer acceptance. In the commercial Bactocatch procedure originally proposed by Holm et al. (1989), raw milk is separated into skim milk and a cream fraction. Skim milk is filtered through ceramic membranes with a nominal pore size of 1.4 μm . Containing a large proportion of microorganisms, the retentate is then either removed or combined with the cream fraction and UHT treated (135–150°C, 2–3 s). After adjusting the fat content, the milk is pasteurized to inactivate remaining vegetative cells. The entire process reduces the bacterial load by a total of 4.6–5.6 log-units. Depending on the storage temperature, shelf lives of 22–29 days at 8°C and even 26–33 days at 6°C are achieved (Elwell and Barbano, 2006; Schmidt et al., 2012; Caplan and Barbano, 2013; Fernández García and Riera Rodríguez, 2014).

The quality of ESL milk at the end of shelf life, however, is subject to strong fluctuations and premature spoilage constitutes a considerable problem. In a study of Schmidt et al. (2012), bacterial counts ranged in between <1 and 8 log cfu/mL and 8% of all analyzed retail packages were already spoiled at the expiration date. Analyses of predominant species revealed that two microbial groups were responsible for premature spoilage: Gram-negative post-pasteurization recontaminants (PPR) and psychrotolerant spore-forming bacteria (PSF), mainly *Bacillus cereus* sensu lato and *Paenibacillus* spp. (Schmidt et al., 2012). This is in line with findings for conventionally pasteurized milk, where PSF are the limiting factor for shelf life as soon as PPR with Gram-negative organisms is sufficiently controlled (Fromm and Boor, 2004; Huck et al., 2008).

To avoid premature spoilage of ESL milk, it is essential to understand and control all potential routes along which PSF may enter the product: as part of the raw milk microbiota or as recontaminants in the dairy plant. The mild conditions of pasteurization do not inactivate spores. Consequently, spore-forming bacteria in conventionally pasteurized milk mainly originate from raw milk and are transmitted through the entire process (Huck et al., 2007; Bartoszewicz et al., 2008). However, in conventionally pasteurized milk recontamination with spore-forming bacteria was found to occur at different stages of

processing (Svensson et al., 1999, 2000; Enero et al., 2001; Salustiano et al., 2009; Kumari and Sarkar, 2016). The entry points for ESL milk are not clear yet; however, microfiltration reduces spore transmission from raw milk. Data concerning the removal of PSF that are naturally present in raw milk is not available to date, but retention rates achieved in spiking experiments ranged between 2.0 and 4.5 log-units (Trouvé et al., 1991; te Giffel et al., 2006; Tomasula et al., 2011). Their small size prevents the complete removal of spores, which makes them an inherent problem.

In this study, we aimed to extend knowledge about the dominant microbiota and spoilage-inducing organisms in microfiltered and pasteurized ESL milk. We also assessed the origin of PSF and possible routes of transmission along the production chain.

MATERIALS AND METHODS

The study consists of three parts (Supplementary Figure S1): (i) analyzing end products, by determining total aerobic bacterial counts (TACs) and predominant microbiota at the end of shelf life; (ii) analyzing bulk tank milk, including counts of mesophilic and psychrotolerant spores, as well as biodiversity of psychrotolerant spores; and (iii) process analysis addressing the removal of psychrotolerant spores along the production chain. We compared isolates obtained from different process stages using species affiliation and strain typing to ultimately investigate possible transmission and recontamination. For each batch of end products, the corresponding bulk tank milk was obtained, stored, and partially used for further analysis. We also included samples of bulk tank milk as well as end products of process analyses in other parts of the study.

Assessment of Microbial Counts and Dominant Species in ESL Milk

To assess the dominant microbiota and bacterial counts in ESL milk, a total of 287 retail packages were analyzed at the end of shelf life. The samples represented 39 batches (different production dates) from four different dairies and included conventional ($n = 103$) and organic ($n = 184$) milk with fat contents of 1.5% ($n = 117$), 3.5% ($n = 151$), and 3.8% ($n = 19$). Of each production batch, several packages containing 1 L ESL milk were analyzed. We examined 111 packages of 14 batches for dairy A, 53 packages of nine batches for dairy B, 74 packages of nine batches for dairy C, and 49 packages of seven batches for dairy D. End products and the corresponding bulk tank milk were obtained for 33 batches (236 packages; Supplementary Figure S1). The end product was analyzed immediately; the bulk-tank-milk samples were stored at –20°C and analyzed later for PSF counts (see Determination of Mesophilic and Psychrotolerant Spore Counts in Bulk Tank Milk). Six batches of end products (51 packages) originated from process analyses described in part Section “Identification of Bacterial Isolates.”

The milk was shipped cooled to between 6 and 8°C via overnight express; end products were incubated at the recommended storage temperature of 8°C until end of shelf

life. Each package's TAC and microbial composition were subsequently analyzed. Serial dilutions were prepared in Ringer solution, plated on tryptic soy agar (TSA; Roth, Karlsruhe, Germany) in duplicates, and incubated for 5 days at 30°C. Resulting colonies were counted and the relative abundance of each distinct colony morphology was estimated. The four to seven most dominant morphologies were subcultured on TSA to determine each package's predominant microbiota. All resulting pure cultures ($n = 1590$) were identified using FTIR spectroscopy and representative isolates of PSF were further identified by 16S rDNA or *rpoB* sequence analyses.

Determination of Mesophilic and Psychrotolerant Spore Counts in Bulk Tank Milk

A total of 360 bulk-tank-milk samples were obtained from the bulk tanks of 12 dairies, including dairies A–D, and from an additional eight dairies that do not produce ESL milk. The milk was shipped cooled via overnight express and analyzed directly upon arrival at our laboratory or after refrigeration at –20°C. To assess seasonal influences on spore counts, the samples were collected over a time span from February 2014 to January 2016. Between 5 and 23 samples were analyzed each month.

To enumerate mesophilic spores, milk samples were held at 80°C for 10 min and plated on TSA in duplicates. Colonies were counted after incubation at 30°C for 2 days ($n = 354$). Due to their low concentration in raw milk, psychrotolerant spores were enumerated using the five-tube most probable number (MPN) method described by McGuigan et al. (2002) with several modifications ($n = 360$). The bulk-tank-milk sample was thoroughly mixed and split into five aliquots of 10, 1, and 0.1 mL each. The final volume of all tubes was set to 10 mL with tryptic soy broth (TSB; Merck, Darmstadt, Germany). An additional sterile tube with 10 mL TSB was included as negative control. The samples were held at 80°C for 10 min and subsequently cooled down in ice water. 100 µL of L-alanine (Merck, Darmstadt, Germany; 10% w/v in phosphate buffer 0.01 M, pH 7.2) were added as germination agent and all tubes were incubated at 6°C for 21 days to allow outgrowth and hence the detection of all psychrotolerant spores present. Afterward, one loopful of each tube was streaked on TSA and plates were incubated at 30°C for 2 days. Tubes were counted positive if at least five colonies of identical morphology were present. The spore counts were subsequently determined using MPN tables (Harrigan and McCance, 1979) taking into account the number of tubes positive for psychrotolerant growth.

Analysis of Biodiversity of Psychrotolerant Spores in Bulk Tank Milk

The biodiversity of psychrotolerant spores was assessed in 28 samples from all three parts of this study (Supplementary Figure S1). Eighteen samples were chosen to analyze the general biodiversity of PSF in bulk tank milk with varying spore counts (0.02–16 MPN/mL) and from different seasons. Six samples originated from process analyses, for which the biodiversity had already been determined (see Identification of Bacterial Isolates).

To assess whether spoilage-inducing PSF were transmitted from raw to ESL milk, four additional samples were analyzed after end products of the same batch tested positive for the growth of psychrotolerant spores. The protocol was identical for all setups, differing only in the desired number of isolates and hence the number of inoculated test tubes. All bulk-tank-milk samples were first analyzed for their MPN count. The bulk tank milk was then divided into 100 (process analyses), 160 (general biodiversity), or 300 aliquots (bulk tank milk of positive end products) containing theoretically 0.9 spores and enriched with 7 mL TSB. All samples were held at 80°C for 10 min and 100 µL of L-alanine (10% w/v) were added. After incubation at 6°C for 21 days, one loopful of each tube was streaked on TSA and incubated at 30°C for 2 days. Isolates showing growth at refrigerated temperatures, as indicated by at least five morphologically identical colonies on one plate, were then subcultured on TSA. The resulting pure cultures were identified by FTIR spectroscopy and representative bacteria were further identified by their 16S rDNA and *rpoB* gene sequence. Selected isolates were also typed at strain level.

Process Analysis of Microfiltered and Pasteurized ESL Milk

In six large-scale process analyses, the efficiency of combined MF and pasteurization for removing psychrotolerant spores was determined and isolates of PSF were obtained for subsequent strain typing. Three productions each of dairy A (A) and dairy B (B) were analyzed. The milk was treated with the Bactocatch procedure: raw milk was degassed and skim milk was filtered through ceramic membranes with a nominal pore size of 1.4 µm. After adjusting the fat content to 1.5% ($n = 5$) or 3.5% ($n = 1$) with UHT-treated cream (B) or UHT-treated cream-retentate mixture (A), the milk was pasteurized and filled. Counts of psychrotolerant spores were determined in bulk tank milk, skimmed milk, permeate, and pasteurized milk and all isolates were identified. Samples that were subjected to UHT treatment were not analyzed further. Large sample volumes were used due to low spore concentrations in bulk tank milk and expected high removal rates during processing. The following aliquots were prepared: 100 x 1 mL (A) or 2 mL (B) of bulk tank milk enriched with 7 mL of TSB, 100 x (A) or 150 x (B) 10 mL of skimmed milk, 75 x 150 mL (A) or 90 x 200 mL (B) each of permeate and pasteurized milk. Bulk tank milk, skimmed milk, and permeate were held at 80°C for 10 min to inactivate vegetative cells and all samples including the pasteurized milk that was not subjected to further heat-treatment were incubated at 6°C for 21 days. One loopful of each tube or flask was subsequently plated on TSA and the plates were incubated at 30°C for 2 days. Spore counts were then determined taking into account the number of units positive for bacterial growth relative to the total sample volume and all isolates were identified by FTIR spectroscopy and 16S rDNA or *rpoB* gene sequencing. Microbial counts and the dominating species in the resulting end products ($n = 51$) were also analyzed at the end of shelf life as described in Section “Assessment of Microbial Counts and Dominant Species in ESL Milk.” For determination of possible transmission of or recontamination by PSF along the production chain, strain typing

was performed with selected isolates originating from different process steps.

Identification of Bacterial Isolates

All isolates obtained in this study were identified using FTIR spectroscopy (Oberreuter et al., 2002; Wenning et al., 2014; von Neubeck et al., 2015). The strains were cultured for 24 ± 0.5 h under the following conditions: spore-forming bacteria on TSA (Oxoid, Wesel, Germany) at 25°C , lactic acid bacteria on All Purpose Tween agar (APT; Merck, Darmstadt, Germany) at 34°C anaerobically, and all other isolates on TSA at 30°C . One loopful of the resulting confluent lawn was suspended in $100 \mu\text{L}$ of sterile water and $25 \mu\text{L}$ of the homogenous suspension were transferred to a 96-well zinc selenide sample carrier. The samples were then dried at 42°C for 45 min to form a continuous film. FTIR spectra were then recorded by a Tensor 27 spectrometer coupled to the HTS-XT device for high sample throughput (both from Bruker Optics, Ettlingen, Germany) and evaluated as described by Oberreuter et al. (2002). Data analysis was performed using OPUS software V7.2 (Bruker Optics, Ettlingen, Germany). Three in-house databases containing approximately 8000 spectra of 240 genera and 1000 species were used to identify isolates.

Representative isolates of psychrotolerant spores were also identified by their 16S rDNA or, if they belonged to the genus *Paenibacillus*, using the more discriminative *rpoB* gene sequence. Isolates were selected separately for each milk sample using hierarchical cluster analysis (HCA) of the FTIR spectra (Wenning and Scherer, 2013). At least one isolate from each cluster was selected for sequencing. The sequencing result was then extrapolated to all isolates of the initial cluster to obtain the actual abundance of all identified species.

Cell lysis and 16S rDNA amplification were performed as described by von Neubeck et al. (2015), with primers 27f ($5'$ -AGAGTTGATCCTGGCTCA- $3'$) and 1492r ($5'$ -CGGCTACCTTGTACGAC- $3'$). For amplification of the *rpoB* gene, primers StreptoF ($5'$ -AARYTIGGMCTGAAGAAAT- $3'$) and StreptoR ($5'$ -TGIARTTTRTCATCAACCATGTG- $3'$) were used, resulting in a 740 bp fragment (Drancourt et al., 2004). Cycling conditions were modified based on the protocol of Durak et al. (2006) with 20 cycles of touchdown PCR consisting of a denaturation step at 95°C for 30 s, annealing from 60 to 50°C for 30 s with temperature decrease of 0.5°C per cycle and elongation at 72°C for 1 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 s and elongation at 72°C for 1 min, with a final elongation at 72°C for 7 min. Sequencing was performed by LGC Genomics (Berlin, Germany), with primers 926r ($5'$ -CCGTCAATTCTTGAGTTT- $3'$) or 1061r ($5'$ -CRRCACGAGCTGACGAC- $3'$) for 16S rDNA and StreptoF for *rpoB* gene sequences. Identification was carried out using the EzTaxon server for 16S rDNA (Kim et al., 2012) and the BLAST algorithm for *rpoB* data (Altschul et al., 1990). For species allocation of *Paenibacillus* isolates, we established a database containing the *rpoB* gene sequences of 44 species that are commonly found in milk, including 12 type strains. Non-type strain *rpoB* sequences were obtained from NCBI or previously identified isolates. Similarity cutoffs for species and genus demarcation were set at 98.65 and 95% sequence identity

for 16S rDNA (Kim et al., 2014) and for species affiliation using the *Paenibacillus* specific *rpoB* sequence at 95% identity. All isolates with similarities below the cutoff value were classified as potential novel species or genera. To confirm phylogenetic allocation, their sequences were aligned with (type) strains of closely related species using MEGA version 6 (Tamura et al., 2013). All presumptive novel species and genera were numbered consecutively from most to least abundant.

Strain Typing of Psychrotolerant Spore-Forming Bacteria

To detect possible transmission or recontamination with psychrotolerant spores along the production chain, isolates obtained from different process stages were further characterized and compared at strain level. Included were isolates from all productions with end products containing PSF as well as process analyses where PSF were detected in more than one of the process steps (Supplementary Figure S1). In total, 10 batches were included and candidate strains for typing were selected using HCA. The relatedness of all representative psychrotolerant spores was determined for each of the 10 batches. If FTIR spectra from isolates of the same species obtained from different process steps clustered closely together, they were considered to be clones and further analyzed by randomly amplified polymorphic DNA (RAPD)-PCR. To capture additional matching strains that did not cluster together, several isolates of identical species from different process steps and *B. cereus* sensu lato as well as *Paenibacillus odorifer* strains from different batches obtained from the same dairy were included in further strain typing. In total, 90 isolates were selected.

Cell lysis for all strains was performed as described previously (von Neubeck et al., 2015). The DNA concentration of cell lysates was determined spectrophotometrically with NanoDrop® ND-1000 (Peqlab, Darmstadt, Germany) and adjusted to $50 \text{ ng}/\mu\text{L}$. Three different primers were used for RAPD analyses that had proven to be very discriminative for sporeformers in previous experiments. OPA7 ($5'$ -GAAACGGGTG- $3'$) and N5 ($5'$ -CGGCCACTGT- $3'$) (Nilsson et al., 1998) were used for every isolate and primer OPB18 ($5'$ -CCACAGCAGT- $3'$) (Woodburn et al., 1995) was only used if with one of the first primers failed to produce a clear band pattern. Amplification was performed with a KAPA2G™ Robust Hot Start DNA Polymerase Kit (Peqlab, Darmstadt, Germany). For each reaction, $5 \mu\text{L}$ Enhancer, $5 \mu\text{L}$ BufferA, $0.5 \mu\text{L}$ dNTPs (10 mM), $2 \mu\text{L}$ primer ($50 \text{ }\mu\text{M}$), $0.1 \mu\text{L}$ DNA polymerase, and $1 \mu\text{L}$ lysate were used. The following reaction conditions were used: 30 cycles of denaturation at 94°C for 30 s, annealing at 30°C (N5), 32°C (OPA7), or 35°C (OPB18) for 40 s and elongation at 72°C for 3 min with an initial denaturation step of 94°C for 3 min and final elongation at 72°C for 3 min. Another strain belonging to the species of interest was also included as outgroup. Amplification products were visualized on 2% agarose gels after gel electrophoresis at 150 V for 2 h in $0.5x$ TBE buffer. As the primers chosen were very discriminative and band patterns obtained differed largely in band number and band intensity (Supplementary Figure S2), they were evaluated visually.

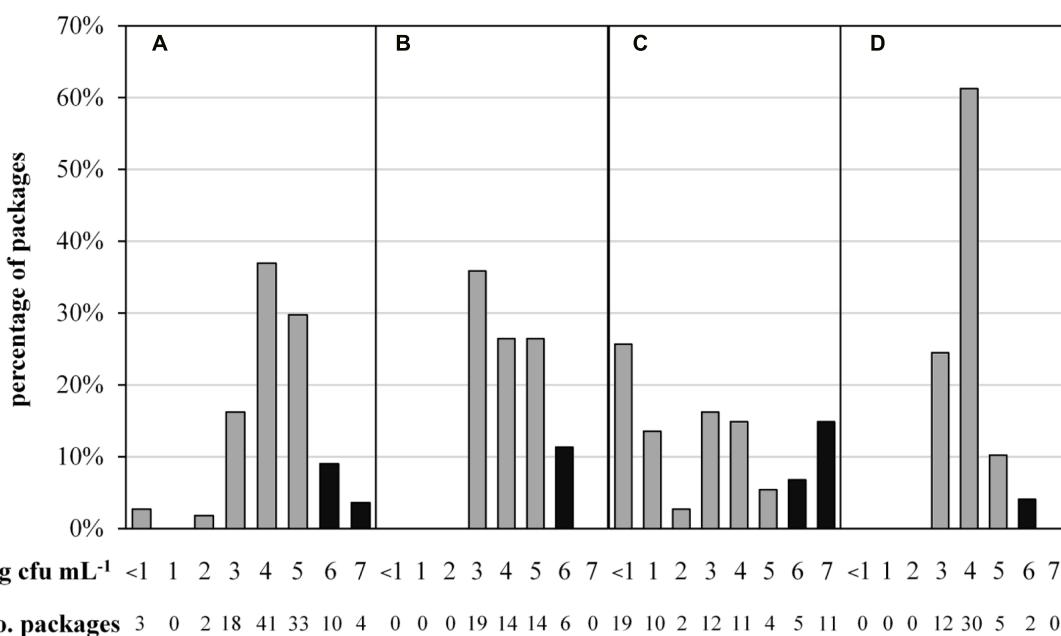


FIGURE 1 | Abundance distribution of aerobic bacterial counts in 287 packages of microfiltered and pasteurized ESL milk at the end of shelf life after storage at 8°C. Samples were obtained from four different dairies (**A**, $n = 111$; **B**, $n = 53$; **C**, $n = 74$; **D**, $n = 49$). Black bars indicate spoilage.

Statistical Analyses

Pearson's chi-squared test was used to test the association (i) between TAC of end products at the end of shelf life (<6 and ≥ 6 log cfu/mL) and prevalence of high G+C Gram-positive bacteria, Gram-negative bacteria and PSF and (ii) of the occurrence of bacterial species in bulk tank milk and ESL milk. To determine seasonal influences on counts of mesophilic and psychrotolerant spores in bulk tank milk, the concentrations determined in each month were tested for differences in central tendencies by the Kruskal-Wallis test. Pairwise comparison of months was then conducted using the Wilcoxon Rank Sum test. All tests were performed using R version 3.1.2. $p < 0.05$ was considered statistically significant and $p < 0.01$ highly significant.

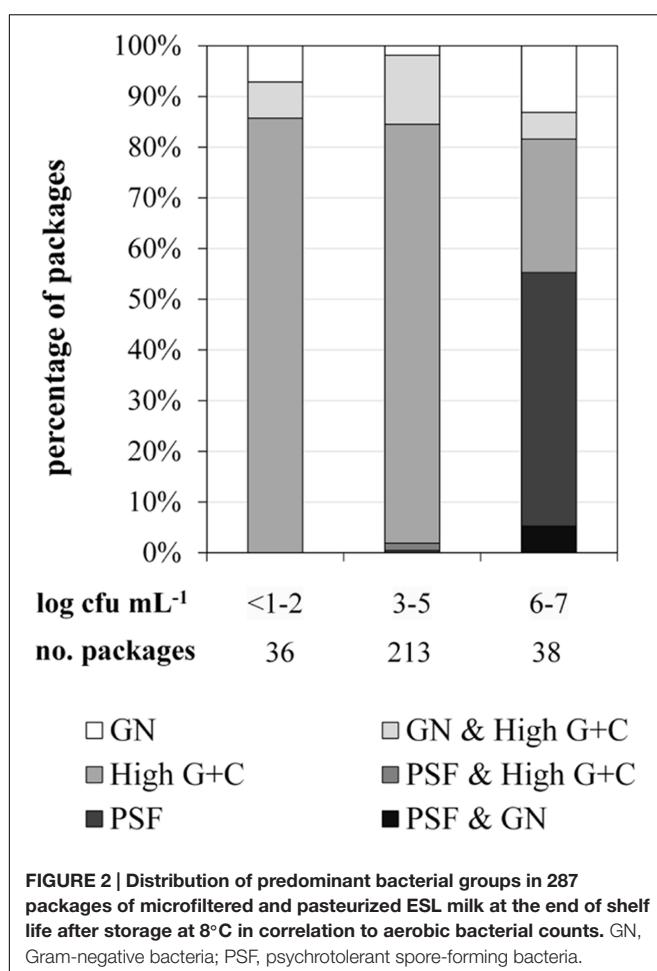
RESULTS

Microbial Composition of ESL Milk at the End of Shelf Life

To determine the microbial status of ESL milk, TAC and the predominant microbiota of 287 retail packages from four dairies were analyzed at the end of shelf life. The maximal TAC in fresh milk is unregulated in Germany, but 6 log cfu/mL is generally regarded as the limit for bacterial spoilage and was also applied in this study. Bacterial counts were highly diverse, ranging from <1.0 to 7.9 log cfu/mL. Even within single batches, TAC varied up to three log-units per mL. The majority of packages showed acceptable microbial counts, containing 3 to 5 log cfu/mL and 13% had very low TAC with as little as <1 to 2 log cfu/mL. However, a total of 15% of packages were spoiled with ≥ 6.0

log cfu/mL. Considerable discrepancies were observed between different dairies (Figure 1). Dairy C not only had the highest proportion of spoiled packages but also the largest spread of TAC and the highest proportion of packages with very low microbial counts. Dairy D's products exhibited the least differences in TAC and the lowest proportion of spoiled end products.

For FTIR-spectroscopy-based analyses of the predominant ESL milk microbiota, a total of 1590 isolates obtained from 287 retail packages were identified. Three bacterial groups were detected: high G+C Gram-positive microorganisms such as *Microbacterium*, Gram-negative recontaminants (e.g., *Moraxella* and *Pseudomonas*), and PSF (*Bacillus* and *Paenibacillus* spp.). The microbiota of packages with TAC ranging in between <1.0 and 5.9 log cfu/mL was clearly dominated by Gram-positive high G+C bacteria (Figure 2). They constituted the predominant group in more than 90% of ESL milk samples regardless whether they had an exceptionally low TAC or were almost spoiled. However, in spoiled packages with TAC >6 log cfu/mL there was a highly significant shift of the microbial composition ($p < 0.01$). The abundance of Gram-positive high G+C bacteria decreased to only 30% whereas 50% of the packages contained exclusively psychrotolerant spores and another 5% a mixed flora of psychrotolerant spores and Gram-negative recontaminants (Figure 2). The percentage of samples dominated by Gram-negative recontaminants (alone or in combination with high G+C Gram-positive bacteria) was found to range between 15 and 20% regardless of TAC. Again, there were remarkable discrepancies between different dairies. Spoiled packages from dairies B and D contained exclusively Gram-positive high G+C bacteria. Among samples from dairy C, only 20% were spoiled by this group and the remaining 80% contained PSF. Spoiled



packages from dairy A were dominated by all three groups with Gram-negative recontaminants having the highest proportion.

To elucidate the risk for milk spoilage associated with each individual group of organisms, we assessed the frequency of spoilage within packages that contained predominantly one of the bacterial groups (mixed populations excluded; **Table 1**). Whereas high G+C Gram-positive bacteria were dominant in 203 packages (71%), only 6% of these samples were spoiled. Gram-negative bacteria were found to be predominant in merely 10 packages (4%); however, half of these samples showed premature spoilage. The most important group was PSF. It was dominant in only 7% of milk samples but proliferated up to ≥ 6 log cfu/mL in 90% of these packages and thereby showed the greatest spoilage potential.

Because it constitutes the most important group of spoilage organisms in microfiltered ESL milk, the prevalence of different PSF species was further analyzed (Supplementary Figure S3). A total of 25 packages from seven batches of two different dairies contained psychrotolerant spores as the predominant bacterial group. To avoid an overestimation, each species was counted only once per positive package. This resulted in 36 representative isolates belonging to nine different species of the genera *Paenibacillus* and *Bacillus*. *B. cereus* sensu lato was by

TABLE 1 | Incidence of predominant bacterial groups in 234 packages of ESL milk at the end of shelf life after storage at 8°C and respective frequency of spoilage.

	No. of packages	Frequency of spoilage
Psychrotolerant spores	21	90%
Gram-negative recontaminants	10	50%
Gram-positive high G+C	203	5%

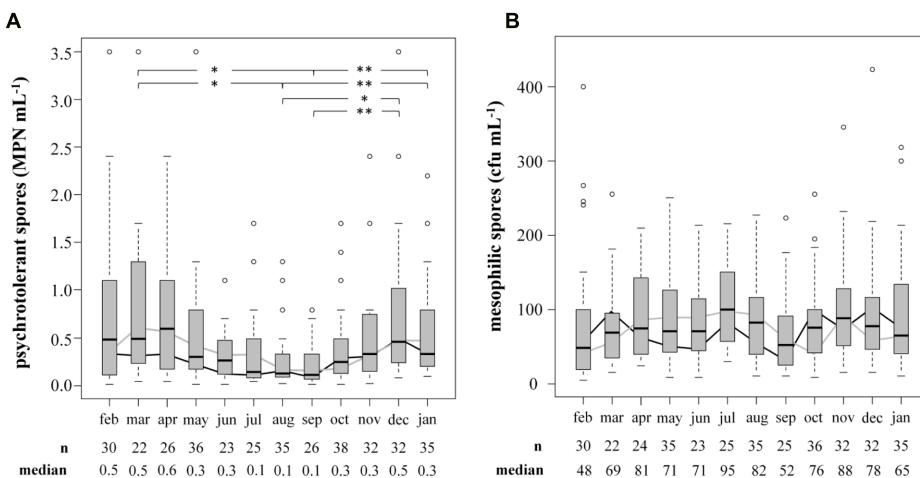
Only packages containing exclusively one bacterial group are included.

far the most abundant species group. It represented 36% of psychrotolerant spores in ESL milk and was detected in 13 packages from four different batches, corresponding to more than half of all units testing positive. Other abundant species were *P. odorifer* (33%), which was isolated from 12 packages from three batches and, interestingly, a presumptive novel species *Paenibacillus* sp. nov. 1 (6%). The latter, however, was found in only one batch. All other species of psychrotolerant spores were isolated from only one or two packages and are therefore of minor relevance.

Prevalence and Biodiversity of Psychrotolerant Spore-Forming Bacteria in Bulk Tank Milk

The prevalence and biodiversity of psychrotolerant spores were investigated to evaluate the importance of bulk tank milk as a potential source of them in ESL milk. Accounting for only 0.57 ± 0.80 MPN/mL, the concentration of psychrotolerant spores in bulk tank milk ($n = 360$) was low. In comparison, mesophilic spores accounted for 90 ± 80 cfu/mL ($n = 354$), reaching a 200-fold higher concentration. To evaluate seasonal influences on spore counts, samples were obtained over 2 years and an average of 15 samples was analyzed each month (**Figure 3**). We found the concentration of psychrotolerant spores in summer and early autumn (August and September) to be significantly lower than that in other months (March, December, January; $p < 0.05$). The median from July to September ranged in between 0.10 and 0.14 MPN/mL. During winter and spring, the spore counts increased 3- to 4-fold and in April the maximum median of 0.59 MPN/mL was detected. This trend was confirmed in both years of analysis. Additionally, not only the concentration but also the variance of psychrotolerant spores between different samples decreased in summer. In contrast, the counts of mesophilic spores showed no seasonality.

For biodiversity analyses, a total of 2634 isolates obtained from 28 bulk-tank-milk samples were identified by FTIR spectroscopy and 875 representative isolates were further identified by their 16S or *rpoB* gene sequence. Overall, a richness of 53 different species belonging to nine genera was detected (Supplementary Table S1). The two most abundant genera were *Paenibacillus* and *Bacillus*. They accounted for 80 and 10% of isolates respectively and were represented by 27 and 9 different species. At species level, *Paenibacillus amyloolyticus/xylanexedens* was clearly dominant. Remarkably, it was isolated 1250 times and thereby represented almost half of all isolates (48%). Other abundant species were *P. odorifer* (10%), *Paenibacillus*



taichungensis/tundrae (8%), the presumptive novel species *Paenibacillus* sp. nov. 1 (7%), and *Bacillus pumilus/safensis* (7%). Most of the species, however, were rare and occurred with fractions of at most 3% each. The 35 least abundant species together accounted for as little as 3.5% of isolates, confirming an uneven abundance distribution. Among the 2634 isolates, there was a considerable number of species and genera that displayed only low similarity of <98.65% for 16S rDNA and 95% for *rpoB* gene sequences to known species. Some strains had a similarity of <95% for 16S rDNA. Although no additional experiments were carried out to confirm the distinctness of those isolates from known species and genera, the genetic data give a strong indication that they belong to hitherto unknown species. In total, 18 potential novel species and three novel genera were detected, which together made up 14% of all isolates. A remarkably great abundance was found for two of the novel species that were assigned to the genus *Paenibacillus*. They represented respectively 6.6 and 3.2% of isolates.

Spore Removal during Processing of ESL Milk

The efficiency of combined MF and pasteurization for removing psychrotolerant spores was analyzed during the production of six batches of organic ESL milk from two dairies. Samples were taken at five different points throughout processing: bulk tank milk, after separation of cream, after MF, after pasteurization, and after filling.

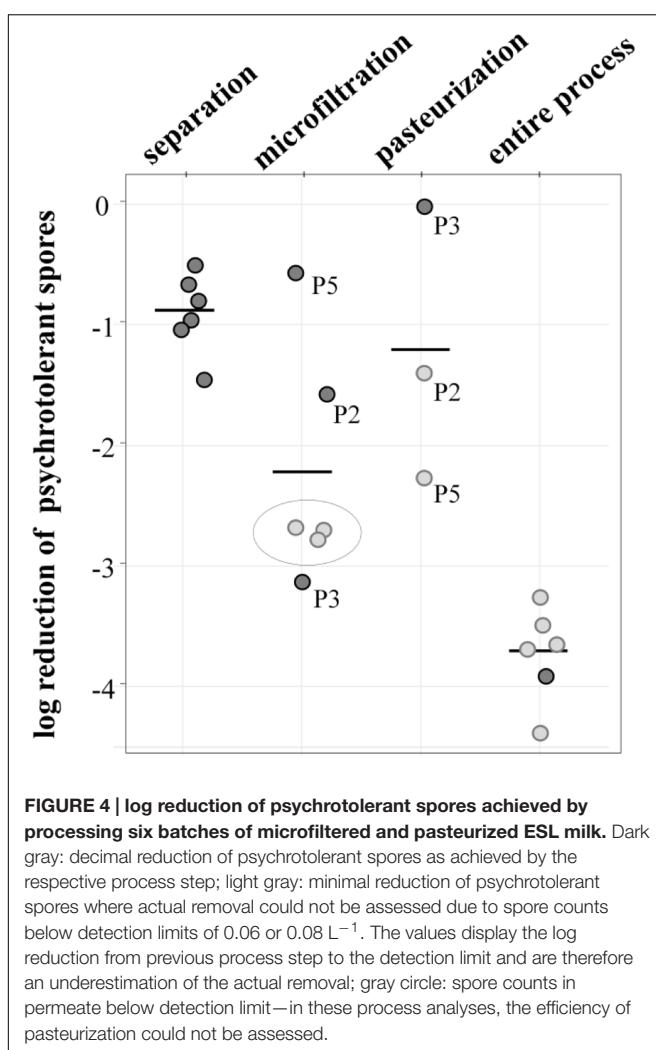
Overall, the process achieved a potent reduction of 3.7 log-units (Figure 4). The initial separation of cream already led to a decrease of 0.9 log-units, but the largest removal rate was attained during MF, accounting for 2.2 log-units on average. Final pasteurization achieved an additional 1.2 log reduction.

However, the available data show differences for independent runs, especially for MF. Here, the values for decimal reduction of psychrotolerant spores ranged from 0.6 or 1.5 log-units in impaired processes, up to 3.1 log-units. Interestingly, we found pasteurization to be more efficient (-1.4 and -2.4 and log-units, respectively) after runs with dysfunctional MF (P2, P5) than after those with fully functional MF (no further removal in P3). This was unexpected, because pasteurization conditions are unable to inactivate spores. Underestimation of removal by MF may be one reason for the high effectiveness determined in the pasteurization step in process analysis P5 (Figure 4). Here, the permeate samples were taken during the first hour of the MF process. Retention of microbial cells in the beginning was possibly less efficient due to a lack of protein layer that becomes established during the first hour of MF and contributes to bacterial retention. In P2 and P3, pasteurized milk was sampled after 3–4 h.

The data generally has some limitations due to very low spore counts as indicated in Figure 4. Despite large sample volumes of up to 18 L, no spores were detected in several permeate and pasteurized milk samples, corresponding to counts of <0.06 or 0.08 MPN/L. In consequence, only minimal or in case of three pasteurizations, no reduction rates could be assessed. The given removal of psychrotolerant spores is therefore underestimated to a certain degree.

Determination of Psychrotolerant Spore-Forming Bacteria's Entry Points along the Processing Chain

To assess the role of raw milk as a possible source of psychrotolerant spores in end products, we compared different production batches of previous analyses in detail. Seven of the 39 milk batches contained end products with PSF. For



six of these, the corresponding bulk tank milk was available and had been analyzed for biodiversity of PSF. The species composition of bulk-tank-milk samples (819 isolates) and their corresponding end products (35 isolates) were initially compared. Conspicuous differences were discovered among the prevalences of all species found in the six ESL milk batches and their abundance in bulk tank milk (Figure 5). Whereas *P. amylolyticus/xylanexedens* was clearly the most dominant species in bulk tank milk, combined MF and pasteurization entirely removed it. Not even a single isolate was obtained from ESL milk. In contrast, *B. cereus* sensu lato represented merely 0.4% of bulk tank milk isolates but 35% of psychrotolerant spores in ESL milk. So the proportion of *B. cereus* sensu lato among the isolates increased almost 90-fold during milk processing. Also, *Bacillus circulans* and *Paenibacillus algorifonticola* were isolated exclusively from end products. For all these species, no association was found between the occurrence in bulk tank milk and that in end products ($p < 0.01$). Several other spore-forming bacteria (e.g., *P. odorifer*, *P. taichungensis/tundrae*, the presumptive novel species *Paenibacillus* sp. nov. 1, and *B. pumilus/safensis*) were detected to a similar degree in raw

milk and end products. This was confirmed by the Pearson's chi-squared test ($p = 0.17$).

To gain further insight into possible points of entry, RAPD typing was performed with 90 strains from different samples and process steps. The presence of identical strains at different stages of the production process provides evidence for the vertical transmission of microorganisms along the production chain. Missing consistencies between strains found in end products and different processing steps would point rather to horizontal transmission (recontamination events).

Transmission of psychrotolerant spores from bulk tank milk to later process stages was observed in 5 of 10 batches of ESL milk (Table 2), but psychrotolerant spores were found to reach the end product from only three batches (batches 1, 4, and 5). It is remarkable that this was attested in two cases for strains that were highly abundant in bulk tank milk: in batches 1 and 5, both having about 10 times higher spore counts in the bulk tank milk than other batches. In batches 2 and 3, the transmission did not extend to the final product, but terminated earlier. *P. odorifer* (batch 2) was found in bulk tank milk, skim milk, and permeate, but it was absent in the end products. As determined during process analysis, spores in this batch were inefficiently removed during the MF step (-0.6 log-units). However, at least partial underestimation of MF efficiency is likely, most probably due to early sampling at the very beginning of the production as already mentioned. Batch 3 constituted an exception. Here, the identical *B. cereus* sensu lato strain was detected in raw, skim, and pasteurized milk although it represented only the 5th most abundant species in bulk tank milk (5%) and the MF was very efficient (-3.1 log-units). Interestingly, in the same batch another *B. cereus* sensu lato strain was isolated exclusively from one end product and not from the bulk tank milk.

A large fraction of all typing results yielded no match with other isolates. Identical strains in different batches from the same dairy would provide evidence for a persisting recontamination source, but all *B. cereus* sensu lato strains that were obtained from two batches from dairy A and *P. odorifer* strains obtained from two dairy-C batches showed unique RAPD profiles and could not be affiliated to the same strain. Remarkably, all 10 packages from one dairy-C batch contained *P. odorifer* and *B. cereus* sensu lato, but these were not detected in the corresponding bulk-tank-milk sample, pointing to a recontamination event.

DISCUSSION

Bacterial Spoilage of ESL Milk

In Germany, shelf life of microfiltered and pasteurized ESL milk is generally regulated to a maximum of 24 days. Under storage at the recommended temperature of 8°C , the product should remain safe and palatable for the entire period. The results of this study, however, clearly demonstrate that microbial growth constitutes a major problem for maintaining the impeccable quality of ESL milk. Of 287 retail packages that were analyzed at the end of shelf life after storage at 8°C , a total of 15% were spoiled, containing $6 \log \text{ cfu/mL}$ up to almost $8 \log \text{ cfu/mL}$. Overall, the microbial counts were higher than in a previous

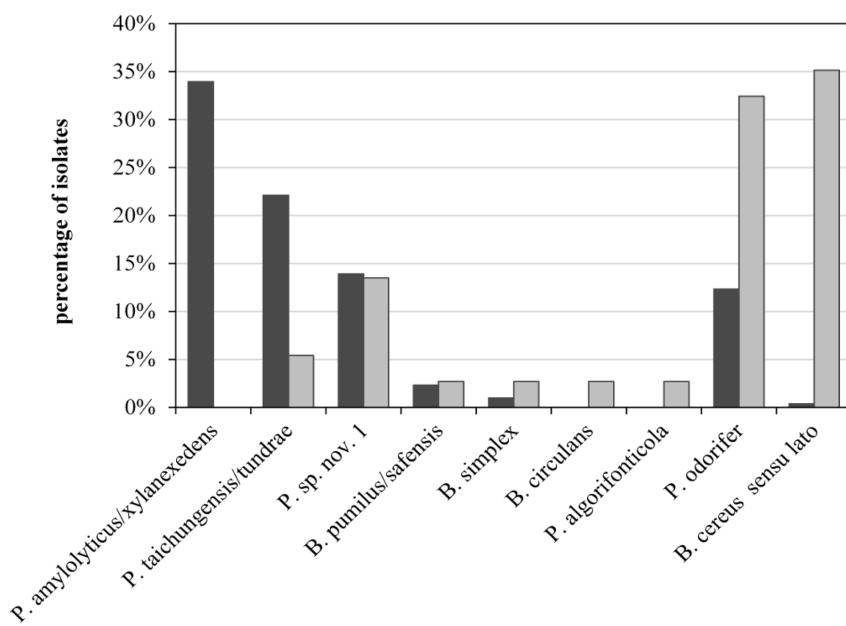


FIGURE 5 | Abundance of psychrotolerant spore-forming bacteria at species level obtained from bulk tank milk (black bars, $n = 819$) and corresponding end products (gray bars, $n = 35$) from six batches of microfiltered and pasteurized ESL milk.

study conducted at our institute (Schmidt et al., 2012), but mainly with milk from different dairies. Here, about 50% of all packages had counts below 3 log cfu/mL. Yet, accounting for 8% of all samples, a large fraction was spoiled. Identifying and eliminating the bacteria contributing to premature spoilage is therefore important for increasing the quality of ESL milk during its entire shelf life.

Three bacterial groups were found to be relevant for microbial spoilage: Gram-negative recontaminants, high G+C Gram-positive bacteria, and PSF. Gram-negative bacteria were isolated from 15% of all packages and were predominant in 20% of spoiled samples, showing that PPR is still not optimally controlled. High G+C Gram-positives were responsible for 31% of premature spoilage. They were by far the most abundant group and isolated as only bacterial group from 203 ESL milk packages. Their spoilage potential however was low. Merely 5% of the positive

samples were spoiled, revealing low growth rates at refrigeration temperature. This confirms the findings of Schmidt et al. (2012), who isolated *Microbacterium* spp. from 65% of packages that all retained TAC <6 log cfu/mL throughout the entire shelf life. The third group, PSF, was most relevant for the quality of ESL milk. Despite their relatively low abundance (7%), *Bacillus* spp. and *Paenibacillus* spp. were isolated from 55% of all spoiled samples. 90% of packages testing positive contained 6 to 7 log cfu/mL, confirming their ability to proliferate well under refrigerated conditions (Meer et al., 1991; Huck et al., 2007). The presence of psychrotolerant spores in milk packages thus implies a 90% risk of premature spoilage. Ranieri et al. (2012) stated that even one *Paenibacillus* spore is sufficient to induce spoilage during shelf life. The results are in line with Schmidt et al. (2012), who reported that *B. cereus* sensu lato and two *Paenibacillus* species were responsible for two thirds of spoiled

TABLE 2 | Summary of all transmission events of psychrotolerant spore-forming (PSF) bacteria from bulk tank milk to later process stages as determined by RAPD typing of 90 strains from 10 batches of ESL milk.

Batch	Species	Isolated from	Bulk tank milk	
			Abundance of species [% of PSF]	PSF count [MPN/mL]
1	<i>P. taichungensis/tundrae</i>	BM SM PE EP	90%	1.84
2	<i>P. odorifer</i>	BM SM PE	5%	0.17
3	<i>B. cereus</i> sensu lato	BM PA	5%	0.63
4	<i>B. pumilus/safensis</i>	BM EP	5%	0.33
5	<i>P. sp. nov. 1</i>	BM EP	42%	1.70
	<i>P. odorifer</i>	BM EP	31%	

In five batches, no transmission was observed (data not shown). For batches 1–3, isolates were obtained from bulk tank milk (BM), skim milk (SM), permeate (PE), pasteurized milk (PA), and end products (EP) — for batches 4 and 5, from bulk tank milk and end products only.

packages. These two genera were also found to be most relevant for product spoilage in traditionally pasteurized milk, as soon as Gram-negative recontamination was avoided (Fromm and Boor, 2004; Huck et al., 2007, 2008). Reduction of PSF counts therefore seems to be an important step toward reducing the incidence of ESL milk spoilage. But unlike Gram-negative bacteria that always result from product recontamination, the source of spore-forming bacteria is much more difficult to determine. Since they are resistant to pasteurization, they are assumed to originate from bulk tank milk. However, contamination of milk in the dairy plant has been reported (Svensson et al., 1999; Eneroth et al., 2001).

Counts and Biodiversity of Psychrotolerant Spore-Forming Bacteria in Bulk Tank Milk

Raw milk constitutes a potential source of PSF in ESL milk. The prevalence of PSF in bulk tank milk was assessed to gain insight into its role in the contamination process. The concentration of psychrotolerant spores was extremely low. They accounted for merely 0.57 MPN/mL, ranging between <0.02 MPN/mL and up to 16 MPN/mL in two outliers. These values accord with previous reports that found counts between 0.001 and 6.300 spores/mL (McKinnon and Pettipher, 1983; Meer et al., 1991; Mayr et al., 1999; McGuigan et al., 2002; Masiello et al., 2014).

Regarding seasonal influences on psychrotolerant spore counts, we detected a marked decrease in summer and early autumn (July to September). Despite large variations between individual samples, these differences were significant for August and September in contrast to March, December, and January ($p < 0.05$). For other months that showed no significance, the trend was still confirmed in two consecutive years of comparative analyses and was further supported by the missing seasonality of mesophilic spores obtained from identical bulk-tank-milk samples. To our knowledge, only three studies so far have investigated the seasonal effects on psychrotolerant spore counts with conflicting results (McKinnon and Pettipher, 1983; Sutherland and Murdoch, 1994; McGuigan et al., 2002). McKinnon and Pettipher (1983) analyzed the concentration of psychrotolerant spores in the bulk tanks of four farms and found no significant differences in between summer and winter. McGuigan et al. (2002) likewise reported that fluctuating spore counts in bulked milk from one processing plant were related neither to meteorological temperature nor to relative humidity. In contrast, Sutherland and Murdoch (1994) detected psychrotolerant spores exclusively in late summer and autumn, when counts in our study were especially low. These contradictory findings may result from the large variety of contamination sources that, depending for example on farm management practices, influence the composition of the milk microbiota to varying degrees. This influence may have changed over time due to altered farm management practices and for the same reason may also depend on the geographical region. The main sources of spores when housing cows are the bedding material (Magnusson et al., 2007), but also the feed (Te Giffel et al., 2002; Vissers et al., 2007). Whenever spores are present

in high numbers, for instance in the silo, they are excreted in the feces and may contaminate the teat surface (Magnusson et al., 2007). Elevated spore levels in winter and spring may result from lower temperatures that inhibit the proliferation of accompanying bacteria and thereby promote the growth of PSF during the storage of bedding material or feed. The fact that we found identical predominant species of psychrotolerant spores independently of the season further suggests that they originate from the same source and only reach higher counts due to improved growth conditions.

Biodiversity analyses of 2634 isolates from 28 bulk-tank-milk samples revealed a broad biodiversity of PSF. Of nine genera that were detected, *Paenibacillus* and *Bacillus* were most frequently isolated. This is in line with previous studies that described these two genera as the most important dairy-associated sporeformers (Coorevits et al., 2008; Ivy et al., 2012; Masiello et al., 2014). At species level, the clear dominance of a single group, namely *P. amylolyticus/xylanexedens*, was detected. It accounted for more than 45% of all isolates. In comparison, a majority (41 species) of the remaining 52 species contributed less than 1% each. An uneven abundance distribution with several predominant species or genera and a large fraction of rare bacteria is frequently described in biodiversity analyses (Quigley et al., 2013; von Neubeck et al., 2015). In the context of dairy-associated psychrotolerant sporeformers for example, Ivy et al. (2012) found that six different species (*B. cereus* sensu lato, *Bacillus licheniformis*, *B. pumilus*, *P. odorifer*, *P. amylolyticus/xylanexedens*, and *Paenibacillus graminis*) represent more than 80% of isolates. The clear dominance of one species to the extent revealed in this study, however, is striking and clearly demonstrates its adaptation to the farm and/or dairy environment and its ability to proliferate under the given conditions. Almost 14% of all isolates were affiliated with presumptive novel species (7%) or even novel genera (3%). This high percentage is in line with findings from previous biodiversity studies (Hantsis-Zacharov and Halpern, 2007; Fricker et al., 2011; Ivy et al., 2012; von Neubeck et al., 2015). Here the authors concluded that the milk microbiota is still underexplored. This is supported by the fact that two of the presumptive novel species, *Paenibacillus* sp. nov. 1 and *Paenibacillus* sp. nov. 2 were among the seven most abundant isolates and are not yet described despite their potential technological relevance. *B. cereus* sensu lato, on the other hand, has been reported to be among the most important PSF in bulk tank milk (Vithanage et al., 2016). In this study though, it accounted for merely 0.6% of all isolates thereby indicating only minor relevance.

Points of Entry of Psychrotolerant Spore-Forming Bacteria into ESL Milk

Bacillus cereus sensu lato was the most important sporeformer in ESL milk. It was isolated from more than half of all positively tested packages and batches and accounted for one third of all isolates from end products. However among bulk tank milk isolates, *B. cereus* sensu lato represented merely 0.6%. In contrast, the third most frequently found species (*Paenibacillus* sp. nov. 1) was almost equally prevalent in end products and bulk tank milk

(Figure 5). It is tempting to assume that both species may have different points of entry into the process chain.

The occurrence of *Paenibacillus* sp. nov. 1 in bulk tank milk and end product was tested to be stochastically dependent (chi-squared), meaning that the prevalence in ESL milk is associated to the prevalence in bulk tank milk. This confirms the expectation that PSF are found in the end product only to the degree they are not removed from bulk tank milk during processing. *P. amylolyticus/xylanexedens* is by far the most prevalent and frequent species in bulk tank milk, but it is not transmitted at all to the end product. This is clear evidence for the high efficiency of spore retention during production, eliminating at least 3.7 log units as determined in this study. For the six batches of ESL milk that were positive for PSF and for which the corresponding bulk tank milk was available, transmission of identical strains could be shown in only half of the cases. In two of these three cases, bulk tank milks had an elevated spore count and transmission was attested for the dominant species only. In both cases *P. amylolyticus/xylanexedens* appeared in the bulk tank milk to a much lesser extent than on average, which explains its absence in ESL milk. Thus, transmission of spores into the end product does occur, but due to high retention during microfiltration it is only relevant in the processing of bulk tank milk with elevated spore counts.

Recontamination along the process chain is likely for species with large discrepancies between abundance in raw and ESL milk, such as *B. cereus* sensu lato and *P. odorifer* (Figure 5). This was confirmed by the Pearson's chi-squared test, which demonstrates that occurrence of both species in bulk tank and ESL milk is stochastically independent ($p < 0.01$). One reason for this is certainly their high prevalence in one production batch from dairy C, which these two species spoiled to 100%. As the bulk tank milk contained only 0.11 MPN/mL and *P. amylolyticus/xylanexedens* was clearly dominant but absent in the end product, *B. cereus* sensu lato and *P. odorifer* most probably entered the product by recontamination. There are plenty of studies reporting on different recontamination sites and the resistance of strains to cleaning and sanitizing conditions. Te Giffel et al. (1997), Faille et al. (2001), and Shaheen et al. (2010) demonstrated that spores of *B. cereus* can adhere to the surfaces of plant equipment and cleaning does not eliminate them. Svensson et al. (2004) isolated identical strains of *B. cereus* from silo tanks over a period of several months and concluded that this was an established in-house flora. Salustiano et al. (2009) was able to relate strains isolated from the surface of the silo tank and filling machine collected after sanitation to strains in the end products. Particular recontamination sites were not analyzed during our study and typing of several strains isolated from ESL milk of the same dairy did not point to persistent recontamination flora. Nevertheless, it is evident from the literature that recontamination is a widespread phenomenon and we consider this to be a probable reason for the large abundance of *B. cereus* sensu lato and *P. odorifer* in the end products of our study.

The counts in the bacterial groups with the highest spoilage potential need to be reduced to improve the milk's keeping

quality. Concerning Gram-negative bacteria, which still represent an important proportion in spoiled ESL milk products, PPR must be avoided. As high PSF counts in bulk tank milk may lead to a transfer of spores into the end product, spore counts in bulk tank milk need to be reduced at the farm level. However, recontamination with PSF is also a major factor and needs to be addressed just like PPR with Gram-negative bacteria by optimizing hygienic conditions in pipes, tanks and the filling process. Aseptic production lines may largely facilitate this task, although accompanied by higher costs.

CONCLUSION

Spore counts in raw milk are low and spores are efficiently reduced during ESL milk production. We therefore conclude that the shelf life of ESL milk is determined by raw-milk-associated factors only to a minor extent. In contrast, both recontamination by spores along the production chain, particularly from the *B. cereus* complex, and post-processing contamination with Gram-negative bacteria are important factors leading to premature spoilage. To improve the shelf life of ESL milk, special attention needs to be paid to plant sanitation and disinfection with particular emphasis on eliminating spores.

AUTHOR CONTRIBUTIONS

MW and SS conceived and designed the study. ED carried out the laboratory work. ED and MW analyzed and interpreted the data and wrote the manuscript. All authors critically revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.00135/full#supplementary-material>

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The Biodiversity of the Microbiota Producing Heat-Resistant Enzymes Responsible for Spoilage in Processed Bovine Milk and Dairy Products

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Raw bovine milk is highly nutritious as well as pH-neutral, providing the ideal conditions for microbial growth. The microbiota of raw milk is diverse and originates from several sources of contamination including the external udder surface, milking equipment, air, water, feed, grass, feces, and soil. Many bacterial and fungal species can be found in raw milk. The autochthonous microbiota of raw milk immediately after milking generally comprises lactic acid bacteria such as *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc* species, which are technologically important for the dairy industry, although they do occasionally cause spoilage of dairy products. Differences in milking practices and storage conditions on each continent, country and region result in variable microbial population structures in raw milk. Raw milk is usually stored at cold temperatures, e.g., about 4°C before processing to reduce the growth of most bacteria. However, psychrotrophic bacteria can proliferate and contribute to spoilage of ultra-high temperature (UHT) treated and sterilized milk and other dairy products with a long shelf life due to their ability to produce extracellular heat resistant enzymes such as peptidases and lipases. Worldwide, species of *Pseudomonas*, with the ability to produce these spoilage enzymes, are the most common contaminants isolated from cold raw milk although other genera such as *Serratia* are also reported as important milk spoilers, while for others more research is needed on the heat resistance of the spoilage enzymes produced. The residual activity of extracellular enzymes after high heat treatment may lead to technological problems (off flavors, physico-chemical instability) during the shelf life of milk and dairy products. This review covers the contamination patterns of cold raw milk in several parts of the world, the growth potential of psychrotrophic bacteria, their ability to produce extracellular heat-resistant enzymes and the consequences for dairy products with a long shelf life. This problem is of increasing importance because of the large worldwide trade in fluid milk and milk powder.

Keywords: microbial dynamics, psychrotrophic, *Pseudomonas*, *Serratia*, peptidase, lipase, heat-resistant enzyme, spoilage

INTRODUCTION

The dairy industry has a long tradition of safeguarding the safety and quality of consumer milk. Two main processes are at the basis of this quality system: cooling of the raw milk to temperatures below 7–10°C until processing and heating the milk in a dairy plant to produce different types of consumer milk depending on the heating process applied: pasteurized, extended shelf life (ESL), ultra-high temperature treated (UHT) or sterilized milk. The most consumed milk worldwide is either pasteurized or UHT. These heating processes eliminate pathogens and increase the shelf life of unopened packages. Pasteurized milk should be stored at refrigeration temperature (4–7°C) for a shelf life of about 2 weeks. On the other hand, UHT milk can be stored for 6–12 months at ambient temperature. However, spoilage can still happen during the predicted shelf life. Spoilage can be considered as any change, which renders a food product unacceptable for human consumption or for business to business trading. Besides physical damage to milk packaging, it is manifested by growth of microorganisms or enzymatic reactions leading to souring, changes in texture, or development of off-flavors.

The spoilage phenomena and mechanisms can be very different in the various types of consumer milk. The predicted and obtained shelf life of pasteurized milk is mainly determined by the presence and growth of aerobic psychrotrophic endospore formers of which members of the *Bacillus cereus* group are the most important spoilers, but other species of the genus *Bacillus* and allied genera are involved as well. Because the endospores resist the pasteurization process, the main spoilage mechanism is their subsequent germination and outgrowth with the production of spoilage enzymes in the pasteurized milk. Gopal et al. (2015) published a recent review for this type of spoilage. Nevertheless, it cannot be excluded that in some dairies post-pasteurization by mainly pseudomonads is still a problem leading to spoiled packages by the production of spoilage enzymes during psychrotrophic growth under refrigeration. Moreover, spoilage enzymes are already produced in the cooled raw milk by psychrotrophs like endospore formers in the vegetative state and pseudomonads. The heat resistance under pasteurization or the role of these enzymes in spoilage is largely unknown and probably of less importance in this type of milk. Native milk proteases and lipases may also be important factors limiting the shelf life of pasteurized milk in particular conditions of low bacterial counts during refrigerated storage (Santos et al., 2003).

For UHT and sterilized milk processed with a low risk of post-heat treatment contamination, the unwanted presence and even outgrowth of micro-organisms is a rare event and restricted to a few particular endospore formers of which *Bacillus sporothermodurans* is the main cause of concern (Scheldeman et al., 2006). However, the most important spoilage problem of UHT and sterilized milk and related UHT dairy products (cream, custard, evaporated condensed milk, chocolate milk, flavored milk, infant formula, drinks based on milk) is caused by enzymes which resist UHT treatment and which are mainly of bacterial origin. These bacteria are psychrotolerants such

as pseudomonads which are able to grow and to produce these thermotolerant enzymes in the cooled raw milk before heat processing. Also milk powder, which is made with a low, medium or high heat process, can contain these thermotolerant enzymes and as a consequence products made with these contaminated milk powders (e.g., desserts, ice mixes, chocolate, confectionery, reconstituted milk) can show a similar spoilage mechanism.

With the current world production and distribution systems of the food industry, there is a real need for high-quality products with ESL. The dairy industry must constantly optimize and improve the processes that result in products that meet business and consumers' demands and which can be exported over long distances and sometimes in unfavorable storage conditions without loss of quality. Despite the further development of the dairy industry in the last century, premature spoilage of milk continues to be a problem and causes considerable environmental and economic losses (Vanetti, 2009). These economic losses are caused by the direct costs of recalls of products and indirectly by the image damage to the companies concerned. A recall of consumer milk typically occurs upon complaints of gelation or sedimentation of milk or sensory deviations before the shelf life has expired. Such a recall depends on the size of the batch of processed raw milk. Recall costs involve the direct sales costs of the recalled goods but also administrative and logistical costs. It can be estimated that total costs are a multitude of the direct costs related to a recall. If the recall pertains to a product containing milk powder, the recall costs may be greater than for consumer milk.

A safe, abundant, and high-quality milk supply should be the goal of every dairy producer in the world. To achieve this, the control strategies must start at the farm and continue throughout processing. To meet increased raw milk quality standards, producers must adopt practices that reduce mastitis and bacterial contamination of raw milk. Raw bovine milk and dairy products are characterized by a wide microbial biodiversity, with more than 150 species identified (Delbès et al., 2007; Vithanage et al., 2014). Various microbial consortia of raw milk have been studied, particularly in relation to the geographical origin in order to maintain and exploit the microbial diversity in traditional dairy products (Boubendir et al., 2016). Furthermore, von Neubeck et al. (2015) estimated that about 18% of isolates from raw milk belong to hitherto unknown species, indicating that a large fraction of the milk microbiota is still unexplored. Nowadays, studies of the structure and the dynamics of milk microbiota based on a polyphasic taxonomic approach as well as culture-independent methods have advanced knowledge. In this review, the most recent findings on the biodiversity of the milk microbiota contributing to spoilage of milk and dairy products with a long shelf life at mostly ambient temperature will be discussed. The biodiversity will be dealt with on the taxonomic and enzymatic level, along with the specific technological problems caused by the heat-resistant or thermotolerant enzymes (peptidases, lipases, and phospholipases) and possible control strategies.

SOURCES OF CONTAMINATION OF RAW MILK

Milk is supposed to be sterile in healthy udder cells. When it leaves the udder it normally contains low numbers of microorganisms, typically ranging from several hundred to a few thousand colony-forming units per milliliter (CFU/mL). De Jonghe et al. (2011) measured a total aerobic plate count around 10,000 cfu/ml at the beginning of storage of the raw milk. But in some countries, raw milk may occasionally be contaminated with much higher numbers of up to 10^7 CFU/mL at the beginning of storage (Machado et al., 2015) depending on the hygienic conditions under which the milk is obtained.

The diversity of raw milk contamination is influenced by handling factors at the production farms. Numerous microorganisms, including bacteria, yeasts, and molds constitute the complex ecosystem present in milk and dairy products. At the farm level, microbial contamination of bulk tank milk occurs via three main sources: bacterial contamination from the external surface of the udder and teats, from mastitis organisms from within the udder and from the surface of the milking equipment (Murphy and Boor, 2000). Air, water, feed, grass, feces, and soil could also represent important sources of milk contamination. Vacheyrou et al. (2011) proved that most of the fungi and bacteria found in milk were also present in the barn and milking parlor environments.

The teat surface may be an important route of milk contamination (Vacheyrou et al., 2011) and a positive association has been found between udder hygiene score and bacterial counts in bulk tank milk (Elmoslemany et al., 2010). Verdier-Metz et al. (2012) have noted that the composition of the microbiota on teat skin varied qualitatively and quantitatively from one farm to another. This can be attributed to different factors including the farming practices as well as dairy breed, type of feed, type of barn, milking system and quality of milking hygiene practices (Monsallier et al., 2012). Mallet et al. (2012) have shown that teat care has more influence on the composition of technologically relevant microbial groups than on the composition of other groups such as *Pseudomonas* and other Gram-negative bacteria in milk.

Braem et al. (2012) showed that the contaminant microbiota of udder is influenced by the infection status of the udder quarters. The contaminant microbiota from non-infected quarters consists predominantly of *Aerococcus*, *Acinetobacter*, *Corynebacterium*, *Jeotgalicoccus*, *Kocuria*, *Staphylococcus*, and *Bifidobacterium* genera (Ryser, 1999; Jost, 2007; Braem et al., 2012). Besides the diversity of bacterial genera found on the teat apex of dairy cows, Braem et al. (2012) highlighted the presence of a variety of different species of *Corynebacterium* and *Staphylococcus*. The udder of dairy cows may be a source of commensal skin associated bacteria, opportunistic pathogenic bacteria, and mastitis-causing pathogens, which could be found in raw milk.

There are some conflicting results on the importance of udder hygiene in the contamination of milk, depending on the type of microorganism. Masiello et al. (2014) showed that the percentage of dirty udders in the milking parlor combined

with the herd size is significantly associated to the raw milk quality (related to psychrotrophic spore formers) and the shelf life of pasteurized milk. On the other hand, Richard et al. (1981) observed that intensive washing of milking equipment and udder preparation (individual washings) results in raw milk that contains a majority of spoilage microorganisms, such as coliforms and *Pseudomonas* spp. In contrast, minimal hygiene around the udder yields raw milk with a majority of useful cheese-making microorganisms including salt-tolerant microbiota such as *Micrococcus*, *Arthrobacter*, *Microbacterium*, *Brevibacterium*, and *Staphylococcus* spp. (Lafarge et al., 2004) and the lactic acid bacteria (LAB) (Desmasures et al., 1997b).

Regarding milking hygiene practices, the cleanliness of milking equipment and storage tanks could affect the introduction and increase in the number of pathogens and other milk quality-affecting bacteria. The contaminant microbiota may persist in water, teat cups, and milking equipment over time indicating a continuous source of microorganisms (Flach et al., 2014; Nucera et al., 2016). This persistence can possibly be explained by biofilm formation and consequent high resistance to disinfection. The milking machine type influences the level of microorganisms in milk, suggesting that these machines are microbiological reservoirs (Mallet et al., 2012). It is well established that the milking machine and storage equipment are commonly colonized by bacterial biofilms (Boari et al., 2009; Marchand et al., 2012; Teh et al., 2012, 2014a). In fact, strains belonging to *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Bacillus licheniformis*, *Serratia liquefaciens*, *Hafnia alvei*, and *Streptococcus uberis* isolated from raw milk tankers are capable of producing biofilms on stainless steel (Teh et al., 2011). In addition to the specific ability of each species or strain, the bacterial adhesion may be affected by the surface roughness and the effectiveness of cleaning processes (Cais-Sokolinska and Pikul, 2008; Vilar et al., 2012). Although biofilm formation within a tanker is of concern, the risk of biofilm development seems to be greater in other areas of a dairy plant (Darchuk et al., 2015).

The quality of water used for cleaning process could affect the contamination level on the surfaces and equipment. A farm water purification system is advised (Garcia Barbero, 1998). In a study performed by Vilar et al. (2008), the bulk-tank bacterial count increased by 12% when non-chlorinated water was used for cleaning. Drinking water and cow feed (including grass silage, soy bean meal, and pasture) are other possible routes for raw milk contamination with *Pseudomonas* spp. through fecal excretion and subsequent contamination of the udder (Marchand et al., 2009a).

Microbial contamination could be transferred from the barn environment, including settled dust and hay, to raw milk. Despite the massive microbiota in the barn, less than a third of this bacterial diversity may be found in milk samples, indicating that there is a partial barrier between barn and milk (Vacheyrou et al., 2011). Differences in housing strategy and feed formulation may contribute to the composition of the bacterial population of milk. Coorevits et al. (2010) demonstrated a greater number of thermotolerant spore-forming bacteria in milk from conventional dairy farms than from organic dairy farms. In the

latter, a higher occurrence of *Bacillus cereus* was attributed to differences in housing strategy. It remains to be investigated whether operational management could also influence other spoilage bacteria.

The wide variety of sources of contamination contribute to the complexity of raw milk microbiota; further investigation is clearly needed to fully understand the routes of raw milk contamination with particular spoilage bacteria like pseudomonads and subsequent control of these microbial sources.

COMPOSITION OF RAW MILK MICROBIOTA AND THE IMPACT OF COLD STORAGE

To understand how the specific spoilage microbiota evolves in raw milk, it is important to know the dynamics of its total microbial composition as a function of the cooled storage time. As raw milk is contaminated during the milking process, several studies have been performed with the aim of identifying the predominant microbiota present in raw cow's milk immediately after milking (**Table 1**). Although the region where milk samples were collected and the methods used for isolation and identification could influence the results obtained for the predominant microbiota in fresh raw milk, the genus *Lactobacillus* was identified within the dominant microbiota in French raw milk using agar-based methods (Vacheyrou et al., 2011) and 16S rRNA gene-based analyses (Delbès et al., 2007). *Lactobacillus delbrueckii* spp. *lactis* and *Lactobacillus casei* as well as *Lactococcus lactis*, also found in Italian milk samples, have a particular importance within the dairy industry (Bertazzoni Minelli et al., 2004; Quigley et al., 2013). *Propionibacterium freudenreichii* and *Corynebacterium* have been detected in raw milk in a recent study (Quigley et al., 2013). Therefore, the technologically relevant Gram-positive bacteria represent the most prevalent bacterial populations in fresh raw milk obtained from healthy cows and under hygienic conditions. However, according to studies described in **Table 1**, some species from the genera *Staphylococcus* and *Streptococcus* are often detected in fresh raw milk as well as members of the Clostridiales. While *Staphylococcus* and *Streptococcus* have been associated with mastitis infections (Todhunter et al., 1995; Zadoks et al., 2001), *Clostridium lituseburense* and *Clostridium glycolicum*, predominant in cow manure and dairy wastewater, are associated with environmental contamination (Liu et al., 2009; St-Pierre and Wright, 2013, 2014).

Cold storage of raw milk is normally applied to reduce the growth of most bacteria. In general, milk is not directly processed after milking and it is stored up to 4 days depending on the legislation of the country (Perin et al., 2012). In dairy processing plants, additional storage until processing is possible (von Neubeck et al., 2015). In an effort to reduce the total aerobic plate count of raw milk, a lower storage temperature (1 to 4°C) is applied, leading to the perception that raw milk could be stored for a longer period before further processing (De Jonghe et al., 2011). Cold storage creates selective conditions

for growth of psychrotrophs and considerable changes in the bacterial communities will occur.

The microbiota of fresh raw milk has been described as predominately Gram-positive, but after cold storage, Gram-negative species become predominant in most studies (**Table 2**). The differences in the predominant microbiota after refrigeration can be explained by the variety of cold storage conditions and the original raw milk microbiota in each study. The dominant Gram-negative microbiota found in raw milk stored at cold temperatures belong to the genera *Pseudomonas*, *Stenotrophomonas*, *Aeromonas*, *Hafnia*, *Acinetobacter*, *Serratia*, and *Chryseobacterium* and Gram-positives include *Bacillus*, *Paenibacillus*, *Lactococcus*, *Enterococcus*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, and *Microbacterium*. Some genera were detected less frequently in raw milk such as *Kocuria* (Lafarge et al., 2004; Mallet et al., 2012; Hanamant and Bansilal, 2013) and *Facklamia* (Rasolofe et al., 2010, 2011).

According to Lafarge et al. (2004), *L. lactis* was the most frequently detected species in French raw milk samples, along with some *Staphylococcus* species. After incubation of the raw milk at 4°C for 24 h, the majority of samples showed decreased representation of *L. lactis* and minority species such as *Lactobacillus plantarum* and *Lactobacillus pentosus* were outcompeted by other species. Despite a wide variance of the predominant groups found in raw milk after cold storage, Lafarge et al. (2004) noted an emergence of psychrotrophic bacteria such as *Listeria* spp. and *Aeromonas hydrophila*. Raats et al. (2011) examined the prevalence of Gram-positive and -negative bacteria in farm-collected raw milk samples and cold-stored dairy plant samples. The farm samples revealed a prevalence of Gram-positive bacteria (bacilli, clostridia, and actinobacteria) while the dairy plant samples were characterized primarily by Gram-negative species (Gammaproteobacteria). In a 16S rDNA based sequencing approach and despite variance in the predominant microbiota according to time and temperature of raw milk storage, Kable et al. (2016) demonstrated that raw milk microbial communities in tanker trucks in California (USA) were similar to each other even when samples were collected from different farms, transported to different locations and sampled at different times of the year. Surprisingly, these authors showed that the core microbiota (i.e., taxa present in all milk samples) of raw milk, consisting of 29 taxonomic groups, contained high proportions of *Streptococcus* and *Staphylococcus* and unidentified members of Clostridiales, but not *Pseudomonas*, which was present in relatively high proportions in some of the milk tested but entirely absent from two of the tankers examined. They also observed that *Pseudomonas*, along with psychrotrophic species of the genera *Lactococcus*, *Streptococcus* and *Acinetobacter*, tended to be present in relatively higher proportions in dairy plant silos than in the tanker trucks.

The psychrotrophic count, approximately 10% of the total count of mesophilic aerobes immediately after milking performed under hygienic conditions, may reach an average of 90% after cold storage (Sørhaug and Stepaniak, 1997; Catania et al., 2012). Rasolofe et al. (2010) noted that the biodiversity of raw milk microbiota decreased over the time of cold incubation

TABLE 1 | Predominant bacterial groups found in fresh raw milk from different countries using culture-dependent and culture-independent methods.

Country	Predominant groups		Reference
	Culture-dependent methods	Culture-independent methods	
France	Halophilic Mesophilic Aerobic <i>Pseudomonas</i> <i>Staphylococcus</i> <i>Acinetobacter</i> <i>Corynebacterium</i> <i>Streptococcus</i> <i>Lactobacillus delbrueckii</i> ssp. <i>lactis</i> <i>Lactobacillus paracasei</i> <i>Lactobacillus plantarum</i> <i>Propionibacterium freudenreichii</i>	<i>Clostridium</i> spp. <i>Clostridium lituseburense</i> <i>Clostridium glycolicum</i> <i>Lactococcus lactis</i> <i>Lactobacillus casei</i> <i>Streptococcus dysgalactiae</i> <i>Turicibacter sanguinis</i> <i>Ralstonia picketti</i> <i>Arthrobacter arilaitensis</i> <i>Corynebacterium confusum</i> <i>Staphylococcus aureus</i> <i>Enterococcus</i> spp. <i>Enterococcus faecalis</i> <i>Leuconostoc lactis</i> <i>Macroccoccus caseolyticus</i> <i>Lactococcus lactis</i> <i>Rothia</i> spp. <i>Staphylococcus</i> <i>Streptococcus</i> <i>Corynebacterium</i> <i>Clostridiales</i>	Michel et al., 2001 Vacheyrou et al., 2011 Delbès et al., 2007 Giannino et al., 2009 Kable et al., 2016
Italy			
The United States			

until psychrotrophic microbiota dominate. However, this group of cold-loving bacteria can represent more than 75% of the initial microbiota of raw milk when collected under conditions of poor hygiene (Hantis-Zacharov and Halpern, 2007; Malacarne et al., 2013).

THE MILK SPOILAGE MICROBIOTA PRODUCING HEAT-STABLE ENZYMES

While pasteurization inactivates most but not all of the bacteria found in raw milk, UHT treatment renders a product free of microorganisms in the vegetative state. However, several of the psychrotrophic microorganisms may secrete hydrolytic enzymes, which can be heat resistant from pasteurization up to UHT level. On the one hand, these hydrolytic enzymes may be an important tool for the food (dairy) industry as these enzymes may contribute to the development of cheese flavor and texture during ripening (Hasan et al., 2006; Tavano,

2013). On the other hand, the hydrolytic enzymes produced by psychrotrophic bacteria are also widely related to technological problems in milk and dairy products. *Pseudomonas* (mainly the *P. fluorescens* group), *Bacillus*, *Serratia*, and *Hafnia* have strong proteolytic potential while other species of *Pseudomonas* (mainly non-fluorescent pseudomonads), *Bacillus*, *Enterobacter*, and *Acinetobacter* are strongly lipolytic (Hantis-Zacharov and Halpern, 2007). According to the studies listed in Table 3, *Pseudomonas* is the predominant spoilage genus isolated from cold raw milk that secretes a heat-stable hydrolytic enzyme. This predominance has been detected at most sampling locations regardless of the approaches used for isolation and identification or time of milk storage.

Studies from the literature agree that *Pseudomonas* is the main genus related to milk spoilage, but within the genus, a diversity of the dominant hydrolytic *Pseudomonas* species isolated from milk samples is observed. Previous older studies focused on *P. fluorescens*, considered the main milk-spoilage species (Makhzoum et al., 1995; Liao and McCallus, 1998;

TABLE 2 | Predominant bacterial groups found in raw milk samples in different countries using culture-dependent and culture-independent methods after cold storage.

Country	Predominant groups		Storage conditions	Reference
	Culture-dependent methods	Culture-independent methods		
Algeria	<i>Stenotrophomonas rhizophila</i>		4°C for 7 days	Boubendir et al., 2016
	<i>Stenotrophomonas maltophilia</i>			
	<i>Chryseobacterium indologenes</i>			
	<i>Lactobacillus pentosus</i>		4°C for 10 days	
	<i>Lactobacillus plantarum</i>			
	<i>Acinetobacter guillouiae</i>		4°C for 21 days	
	<i>Pseudomonas fluorescens</i>		2°C for 10 days	
	<i>Bacillus cereus</i>			
	<i>Bacillus weihenstephanensis</i>			
	<i>Bacillus circulans</i>			
Australia	<i>Pseudomonas</i>		4–10°C for 10 days	Vithanage et al., 2016
	<i>Acinetobacter</i>			
	<i>Hafnia</i>			
	<i>Bacillus</i>			
	<i>Lactococcus</i>			
	<i>Microbacterium</i>			
	<i>P. fluorescens</i>		4°C for 2 days	
	<i>Pseudomonas putida</i>			
	<i>Pseudomonas stutzeri</i>			
	<i>Serratia liquefaciens</i>			
Brazil	<i>Serratia odorifera</i>			Pinto et al., 2015
	<i>Bacillus amyloliquefaciens</i>			
	<i>Bacillus subtilis</i>			
	<i>Bacillus</i> sp.			
	<i>Paenibacillus alvei</i>			
	<i>Paenibacillus macerans</i>			
	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>			
	<i>Lactococcus</i> sp.			
	<i>Enterococcus faecalis</i>			
	<i>Enterococcus faecium</i>			
	<i>P. fluorescens</i>		NI	
	<i>Acinetobacter</i> spp.			
	<i>Aeromonas hydrophila</i>			
	<i>Acinetobacter</i> sp.		4°C for 3 days	
	<i>Acinetobacter calcoaceticus</i>			
	<i>Staphylococcus aureus</i>			
Canada	<i>Staphylococcus equorum</i>			Arcuri et al., 2008
	<i>Facklamia tabacinasalis</i>			
	<i>Enterococcus faecium</i>			
	<i>Lactococcus lactis</i>			
	<i>Streptococcus uberis</i>			
	<i>Pseudomonas fluorescens</i>			
			4°C for 7 days	
			2–4°C for 24–48 h	
France	<i>Staphylococcus haemolyticus</i>			Mallet et al., 2012
	<i>Staphylococcus aureus</i>			
	<i>Staphylococcus saprophyticus</i>			
	<i>Staphylococcus hominis</i>			
	<i>Staphylococcus epidermidis</i>			
	<i>L. lactis</i>			
	<i>Enterococcus faecalis</i>			
	<i>Kocuria rhizophila</i>			
	<i>Stenotrophomonas maltophilia</i>			

(Continued)

TABLE 2 | Continued

Country	Predominant groups		Storage conditions	Reference
	Culture-dependent methods	Culture-independent methods		
Germany	<i>Acinetobacter johnsonii</i>			
	<i>Pseudomonas</i>		5°C for 24–48 h	Richard and Houssu, 1983
	<i>Fravobacter-Cytophaga</i>			
	<i>Coliforms</i>		8–12°C for 24–48 h	
	<i>Pseudomonas</i>			
	<i>Pseudomonas</i>		4°C for 24 h	Desmases et al., 1997a
	<i>Lactococci</i>			
	<i>Micrococcaceae</i>			
	<i>Pseudomonas proteolytica</i>		4–5°C for 3–4 days	von Neubeck et al., 2015
	<i>L. lactis</i>			
Israel	<i>Acinetobacter</i> sp. nov.			
		<i>Pseudomonas</i>	4°C for 22–102 h	Raats et al., 2011
		<i>Acinetobacter</i>		
The United States	<i>Pseudomonas</i>			
	<i>Klebsiella</i>		7°C for 12–18 h	Jayarao and Wang, 1999
	<i>Enterobacter</i>			
	<i>Escherichia</i>			
Tunisia	<i>Pseudomonas</i>		4°C for 24–96 h	Mankai et al., 2003
	<i>Aeromonas</i>			

NI – The storage conditions were not informed.

Matselis and Rouissi, 1998; Ahn et al., 1999; Kumeta et al., 1999; Woods et al., 2001). However, the taxonomy of the genus *Pseudomonas* is very complex and many new species have been described in the *P. fluorescens* group for which phenotypic methods lack discriminatory power, so the role of *P. fluorescens* in milk spoilage has been overestimated (Marchand et al., 2009a). Even with the application of the sequencing of 16S rDNA and housekeeping genes (*rpoB*, *gyrB*) and comparison with an up to date in house database for *Pseudomonas*, a recent study on different food matrices could not identify all isolates to the exact species status with many of them classified as closely related to a known species (referred to as the species name + ‘-like’) (Caldera et al., 2016). In that study, besides the species *P. fragi*-like) and *P. gessardii*-like known as milk spoilers (Marchand et al., 2009b; De Jonghe et al., 2011), several other species as *P. proteolytica*, *P. brenneri*, and *P. rhodesiae* were found in raw milk, and *P. peli*-like in pasteurized milk. Mostly after applying culture-independent methods for identifying the spoilage microbiota, other species belonging to *Pseudomonas* genus have been identified and characterized (von Neubeck et al., 2016). The peptidase producer *Pseudomonas lundensis* was isolated from raw milk samples from Belgium (Marchand et al., 2009a,b), from Germany (von Neubeck et al., 2015) and from Brazil (Machado et al., 2015). Two novel species, *Pseudomonas helleri* and *Pseudomonas weihenstephanensis*, isolated from cow milk, were characterized based on genetic, phylogenetic, chemotaxonomic, physiological, and biochemical data (von Neubeck et al., 2016). Other studies have demonstrated the (UHT) heat resistance of enzymes produced by *P. weihenstephanensis*, *Pseudomonas*

proteolytica, and *Pseudomonas panacis* (Baur et al., 2015b; Stoeckel et al., 2016a).

Acinetobacter (like *Pseudomonas* also member of Gammaproteobacteria) is frequently detected in cold raw milk samples (Table 2). Strains belonging to this psychrotrophic genus may produce enzymes (Snellman et al., 2002; Salwan and Kasana, 2013) which could potentially lead to milk spoilage. Although some studies have detected hydrolytic strains of *Acinetobacter* in raw milk samples (Nörnberg et al., 2010; von Neubeck et al., 2015; Vithanage et al., 2016), the heat resistance of these enzymes and the spoilage potential from this genus is not well characterized and requires further investigation. *Chryseobacterium* (previously classified in *Flavobacterium*) also appears as a dominant member of Algerian cold raw milk (Table 2) and some species like *Chryseobacterium joostei* have been described recently as showing an even greater spoilage capacity than *P. fluorescens* in milk on the basis of growth rate, proteolytic and lipolytic activity (Bekker et al., 2015, 2016). However, besides proteolytic enzymes being resistant to pasteurization, resistance of these enzymes to UHT is not known.

The wide biodiversity of the microbiota of cold raw milk has led to less frequent reporting of several spoilage species. Although the predominance of *Pseudomonas* is well known, the importance of *Serratia* has been described more recently. Along with strains belonging to *Pseudomonas*, *Serratia* was also detected and characterized as a predominant milk spoiler in Australian, Brazilian, and Italian samples (Table 3). Teh et al. (2011) and Cleto et al. (2012) have detected *Serratia* in milk-processing plants and raw milk road tankers, respectively, while Lo et al.

(2016) reported that *P. fluorescens* and *Serratia* were responsible for spoilage of raw milk stored at 4°C for 7 days. Lo et al. (2016) also showed that *Serratia* was slightly more dominant than *P. fluorescens* (50% vs. 42%) in a raw milk sample collected in a small Australian farm during the autumn. Besides *Serratia*, other psychrotrophic bacteria belonging to Enterobacteriaceae have been isolated from cold raw milk and have been identified as potential milk spoilers due to heat-resistant enzymes. Tondo et al. (2004) described the extensive coagulation of milk proteins after incubation with the heat-resistant peptidase of *Klebsiella oxytoca*. Other examples of the enteric group often detected in raw milk samples are *H. alvei*, *Hafnia paralvei*, and *Enterobacter aerogenes*, which are not only predominant species in raw milk, but also the most enzymatically active genera (Chen et al., 2011; Vithanage et al., 2016).

Although spoilage microbiota in raw milk is mostly Gram-negative and psychrotrophic, some Gram-positive genera have been highlighted, including *Bacillus*, *Paenibacillus* (both containing psychrotrophic members) as well as thermophilic *Geobacillus*. *Paenibacillus polymyxa*, *B. cereus*, *B. licheniformis*, and *Bacillus subtilis* are frequently linked to milk spoilage (Ternström et al., 1993; De Jonghe et al., 2010; Hanamant and Bansilal, 2012; Ranieri et al., 2012; Gopal et al., 2015) together with *Geobacillus thermoleovorans* and *Geobacillus stearothermophilus* (Sadiq et al., 2016). According to De Jonghe et al. (2010) and Sadiq et al. (2016), they are the producers of spoilage enzymes more particularly heat-stable lipase (Vithanage et al., 2016) that may adversely affect the quality of milk powder

and dairy products made with milk powder (Chen et al., 2003). At refrigeration temperatures (e.g., 5–7°C), spores without heat activation do not germinate and remain stable in milk (De Jonghe et al., 2010) and unless vegetative cells are present in biofilms on the milking or dairy equipment with the release of spoilage enzymes prior to heat treatment (Chen et al., 2004), it is questionable whether these spore formers play a role in spoilage of milk with long shelf life.

HEAT-STABLE SPOILAGE ENZYMES PRODUCED BY PSYCHROTROPHIC MICROORGANISMS

In general, the majority of psychrotrophic microorganisms isolated from milk have the ability to produce hydrolytic enzymes that break down the major milk constituents such as protein, fat and lecithin (Sørhaug and Stepaniak, 1997; Baur et al., 2015a; von Neubeck et al., 2015). Several peptidases, lipases and phospholipases produced by psychrotrophic bacteria isolated from milk have been described in the literature (Sørhaug and Stepaniak, 1997; Chen et al., 2003; Samaržija et al., 2012). Many of these hydrolytic enzymes are heat resistant and consequently retain part of their activity after conventional heat treatment applied in dairy industries such as pasteurization and UHT treatment. Regarding quality and economic aspects, the thermostable hydrolytic enzymes have the most significant effect in dairies since these enzymes lead to flavor defects and

TABLE 3 | Predominant spoilage species isolated from cold raw milk using culture-dependent and culture-independent methods.

Country	Predominant groups		Reference
	Culture-dependent methods	Culture-independent methods	
Australia		<i>P. fluorescens</i> <i>Serratia</i>	Lo et al., 2016
Belgium	<i>Pseudomonas lundensis</i> <i>Pseudomonas fragi</i>		Marchand et al., 2009a
Brazil	<i>Pseudomonas</i> spp. <i>Serratia liquefaciens</i>		Machado et al., 2015
Italy	<i>Pseudomonas</i> spp. <i>Enterobacter cloacae</i> <i>Hafnia alvei</i> <i>Serratia marcescens</i> <i>Citrobacter freundii</i>		Decimo et al., 2014
Germany		<i>P. proteolytica</i> <i>Pseudomonas</i> sp. nov. (1) <i>P. lundensis</i> <i>P. fragi</i> <i>Acinetobacter</i>	von Neubeck et al., 2015
Sweden and Norway	<i>P. fluorescens</i> biovar I <i>P. fluorescens</i> biovar III <i>P. lundensis</i> <i>P. fragi</i>		Ternström et al., 1993
The United States	<i>P. fluorescens</i> <i>P. putida</i>		Dogan and Boor, 2003

technological problems such as sedimentation and gelation in UHT milk, rancidity and flavor defects in milk powder and cheese during their shelf life (Sørhaug and Stepaniak, 1997).

The Main Proteolytic Enzymes Found in Raw Milk

The term proteolytic enzyme includes all the hydrolases that act on proteins, or further degrade the fragments of them. A few synonyms of proteolytic enzymes such as peptide-bond hydrolase, peptidase or protease could be found in the literature albeit the International Union of Biochemistry and Molecular Biology (IUBMB) recommend the term peptidase (Barrett, 2001). The main problem of the peptidases secreted by psychrotrophic bacteria is that they are heat-stable, which means that they resist at least pasteurization but it is not always described to what extent these enzymes also resist the higher temperatures of UHT.

AprX is the most studied heat-stable peptidase produced by the microbiota found in raw milk, although other species isolated from milk samples may also produce peptidases different from AprX, such as *Klebsiella oxytoca* (Tondo et al., 2004) or *Serratia liquefaciens* (Decimo et al., 2014). *Bacillus* spp. show more diverse proteolytic activity than *Pseudomonas* spp., and many species may secrete more than one type of extracellular and intracellular peptidase (Nabrdalik et al., 2010). The majority of heat-stable spoilage peptidases found in milk samples maintained at refrigeration conditions are produced by Gram-negative bacteria. This section therefore focuses on heat-stable peptidases from *Pseudomonas* and *Serratia*.

Peptidase from *Pseudomonas* Isolated from Milk and Dairy Products

The misidentification within the *P. fluorescens* group and an overestimation of the relevance of *P. fluorescens* in milk and dairy products spoilage has led to a large number of works focused on purification and characterization of heat-resistant peptidase produced by the so-called species *P. fluorescens* (Azcona et al., 1989; Kohlmann et al., 1991; Kim et al., 1997; Liao and McCallus, 1998; Matselis and Roussis, 1998; Costa et al., 2002; McCarthy et al., 2004; Maunsell et al., 2006; Dufour et al., 2008; Mu et al., 2009; Martins et al., 2015; Zhang et al., 2015). Although acknowledging this problem, the species name as described in the, respectively, cited literature will be retained in this review.

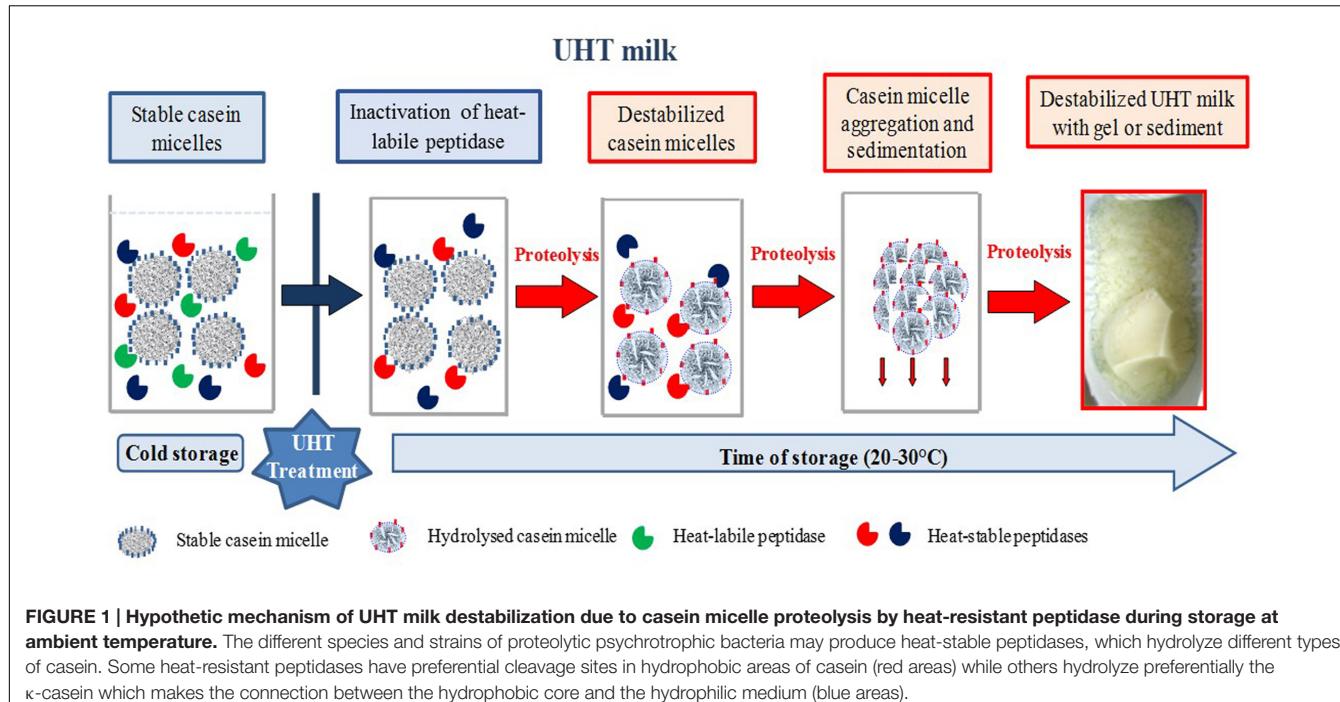
The number of the different peptidases produced by the genus *Pseudomonas* is heterogeneous and varies according to the species and the strains (Nicodème et al., 2005). Most of the studies mentioned in the paragraph above show that the strains of *Pseudomonas* spp. isolated from raw milk secrete at least one monomeric peptidase with molecular weight varying between 23 and 56 kDa. The metallopeptidase AprX is the main heat-resistant peptidase in the genus *Pseudomonas* related to milk spoilage targeted in the literature (Liao and McCallus, 1998; Dufour et al., 2008; Marchand et al., 2009b; Baglinière et al., 2013; Martins et al., 2015). This enzyme is mainly secreted by the species *P. fluorescens*, but this peptidase has also been detected in various other species found in raw milk belonging to the genus *Pseudomonas* such as *P. fragi*, *P. tolaasii*, *P. rhodesiae*, *P. gessardii*,

P. proteolytica, *P. brenneri*, or *P. chlororaphis* (Martins et al., 2005; Marchand et al., 2009b; Caldera et al., 2016). Although no *aprX* sequence has yet been obtained, *P. lundensis* produces a similar enzyme, as evidenced by a few peptides of *P. lundensis* retrieved by mass spectrometry, which display similarity with the other *Pseudomonas* AprX proteases (Marchand et al., 2009b).

AprX is a peptidase of 45 to 50 kDa encoded by the *aprX* gene located on the *aprX-lipA* operon, which contains eight genes and spans 14 kb (McCarthy et al., 2004). In general, AprX is rich in alanine and glycine residues and poor in cysteine and methionine residues (Dufour et al., 2008). The lack of cysteine residues allows avoidance of steric constraints due to disulphide bonds and increases its flexibility (Matéos et al., 2015). The presence of Ca^{2+} (GGXGXDXUX) and Zn^{2+} (HEIGHTLGLAHP) binding motifs confirms its dependence of divalent-cations (Dufour et al., 2008). The AprX protein is highly conserved within *Pseudomonas* species (76–99% similarity for AprX of *P. fluorescens* group), but is more heterogeneous between species (57–69% similarity for AprX between strains of *P. fluorescens* and *P. fragi*) (Marchand et al., 2009b; Matéos et al., 2015). In addition to the four AprX sequence groups (with one group split into two subgroups) identified within *Pseudomonas* raw milk isolates by Marchand et al. (2009b), a fifth group was added recently including Mozzarella isolates (Caldera et al., 2016).

AprX exhibits activity in a large range of pH (4.5–10) with an optimum activity between 7.5 and 9, which proves that AprX is an alkaline peptidase. AprX generally exhibits activity in a large range of temperatures (0–55°C) with optimal activity between 37 and 47°C (Dufour et al., 2008; Martins et al., 2015; Matéos et al., 2015). Inhibition studies revealed that AprX was inhibited by typical divalent-ion chelators such as EDTA (Ca^{2+} and Zn^{2+} chelator), EGTA (Ca^{2+} chelator), o-phenanthroline (Zn^{2+} chelator) while serine peptidase inhibitors (PMSF and leupeptin) did not affect activity of the enzyme (Liao and McCallus, 1998; Dufour et al., 2008; Matéos et al., 2015). It was shown for an alkaline metallopeptidase isolated from a *Pseudomonas* sp. isolated from refrigerated milk, that Ca^{2+} stabilizes the enzyme and improves its activity (Ertan et al., 2015), while Zn^{2+} is essential in the active site (Wu and Chen, 2011).

AprX may hydrolyze the four types of casein (α_{s1} , α_{s2} , β , and κ) with a large activity spectrum (Baglinière et al., 2013). Matéos et al. (2015) have shown that cleavage sites are mainly found in hydrophobic areas of casein. The extracellular peptidase produced by *P. fluorescens* hydrolyzes milk caseins preferentially in the following order κ - > β - > α_{s1} -caseins (Fairbairn and Law, 1986; Mu et al., 2009; Pinto et al., 2014; Zhang et al., 2015). However, Baglinière et al. (2012) described the preferential proteolysis of β -casein by AprX. This difference in preferential proteolysis between the different studies could be attributed to the differences in the species and strain used. In Figure 1, a hypothetical mechanism of UHT milk destabilization due to casein micelle proteolysis by heat-resistant protease during storage at ambient temperature is shown. The intensity of proteolytic activity is dependent on species and strains. Marchand et al. (2009a) and Baglinière et al. (2012) revealed a large heterogeneity, respectively, in the proteolytic activity within the *Pseudomonas* genus and in effect



on destabilization and flavor defects of UHT milk inoculated with *P. fluorescens* strains and other *Pseudomonas* species. Caldera et al. (2016) observed a high total proteolytic activity (without prior heat treatment) for all *P. proteolytica* isolates (4 and 12 μ mol glycine equivalent/mL, (as measured with the 2,4,6-trinitrobenzenesulfonic acid [TNBS] method), the major part of *P. gessardii*-like isolates (2 and 16 μ mol glycine equivalent/mL), and for 36% of *P. fragi*-like isolates (5 and 14 μ mol glycine equivalent/mL). The high variability of *Pseudomonas* strains regarding the proteolytic activity may be a consequence of heterogeneous enzyme expression, regulation by quorum sensing (QS), effect of temperature, iron content, and bacterial growth phase (Woods et al., 2001; Nicodème et al., 2005; Marchand et al., 2009a).

Although AprX has been reported as the main heat-stable peptidase encountered in *Pseudomonas* spp. isolated from raw milk in several recent studies (Marchand et al., 2009b; Baglinière et al., 2013; Matéos et al., 2015), some authors showed that *P. panacis* and also *P. fluorescens* can secrete another heat-stable peptidase AprA (Maunsell et al., 2006; Baur et al., 2015b). According to Baur et al. (2015b), the peptidase AprA secreted by a strain of *P. panacis* isolated from raw milk was able to withstand a UHT process. In the same study, the authors showed that the peptide sequence of AprA was 98% similar to the peptidase AprX secreted by a strain of *P. fluorescens*. As AprX, AprA is a metallopeptidase of about 50 kDa belonging to the serralysin family and presents in its primary structure the binding motifs for Ca^{2+} and Zn^{2+} (Takahashi, 2013; Baur et al., 2015b). According to Ma et al. (2003), there is a nomenclatural problem in the Apr protease system of *Pseudomonas*. According to those authors, AprA should be considered the main alkaline peptidase and AprX, lacking both the conserved Zn^{2+} -binding sequence

and the glycine-rich motif of AprA, is produced together with AprA by *P. aeruginosa*. However, the alkaline metalloprotease of *P. fluorescens* responsible for milk spoilage was first described as AprX by Dufour et al. (2008) and has been named as such in most studies since then. AprA and AprX produced by the *Pseudomonas* strains responsible for milk spoilage are the same enzyme because of their high sequence similarity and presence of the conserved motifs, while AprX produced by *P. aeruginosa* is a different enzyme.

A recent study conducted by Stuknytė et al. (2016) detected two other thermostable proteolytic bands with molecular masses of approximately 15 and 25 kDa after zymography analysis from *P. fluorescens* PS19 supernatant. The 25-kDa fragment did not show homology to AprX, indicating that this strain is able to secrete a heat-stable peptidase other than AprX or AprA.

Heat-Stable Peptidase from *Serratia* Isolated from Milk and Dairy Products

The importance of *Serratia* as a milk-spoilage microorganism has been shown recently (Cleto et al., 2012; Decimo et al., 2014; Machado et al., 2015), although previous studies have described and/or characterized peptidases from *S. proteamaculans* (Christensen et al., 2003; Demidyuk et al., 2006; Eom et al., 2014), *S. marcescens* (Matsumoto et al., 1984; Letoffe et al., 1991; Jayaratne, 1996; Romero et al., 2001; Tao et al., 2007; Nam et al., 2013) and *Serratia* sp. E-15 (Hamada et al., 1996).

The number of peptidases produced by *Serratia* is variable. This characteristic could either be species dependent or variable, depending on the method used for peptidase detection. According to Matsumoto et al. (1984), *S. marcescens* kums3958 produced four peptidases as detected by polyacrylamide gel electrophoresis. These peptidases presented a molecular weight

of 56, 60, and 73 kDa wherein the 73 kDa-peptidase has been separated in two peptidases after isoelectric focusing. Nevertheless, Romero et al. (2001) detected only two peptidases when *S. marcescens* was inoculated into reconstituted whey. The molecular masses of both peptidases estimated on SDS-PAGE were 53.5 and 66.5 kDa for the metallopeptidase and the serine peptidase, respectively. Those authors did not detect the 73 kDa-peptidase. This result could be explained by the different growth conditions and strains used.

The metallopeptidase from *S. marcescens* S3-R1, which has a molecular weight of approximately 50.3 kDa, has been characterized by Nam et al. (2013). Those authors showed that this peptidase presents its optimal activity at pH 7–9 and at 40–50°C.

Unfortunately, there is no information in the literature about the characterization of the heat-stability of these peptidases. However, Glück et al. (2016) observed, for two strains of *S. marcescens* isolated from raw milk, an extracellular peptidase residual activity of 71 and 91% after a heat-treatment of 95°C for 5 min, highlighting the secretion of heat-stable peptidase by this species. Nevertheless, the authors did not identify the peptidase responsible for this residual activity. Worth noting is that *S. marcescens* is an opportunistic pathogen for human and insects (Ishii et al., 2014; Hagiya et al., 2016), which justifies most studies focused on peptidases produced by this species, while the characterization of *S. liquefaciens* peptidases have been discussed by few authors only (Kaibara et al., 2010, 2012; Machado et al., 2016).

Serratia liquefaciens FK01 produces two serralysin-like metallopeptidases (Kaibara et al., 2010, 2012). These peptidases are encoded by *ser1* and *ser2* genes. Both peptidases showed molecular mass of approximately 50 kDa and presented Zn²⁺ binding motif (HEXXHXUGUXH), Ca²⁺ binding motif (GGXGXDXUX), and ABC exporter motif (DXXX) (Kaibara et al., 2010, 2012; Machado et al., 2016). The difference between both peptidases produced by *S. liquefaciens* seems to be heat resistance. According to Machado et al. (2016), only Ser2 withstood the heat treatment of 95°C for 8:45 min. Those authors highlighted that proteolytic activity of Ser2 was highly variable depending on the incubation conditions and on the *S. liquefaciens* strain inoculated into the milk samples.

Technological Problems Resulting from the Residual Activity of Peptidases after Heat Treatment

Heat-resistant peptidases can lead to serious problems for the dairy industry. Since *Pseudomonas* has been widely studied, there are several studies focused on technological problems caused by peptidases from *Pseudomonas* (Celestino et al., 1997; Sørhaug and Stepaniak, 1997; Belloque et al., 2001; Datta and Deeth, 2001, 2003; Chen et al., 2003; Baglinière et al., 2013), however, there are no studies yet regarding the consequences of peptidase from *Serratia* in dairy products.

After raw milk storage for prolonged time, UHT processing can be compromised because of destabilization of the milk, resulting in clogging of the heating exchanger (Figure 2). Pinto et al. (2014) showed that α-, β-, and κ-casein from milk inoculated with *P. fluorescens* were completely hydrolyzed after 4 days

incubation at 4°C. The proteolysis of casein contributes to destabilization of UHT milk and to protein sedimentation during its storage (Gaucher et al., 2011; Baglinière et al., 2013; Matéos et al., 2015). A visual destabilization of UHT milk by AprX from *P. fluorescens* F was observed after 7 days of storage when 0.2 mg/mL of peptidase had been added in raw milk before UHT treatment (Baglinière et al., 2013). The protein sedimentation could be observed after 2 weeks of storage in UHT milk samples when peptidases from *P. panacis* where inoculated at a final concentration of 1 picokatal/mL (Baur et al., 2015b; one katal of an enzyme is that amount which breaks a mole of peptide bonds per second under specified conditions).

The proteolysis of milk protein can also lead to bitter off-flavor of some dairy products such as UHT milk. This is caused by the generation of hydrophobic peptides by hydrolysis of casein (Chen et al., 2003; Datta and Deeth, 2003). Valero et al. (2001) showed that new flavor and volatile components appeared in skimmed milk samples after 65 days storage related to proteolysis and the Maillard reaction. The proteolytic activity might increase the number of free amino groups, which can participate with the reducing sugars in Maillard reactions (Valero et al., 2001). In UHT-milk spiked with one each of six isolates representing the different *Pseudomonas* peptidase groups, a casein hydrolysis of >1.5 μmol glycine equivalents/mL (as measured with the TNBS method) was the threshold for the taste panel to detect off-flavor, but no clear correlation was found between the onset of off-flavors and the rate of protein hydrolysis (Marchand et al., 2017). The degree of proteolysis (as measured with the TNBS method) of the UHT-milk samples in which off-flavors were significantly tasted, were different for each *Pseudomonas* peptidase under evaluation (Figure 3). *P. fragi* peptidase was capable in generating off-flavors after very limited proteolysis (a raise in TNBS-value of 0.15 glycine equivalents/mL). Therefore, it can be speculated that not all *Pseudomonas* peptidases have the same specificity for their casein substrates.

However, a large difference in heat resistant or total proteolytic activity was observed within *P. fragi* by Marchand et al. (2009a) and Caldera et al. (2016), respectively, with only 36% of *P. fragi*-like isolates (from diverse sources including dairy and raw milk) showing total proteolytic activity according to the latter authors. Nevertheless, when positive for proteolytic activity, the *P. fragi* group exhibited a significantly higher heat-resistant proteolytic activity than the *P. lundensis* and the *Pseudomonas* spp. group (Marchand et al., 2009a), which suggests that the presence of proteolytic strains of *P. fragi* prior to UHT-processing will severely compromise the shelf life of derived dairy products.

Another technological problem in the dairy industry caused by proteolytic activity from psychrotrophic microorganisms is the yield reduction in cheese manufacturing (Cardoso, 2006; Mankai et al., 2012). Cardoso (2006) showed a reduction of 6.38% in total solids in fresh Minas cheese produced using a raw milk stored for four days under cold temperatures, which promotes psychrotrophic growth.

Lipolytic Enzymes

Lipolytic enzymes in cow's milk from endogenous or exogenous sources and the action of these enzymes on the milk substrate is

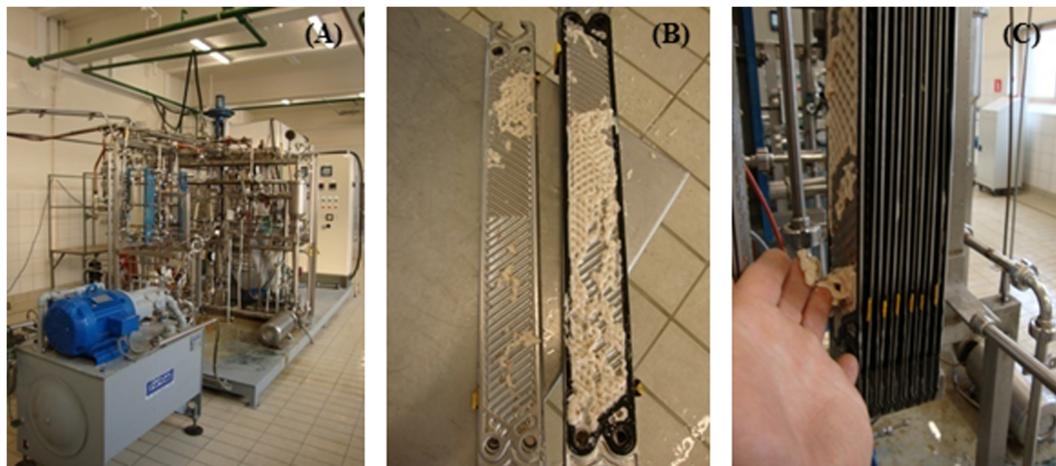


FIGURE 2 | Clogging of the heating exchanger due to processing of milk spiked with *Pseudomonas* and stored for 5 days at 6.5°C. (A) UHT Process Pilot Plant (ILVO, Belgium), **(B)** heat exchanger, **(C)** detail of the heat exchanger with clotting of milk.

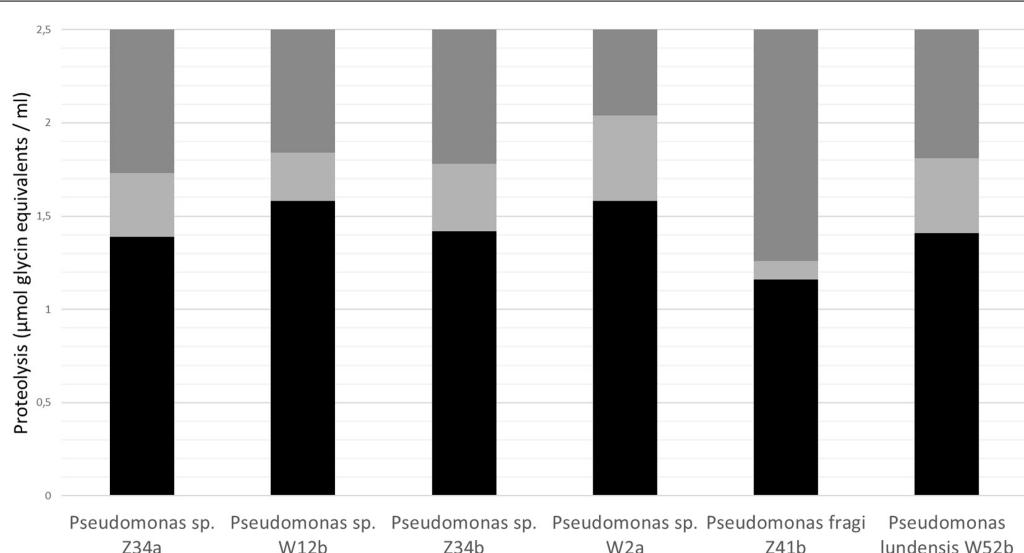


FIGURE 3 | Correlation between off-flavors and protein hydrolysis by six different *Pseudomonas* peptidase groups in UHT-milk, as described by Marchand et al. (2009b). Raw milk was pasteurized, inoculated with one of the *Pseudomonas* strains to a final concentration of 3 log CFU/mL, and stored for 3 to 5 days at 6.5°C until skimming and indirect UHT-processing (5 s at 140°C). Processed milk was aseptically filled in high-density polyethylene bottles of 0.5 L and stored at 37°C to accelerate proteolysis. The six *Pseudomonas* peptidase milk samples were compared with the reference control milk by an experienced taste panel of 35 people at the Institute for Agricultural and Fisheries Research (ILVO), Belgium in a sensorial cabinet equipped with individually partitioned booths. Milk with off-flavor was diluted as follows: (A) *Pseudomonas* milk undiluted, (B) 2/3 *Pseudomonas* milk + 1/3 control milk, (C) 1/3 *Pseudomonas* milk + 2/3 control milk, (D) Control milk undiluted. The taste panel was asked to rank the milk samples (A–B–C–D) according to preference. Statistical evaluation of the results was based on the Rank Test to KRAMER (Kramer, 1960) for $\alpha = 0.05$. Simultaneously, proteolysis (expressed as μmol glycine equivalents mL^{-1} milk) was determined in each milk dilution (A–B–C–D) by the trinitrobenzenesulfonic acid (TNBS) method (Polychroniadou, 1988). No significant proteolysis off-flavors is indicated by black bars, the uncertainty range by light gray bars (the panel did not reject the milk samples; the lower limit of the bar is determined by the TNBS-value of the most diluted sample that was not rejected by sensory analysis) and the significant proteolysis off-flavors by dark gray bars (panel rejected the milk samples and tasted off-flavors; the lower limit of the bar is determined by the TNBS-value of the least diluted sample). This figure was reproduced from Marchand et al. (2017).

considered as limited by its natural organization in the form of fat droplets (Bourlieu et al., 2012). Undesirable lipolysis of milk and dairy products has not been studied as much as undesirable proteolysis. Exogenous lipases produced by psychrotrophic bacteria can hydrolyze milk fat and release free fatty acids (FFAs),

mono- and di-acylglycerols and glycerol. The lipolytic activity of psychrotrophs is species-specific. According to Decimo et al. (2016) bacterial triacylglycerol hydrolysis may occur to a greater or lesser extent, but the type and amount of released FFAs are not easily predictable. The lipolytic activity generates undesirable

product flavors such as rancid, unclean, soapy, or bitter, making the product barely acceptable to the consumer (Deeth, 2006).

Among the lipolytic psychrotrophic bacteria, *Pseudomonas* spp. is the predominant Gram-negative group found whereas *Bacillus* spp. is the predominant Gram-positive group (Chen et al., 2003; Decimo et al., 2014; Vithanage et al., 2016). However, other genera isolated from raw milk, may also produce lipolytic enzymes, such as *Serratia*, *Hafnia*, *Acinetobacter*, *Microbacterium*, and *Enterobacter* (Hantis-Zacharov and Halpern, 2007; Decimo et al., 2014; Vithanage et al., 2016).

Lipolytic enzymes are defined as carboxylesterases that hydrolyze acylglycerols (Jaeger et al., 1994, 1999; Chen et al., 2003). Carboxylesterases can be divided in two groups, the lipase or triacylglycerol acylhydrolases (EC 3.1.1.3) and the esterase or carboxylases (EC 3.1.1.1). Lipases are active at lipid–water interfaces rather than in the aqueous phase and preferentially hydrolyze acylglycerols with more than 10 carbons ($>C_{10}$). Esterases are active in aqueous solutions and are only able to hydrolyze acylglycerols with fewer than 10 carbons ($<C_{10}$) (Anthonsen et al., 1995). Lipase is also capable of hydrolyzing acylglycerols $<C_{10}$ (Jaeger et al., 1994, 1999) and intact milk fat globules (MFG). Without hydrolysis of the fat globules, the lipolytic enzyme does not have access to triglycerides (Deeth, 2006). In terms of spoilage potential, this difference between lipase and esterase hydrolysis could explain most of the lipolytic enzymes studied are secreted lipase. Another enzyme able to hydrolyze MFG is phospholipase. However, this enzyme cannot hydrolyze triacylglycerol. Therefore, this review focuses on lipase secreted by the genera *Pseudomonas* and *Bacillus*, particularly heat-stable lipase.

Lipase from *Pseudomonas* Isolated from Milk and Dairy Products

Among the lipolytic species of *Pseudomonas*, *P. fluorescens* is the species more often found in raw milk. Nevertheless, some recent studies showed the presence of lipolytic strains of *P. aeruginosa*, *P. putida*, *P. fragi*, and *P. gessardii*-like in raw milk or *P. peli*-like in pasteurized milk (Munsch-Alatossava et al., 2013; Decimo et al., 2014; Caldera et al., 2016). *Pseudomonas* spp. produce a large variety of lipolytic enzymes classified into six groups corresponding to family and subfamily (Arpigny and Jaeger, 1999; Chen et al., 2003). However, most of lipases secreted by the species found in raw milk such as *P. aeruginosa*, *P. fluorescens*, and *P. fragi* belong to the sub families I.1 and I.3 (Arpigny and Jaeger, 1999). Most of them have specificity for the sn-1 and sn-3 positions of triacylglycerols, and some hydrolyze diacylglycerols and monoacylglycerols faster than triacylglycerols (Chen et al., 2003). The classification of these enzymes is based on their amino acid homologies and some biological properties. These lipases present the consensus pentapeptide G-X-S-X-G in the amino acid sequence, corresponding to the catalytic site.

Subfamily I.1 corresponds to the secreted lipases with a molecular weight of approximately 30 kDa, which present two aspartic residues involved in the Ca^{2+} binding site (Arpigny and Jaeger, 1999). These lipases are mainly secreted by *P. aeruginosa* and *P. fragi* (Arpigny and Jaeger, 1999). However, the species *P. fluorescens* is also able to secrete a lipase belonging to this

subfamily (Beven et al., 2001). Subfamily I.3 corresponds to lipase with a molecular mass of 50 to 65 kDa. The most studied lipase from this group is LipA from *P. fluorescens* encoded by the *lipA* gene located in the same operon as the peptidase AprX, the operon *aprX-lipA*. Similar to the peptidases Ser2 and AprX, this lipase presents in the amino acid sequences the binding motif to fix Ca^{2+} suggesting the need of this ion for its stability. The lipases of *P. fluorescens* 33 (Kumura et al., 1993a,b), *P. fluorescens* 041 (Martins et al., 2015), *P. fluorescens* SIK W1 (Son et al., 2012) isolated from milk and one of the lipases of *P. fluorescens* C9 (Beven et al., 2001) belong to this family.

Numerous older studies have shown the heat-stability of the lipolytic activity of *Pseudomonas* species. Law et al. (1976) showed that after a heat treatment of 63°C during 30 min in raw milk, the extracellular residual lipolytic activities of various strains of *Pseudomonas* isolated from raw milk were 55 to 100%. Fitzgerald et al. (1982) observed that lipases from *Pseudomonas* isolated from raw milk presented 75 to 100% of residual lipolytic activity after heating skim milk at 100°C for 30 s. Moreover, Andersson et al. (1979) reported a D-value of 23.5 min (calculated time required for a 90% reduction of the initial activity) for *P. fluorescens* SIK W1 lipase after heat-treatment of skim milk at 100°C. As described by Fox and Stepaniak (1983), lipase from *Pseudomonas* seems to be more heat-stable in synthetic milk salt solutions than in phosphate buffer. This better heat-stability in milk salt solutions is probably due to the presence of calcium (Andersson et al., 1979). Recent studies, however, show that not all lipases secreted by the genus *Pseudomonas* are heat-stable. The residual lipase activity of the purified LipM of *P. fluorescens* 041 isolated from Brazilian raw milk was only 25.4% after heat-treatment of 72°C for 20 s in buffer (Martins et al., 2015). It is noteworthy that Vithanage et al. (2016) showed that more than 30% of *Pseudomonas* strains isolated from raw milk presented 50 to 75% of residual lipase activity after a heat treatment of 4 s at 142°C (UHT treatment). Those authors observed more strains producing heat-stable lipases than strains producing heat-labile lipases among *Pseudomonas* strain isolated from raw milk.

These studies confirm that many lipases from the genus *Pseudomonas* can resist heat-treatment used in dairy industries such as pasteurization and/or UHT treatment. No heat treatment is available that may inactivate these lipases without altering the milk's sensory and nutritional qualities.

Lipase from *Bacillus* spp. Isolated from Milk and Dairy Products

The presence of the thermophilic species *Geobacillus stearothermophilus* as a lipolytic enzyme producer in raw milk and milk powder has been reported by various authors (Chopra and Mathur, 1984, 1985; Chen et al., 2004). The principal characteristic of *Bacillus* lipases is the substitution of the first glycine by alanine in the conserved pentapeptide A-X-S-X-G (Arpigny and Jaeger, 1999). Most *Bacillus* lipases show the highest catalytic activities at temperatures ranging from 60 to 75°C (Chen et al., 2003). The lipase of *Bacillus* can be classified in two groups: subfamilies I.4 and I.5. The lipase of *B. subtilis* (molecular mass about 20 kDa) is the smallest true lipase known from bacteria found in raw milk (Arpigny

and Jaeger, 1999). This lipase, belonging to subfamily I.4, is also secreted by *B. licheniformis*, frequently encountered in raw milk as a lipolytic enzyme producer (Baur et al., 2015a). With a molecular mass of 45 kDa, the lipase secreted by the species *G. thermocatenulatus* and *G. stearothermophilus* belongs to subfamily I.5 and shows optimal activity at pH 9.0 and 60–65°C (Schmidt-Dannert et al., 1996; Kim et al., 1998; Arpigny and Jaeger, 1999).

As described above for *Pseudomonas*, many *Bacillus* (or *Geobacillus*) lipases remain stable during heat-treatments used in dairy industries (pasteurization and/or UHT treatment) and can therefore affect milk and dairy products during storage. Chen et al. (2003) calculated a $t_{1/2}$ of 690 min at 70°C in buffer at pH 7.0 for the lipase produced by a strain of *G. stearothermophilus*. Considering all lipases secreted by *Bacillus*, lipases from strains isolated from milk powder production presented a higher residual activity after pasteurization at 72°C for 2 min in milk (Chen et al., 2004). In addition, a recent study showed that more than 38% of *Bacillus* strains isolated from raw milk presented 50 to 75% of residual lipase activity after a heat treatment at 142°C for 4 s (Vithanage et al., 2016).

Phospholipase C

The production of different phospholipases has been reported for Gram-negative and Gram-positive psychrotrophs (Sørhaug and Stepaniak, 1997). The phospholipase most studied is phospholipase C, which can be either hemolytic or non-hemolytic. Phospholipase C activity has been detected in the genera *Pseudomonas*, *Bacillus*, *Serratia*, *Hafnia*, *Acinetobacter*, and *Microbacterium* in raw milk by Vithanage et al. (2016). However, according to De Jonghe et al. (2010), in the genus *Bacillus* only the species *B. cereus* is able to produce the phospholipase C. The presence of this enzyme was not observed for the species *B. licheniformis* or *B. subtilis*. This enzyme is particularly heat stable (Sørhaug and Stepaniak, 1997) and disrupts the integrity of the MFG membrane (Craven and Macauley, 1992; Shah, 1994).

The phospholipase C of *P. fluorescens* is well known as a heat-stable enzyme, presenting high residual activity after pasteurization and UHT treatment (Sørhaug and Stepaniak, 1997). Vithanage et al. (2016) observed that about 25% of *Bacillus* and *Pseudomonas* strains isolated from raw milk presented 50 to 75% of residual phospholipase C activity after heat-treatment of 140°C during 4 s.

Technological Problems Resulting from the Residual Activity of Lipases after Heat Treatment

The presence of bacterial lipase could affect the quality of fluid milk, dry whole milk, cheese, and butter (Sørhaug and Stepaniak, 1997; Chen et al., 2003). However, it seems that the modifications induced to milk lipids are highly dependent on the lipase specificity and also on the fat condition. Excessive shaking, addition of air, repeated thermal shocks, and homogenization, all of which can occur at different stages of production and processing, adversely affect the integrity of the fat globule, modify the interfaces between the fat and non-fat phase and lead to an increase of lipolysis (Kim et al., 1983). The action of lipase

on milk fat can release short-chain fatty acids (C4:0 to C8:0), medium-chain fatty acids (C10:0 and C12:0) and long-chain fatty acids (C14:0 to C18:0). Short-chain fatty acids (e.g., butyric, caprylic, and caproic acids) have strong flavors and can impart unpleasant flavors variously known as rancid, bitter, butyric, unclean, astringent, or ‘lipase’ (Deeth, 2011), whereas medium-chain fatty acids are responsible for a soapy taste (Chen et al., 2003). Long-chain fatty acids contribute little to flavor. Moreover, as described by Chen et al. (2003), an oxidized flavor can be generated by the oxidation of free unsaturated fatty acids to aldehydes and ketones. Due to presence of heat-stable lipases in raw milk, these undesirable flavors, such as rancidity, can occur in UHT-milk (Adams and Brawley, 1981). The mono- and diacylglycerols which are the other products of lipase action have surface-active properties that can affect some products such as steam-foamed milk used in coffee-based drinks (Deeth, 2011).

Whole milk powder can be also affected by residual heat-resistant lipase, because most enzymes are more stable when water activity decreases. Indeed, some authors showed that lipase of *P. fluorescens* in spray-dried powder did not lose activity at 20°C for up to 60 days (Shamsuzzaman et al., 1986). According to Andersson (1980), lipases retain more activity than peptidases in milk powder during prolonged storage. Moreover, residual lipase activities may be detected when dry whey products and skimmed milk powder are added as ingredients to fatty products (Stead, 1986).

Lipase and Peptidase Regulation

Understanding of the regulation of peptidases and lipases produced by psychrotrophic bacteria in milk samples is still limited. Compared to the other psychrotrophic genera, the regulation of enzymes secreted by the genus *Pseudomonas* has been the most studied, especially the operon *aprX-lipA* regulation. However, the complex production process of these two enzymes is not completely understood. The following section will be focused on the regulation of extracellular enzymes produced by the genus *Pseudomonas*. The factors involved in this regulation are described briefly.

Many factors are involved in the lipase and peptidase production by psychrotrophic bacteria, such as temperature (Burger et al., 2000; Nicodème et al., 2005), phase of growth (Chabeaud et al., 2001; van den Broeck et al., 2005), QS (Givskov et al., 1997; Christensen et al., 2003; Juhas et al., 2005; Liu et al., 2007; Pinto et al., 2007) or iron content (McKellar, 1989; Woods et al., 2001).

In *P. fluorescens*, enzyme production seems to be strongly related to cell density. According to Bai and Rai (2011), the production of extracellular peptidases in *P. fluorescens* is associated with the high cell density that is typically encountered at the end of the exponential phase of growth. One hypothesis is that this regulation by cell density may be mediated by QS. Indeed, bacteria may communicate by QS using signaling molecules called *N*-acyl-homoserine lactones (AHLs). These molecules are produced by numerous Gram-negative bacteria such as *Pseudomonas* (Liu et al., 2007) or *Serratia* (Givskov et al., 1997) and are implicated in the genetic control of a wide range of phenotypic attributes such as cell differentiation,

biofilm formation, sporulation, toxins, and enzyme secretion (De Oliveira et al., 2015). Their production is strongly dependent on a specific cell density (Fuqua et al., 1994; Liu et al., 2007). A remarkable seasonal variation on heat resistant proteolytic activity of *Pseudomonas* strains from raw milk was observed (Marchand et al., 2009a). This effect could be related to growth rate: in milk samples with proteolytic bacteria, the proteolytic psychrotolerant counts were significantly higher in samples collected in winter than in summer and winter isolates displayed better growth characteristics and peptidase production than the summer isolates.

Pinto et al. (2007) showed that more than 80% of psychrotrophic proteolytic strains isolated from cooled raw milk were able to produce AHLs in raw milk and pasteurized milk, suggesting that QS may play a role in the spoilage of milk. Liu et al. (2007) reported that the proteolytic activity of the strain 395 of *P. fluorescens* was stimulated by the addition of AHL. Those authors concluded that the *aprX* gene was regulated at a transcriptional level by AHL during the end of the exponential phase of growth. In contrast, Pinto et al. (2010) did not observe any effect on the growth and proteolytic activity after adding synthetic AHL in the culture of the proteolytic strain *P. fluorescens* 07A that secretes AprX but does not produce AHL. The extracellular peptidase activity was detected only when the cell population reached 10^8 CFU/mL. They concluded that peptidase activity of this strain was not regulated by QS via AHLs but could be related to cell density. The regulation of enzyme production by QS via AHLs seems thus to be strain dependent in the species *P. fluorescens*. According to Siddiqui et al. (2005), in *P. fluorescens* CHA0, the expression of *aprA* gene seems to be also cell density dependent. In addition, the authors showed that the expression of this protease was positively regulated by the two-component system GacS/GacA, which controls the expression of secondary metabolism and protein secretion in a wide variety of bacterial species.

Relating to other genera implicated in milk spoilage, the relationship between QS, cell density and enzyme production has been observed among members of the *Serratia* genus. It seems that the operon *slaA-lipB* of *S. proteamaculans*, which is required for the secretion of several unrelated and potentially food-quality-relevant proteins and the exoenzyme production and its homolog in *S. liquefaciens*, is under the transcriptional control of QS (Givskov et al., 1997; Christensen et al., 2003). Christensen et al. (2003) demonstrated that the activities of several exoenzymes including peptidases from *S. proteamaculans* B5a are affected by *N*-(3-oxo-hexanoyl)-L-homoserine lactone, which is a signal molecule of QS system in Gram-negative bacteria. Although Ser2 is a heat-resistant peptidase that may compromise milk product quality, no information about regulation of Ser2 expression is available.

Numerous studies show that the enzymes of *P. fluorescens* are regulated by the temperature of growth. Optimal peptidase production occurs when the temperature of growth is slightly above the optimal temperature of growth, while above this temperature peptidase production is severely repressed (Burger et al., 2000; Woods et al., 2001; McCarthy et al., 2004). In contrast, optimal lipase production occurs when the temperature

of growth is well below the optimal growth temperature, suggesting the contribution of low temperature-dependent regulation system (Andersson, 1980; Merieau et al., 1993; Woods et al., 2001). However, a study carried out by Woods et al. (2001) showed that the temperature does not regulate *AprX* and *LipA* production at the transcriptional level (operon *aprX-lipA*) suggesting that the regulation is post-transcriptional or post-translational.

Relating to mineral content, the expression of the *aprX-lipA* operon is negatively regulated at the transcriptional level by iron (III) (Woods et al., 2001). Moreover, McCarthy et al. (2004) observed that the operon *aprX-lipA* is under transcriptional control of the two-component regulatory system homologous to the *E. coli* two-component system called EnvZ-OmpR. However, those authors observed that lipase production was more affected by this regulatory system than peptidase production. The distal locations between the genes *aprX* and *lipA* on the operon could explain that this difference of regulation may be related to their proximal and distal position, respectively, within the *aprX-lipA* operon (McCarthy et al., 2004).

CONTROL OF SPOILAGE BY HEAT-RESISTANT BACTERIAL ENZYMES

Reducing the activity and/or limiting the secretion of heat-resistant hydrolytic enzymes of psychrotrophic bacteria is a scientific challenge. Once the enzymes are formed, reducing their activity by heating seems to be very difficult. A recent review (Stoeckel et al., 2016b) summarizes available data on inactivation of *Pseudomonas* proteases and proposes heat treatments that reduce the protease activity in the final product to extend the shelf life of UHT products. For UHT products intended for export, UHT heating combined with prolonged preheating (e.g., 90–95°C for 180–90 s) is suggested to reduce 99.99% of the indigenous milk plasmin activity. *Pseudomonas* proteases show also an irreversible low temperature inactivation behavior due to unfolding of the tertiary structure of the enzyme at a temperature range of 45–65°C, rendering it susceptible to autoproteolysis. This inactivation was recently shown between 42 and 48°C for a new broad specificity metalloprotease from a *Pseudomonas* spp. isolated from refrigerated milk (Ertan et al., 2015). However, this effect seems much lower in milk compared to buffer systems because of protective effects of milk components, and thus a low preheating step is not effective to sufficiently reduce this bacterial proteolytic activity. The development of other heating processes (e.g., heat-treatment at 125–130°C) for long holding times (>150 s) has been suggested (Stoeckel et al., 2016b). However, such treatment may result in color changes and degradation of lysin, thiamin, and riboflavin (Kessler, 1996).

Another option for spoilage control is to prevent the production of heat-resistant enzymes by limiting the growth of psychrotrophic bacteria in raw milk. This could be realized by modifying the composition of the atmosphere surrounding the milk. N₂ gas flushing of cold-stored raw milk (6°C) has been shown to strongly inhibit bacterial growth (Gschwendtner et al., 2016), as 3–4 log fewer bacteria were counted after

7 days compared to non-flushed milk. Furthermore, analysis of the bacterial population by next generation sequencing (NGS) of 16S rRNA transcripts showed a relatively lower number of *Pseudomonas* reads in the N₂ gas flushed milk, indicating selective inhibition of *Pseudomonas* growth. Changing the atmosphere by N₂ may thus have potential as a control measure to prevent outgrowth of *Pseudomonas* spp. during cold storage of raw milk. However, information in the literature about the ability of facultative anaerobic species of the genus *Bacillus* and its allied genera and of the facultative anaerobic genus *Serratia* to produce extracellular enzymes under anaerobic conditions is lacking at this time. Furthermore, practical applications must be evaluated at dairy farm and industrial level. CO₂ treatment of raw milk has also been shown to reduce the microbial growth in raw milk (Ma et al., 2003; Vianna et al., 2012; Lo et al., 2016). Lo et al. (2016) observed that in CO₂-treated raw milk samples a clear inhibition of bacterial growth compared to non-treated samples, resulting in a delay of spoilage by at least seven days. Using NGS, a relatively lower number of *Pseudomonas* and *Serratia* reads were found in three out of five CO₂-treated raw milk samples, indicating selective growth inhibition of these genera. However, a disadvantage of CO₂ treatment is that it reduces the pH of the milk (Ma et al., 2003) which may result in changes of (heat)stability.

A third and probably the most cost-effective option for control of spoilage caused by heat-resistant bacterial enzyme activity is hygiene: preventing the contamination of raw milk with psychrotrophic bacteria. Good hygiene practices during milking, cold-storage and transport of the raw milk may reduce the risk of contamination, as these bacteria mainly originate from the udder, milking equipment and milk storage tanks. Wash water used to clean milking equipment has also been shown to be an important source of *Pseudomonas* contamination of raw milk, indicating the need for attention to water quality (Perkins et al., 2009; Leriche and Fayolle, 2012). Specific pre-milking teat cleaning strategies have been shown to reduce the spore count in milk by >1 log (Magnusson et al., 2006). It remains to be investigated whether such udder hygiene management strategies are also effective to reduce the psychrotrophic non-spore forming count in view of the study of Mallet et al. (2012) which showed that teat care has more influence on the composition of technologically relevant microbial groups than on the composition of other groups such as *Pseudomonas* and other Gram-negative bacteria. This may indicate that control of biofilms in milk production and processing environments is maybe more important (reviewed by Marchand et al., 2012; Aswathanarayan and Vittal, 2014; Nucera et al., 2016), as release of vegetative cells or spoilage enzymes from these biofilms may compromise the quality of UHT products (Flach et al., 2014; Teh et al., 2014b). Prevention of biofilm formation may possibly be achieved by specific coating of stainless steel surfaces of milk equipment and milk storing tanks with spoilage bacteria, as was recently shown for milk spore-formers on plate heat exchanger surfaces (Jindal et al., 2016). Biofilms are difficult to remove because the bacteria are protected from disinfectants due to the presence of extracellular polymeric substances. The hygienic design of milking and milk storage equipment as well as effective cleaning and disinfection

procedures and proper application are all important factors in the control of biofilms in the dairy industry (Marchand et al., 2012). To remove bacterial biofilms on stainless steel surfaces, the use of specific disinfecting agents, such as products based on hydrogen peroxide and peroxyacetic acid, as well as higher concentrations and longer contact times may be required (Królasik et al., 2010).

Recently, some novel strategies based on the reduction of the bacterial contamination of the raw milk have been proposed as potential measures to extend the shelf life of UHT products, such as the use of microfiltration and the application of lytic bacteriophages. Microfiltration (1.4 μm pore diameter) resulted in 1–2 log reduction of the psychrotrophic bacterial count of the raw milk and ESL by 21 to 63 days of the UHT-treated milk for low and high somatic cell count (SCC) raw milk, respectively (Zhang et al., 2016). The practical application of microfiltration at the farm is questionable, however, since the cream must be removed from the raw milk before such treatment is possible. Application of a lytic phage cocktail against *Pseudomonas* was shown to result in a 1-log reduction of the psychrotrophic bacteria of raw milk after 5 days at 4°C (Hu et al., 2016). However, the rather limited effect and the fact that the use of bacteriophages in food is strictly regulated in many countries will probably hamper practical application.

CONCLUSION

The combination of psychrotrophic growth in cooled raw milk with the concomitant production of heat-resistant spoilage enzymes presents a formidable challenge to the dairy industry, which relies on refrigerated storage of the raw milk supply and high temperature treatment to produce long shelf life products like UHT-milk and other related dairy products and milk powder. The predominant genus responsible for milk spoilage worldwide found in cold raw milk is *Pseudomonas*, although in specific regions *Serratia* is considered a predominant genus responsible for milk spoilage appearing in cold raw milk. Further research using cultivation-independent metagenomics studies should be performed to exclude possible cultivation biases in most of the studies performed up to now. But bacterial isolates will remain necessary to establish whether they truly produce heat resistant enzymes relevant for spoilage of UHT-milk and related products. The current scientific knowledge on peptidase and lipase enzyme production and activity in these microorganisms still gives no viable possible control options. At present, these microorganisms should be controlled as much as possible at each step of the dairy production chain taking into account an optimal hygiene and cooling management. However, a taxonomically exact and region tailored knowledge of the heat-resistant spoilage enzyme producing microbiota in raw milk will help to trace the contamination sources in the supply and production chain in order to prevent their entrance. The data on the microbiota composition in raw milk presented in this review on a worldwide scale may offer the necessary points of view to look for specific as well as common patterns of contamination with these spoilage microorganisms. On the other hand, a first fast screening at the dairy processing plant of the incoming raw milk for potential

heat-resistant spoilage enzymatic activity would be helpful to steer the milk flow toward processing for long shelf life milk products such as UHT-milk or toward other shorter shelf life products. No such test, which would require very high sensitivity, is currently available; its development represents an enormous scientific challenge.

AUTHOR CONTRIBUTIONS

SGM, FB, MH, and MV contributed substantially to the conception of this review. SM, JD, and MH have participated

in the acquisition of data about the relationship between off-flavors and proteolysis in milk samples. EV contributed to the section on control of spoilage by heat-resistant bacterial enzymes. MV, EV, and JD also provided a critical review of the manuscript.

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The Evolving Role of Coliforms As Indicators of Unhygienic Processing Conditions in Dairy Foods

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Testing for coliforms has a long history in the dairy industry and has helped to identify raw milk and dairy products that may have been exposed to unsanitary conditions. Coliform standards are included in a number of regulatory documents (e.g., the U.S. Food and Drug Administration's Grade "A" Pasteurized Milk Ordinance). As a consequence, detection above a threshold of members of this method-defined, but diverse, group of bacteria can result in a wide range of regulatory outcomes. Coliforms are defined as aerobic or facultatively anaerobic, Gram negative, non-sporeforming rods capable of fermenting lactose to produce gas and acid within 48 h at 32–35°C; 19 genera currently include at least some strains that represent coliforms. Most bacterial genera that comprise the coliform group (e.g., *Escherichia*, *Klebsiella*, and *Serratia*) are within the family Enterobacteriaceae, while at least one genus with strains recognized as coliforms, *Aeromonas*, is in the family Aeromonadaceae. The presence of coliforms has long been thought to indicate fecal contamination, however, recent discoveries regarding this diverse group of bacteria indicates that only a fraction are fecal in origin, while the majority are environmental contaminants. In the US dairy industry in particular, testing for coliforms as indicators of unsanitary conditions and post-processing contamination is widespread. While coliforms are easily and rapidly detected, and are not found in pasteurized dairy products that have not been exposed to post-processing contamination, advances in knowledge of bacterial populations most commonly associated with post-processing contamination in dairy foods has led to questions regarding the utility of coliforms as indicators of unsanitary conditions for dairy products. For example, *Pseudomonas* spp. frequently contaminate dairy products after pasteurization, yet they are not detected by coliform tests. This review will address the role that coliforms play in raw and finished dairy products, their sources and the future of this diverse group as indicator organisms in dairy products.

Keywords: coliform, dairy foods, indicator organisms, *Pseudomonas*, pathogens

INTRODUCTION

In microbiological testing, an "indicator organism" is defined as a marker that reflects the general microbiological condition of a food or environment (Chapin et al., 2014). In contrast, an "index organism" is a marker that reflects the possible presence of ecologically similar pathogens, suggesting a potential public health risk (Chapin et al., 2014). For nearly a century, coliforms

have been used as indicator organisms, first in evaluating water for fecal contamination and later in identifying unsanitary conditions in pasteurized dairy products and other foods. Indeed, coliform testing of pasteurized milk was recommended by the U.S. Public Health Service in the earliest edition of the Grade "A" Pasteurized Milk Ordinance (PMO) published in 1924 (Tortorello, 2003). Currently, the PMO limits coliforms in Grade "A" pasteurized milk and milk products to 10 or fewer CFU per mL (FDA, 2015). Coliforms, defined as aerobic or facultatively anaerobic, Gram-negative, non-spore-forming rods capable of fermenting lactose with the production of acid and gas at 32–35°C (Davidson et al., 2004), were originally considered to represent only strains from the genera *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella*. Classification of coliforms has been a difficult issue for decades. Coliform differentiation was originally primarily based on the fermentation of sucrose and dulcitol, production of indole and acetyl methyl carbinol, and gelatin liquefaction. Later, Parr established the IMViC formula, which involved indole production, methyl red reaction, Voges-Proskauer test, and citrate utilization (Parr, 1938). Even with these methodological improvements, some strains were still not detected as part of the coliform group.

As taxonomic classification methodologies have improved over the decades, it has become clear that coliforms, as defined solely by the method used to detect them, are a much broader and more diverse group of bacteria (Leclerc et al., 2001). Currently, 19 genera have member strains that fall into the coliform group, mostly encompassed in the family Enterobacteriaceae, however, strains of *Aeromonas*, in the family Aeromonadaceae, also have been identified as coliforms (Abbott et al., 2003) because of their ability to ferment lactose to form gas and acid within 48 h at 32–37°C, although it should be noted that there is some disagreement regarding whether *Aeromonas* should be considered a coliform. Importantly, because of the method-defined nature of this group, it is not uncommon for some species or strains within a genus to be coliform-positive while others are coliform-negative. Such variability within genera complicates classification and understanding of these microorganisms.

In an effort to increase functional differentiation within the diverse coliform group, Leclerc et al. (2001) proposed three categories of coliforms based on taxonomic and physiological traits: "thermophilic," which include *Escherichia coli* of fecal origin; "thermophilic and ubiquitous" and; "psychrotrophic," which are purely environmental. Of the "thermophilic" coliforms, which are characterized by their ability to grow and ferment lactose at 44–45°C, the only reliable indicator of fecal contamination is *E. coli*. This organism does not survive well in environments outside of the intestinal tract of warm-blooded animals, hence, it is not an environmental contaminant. However, while others in this group, including some species of *Klebsiella*, *Enterobacter*, and *Citrobacter*, may originate from fecal matter, they also can originate from environmental sources, making them unreliable indicators of fecal contamination. In contrast, "psychrotrophic" environmental coliforms have the ability to grow and ferment lactose at refrigeration temperatures, but generally do not grow above 38°C, which

distinguishes them from the thermophilic group. Members of the genera *Klebsiella*, *Enterobacter*, *Serratia*, and others are considered environmental coliforms (Leclerc et al., 2001). Finally, "thermophilic and ubiquitous" coliforms originate from various natural environments including soil, water, vegetation, insects, farm produce, wooden reservoirs, grass, silages, and fresh vegetables (Seidler et al., 1975). Members of this group of "ubiquitous" coliforms are found within the genera *Klebsiella*, *Enterobacter*, and *Citrobacter*.

As a consequence of the improved understanding of the environmental sources of many microorganisms that test positive as coliforms, many industries have moved away from using detection of total generic coliforms for food and water testing (Leclerc et al., 2001; Busta et al., 2006) as they are poor indicators of fecal contamination and overall hygienic conditions. However, coliform testing remains a cornerstone of microbial testing in the U.S. dairy industry, from raw milk testing to processed dairy product testing. Recent studies provide evidence that coliform testing should be reconsidered as a marker for unsanitary conditions in the dairy industry as further understanding of this diverse group of microbes is achieved.

Coliforms Represent a Common Raw Milk Contaminant that Originates from Various Environmental and Fecal Sources

Coliforms are among the many groups of microorganisms that are normally present in raw milk, i.e., 96% of all bulk tank milk samples collected during a 2002 study in the U.S. were coliform-positive (Van Kessel et al., 2004). California has established the only regulatory limit for coliforms in raw milk intended for Grade "A" dairy products in the U.S. (not to exceed 750 CFU/mL; California Department of Food and Agriculture [CDFA], 2016). Reported coliform levels in raw milk vary greatly, with mean coliform counts for milk sampled in the U.S. ranging from 31 cfu/mL (Boor et al., 1998) to 2,570 cfu/mL (Jayarao and Wang, 1999). Similar results have been reported by others (D'Amico et al., 2008; Pantoja et al., 2011; Jackson et al., 2012). Common coliform genera in raw milk include *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella* (Jayarao and Wang, 1999), which can originate from a variety of sources in the dairy farm environment including water, plant materials, equipment, dirt, and fecal sources (Kagkli et al., 2007). High levels of coliforms (e.g., >1,000 cfu/mL) in raw milk may indicate unsanitary practices on the farm, inadequate refrigeration, or the presence of coliform mastitis (Jayarao and Wang, 1999; Hogan and Smith, 2003, Pantoja et al., 2011). Additionally, certain management practices at the farm level, including milking machine wash failures, rate of cluster washes and rate of milking unit fall-off during milking also correlate to variations in levels of coliforms in raw milk (Pantoja et al., 2011).

Milking mastitic cows can introduce coliforms into bulk tank raw milk, hence somatic cell counts (SCC) also can be

correlated with the presence of coliform bacteria. Coliform genera recognized as causing mammary infections include *Escherichia*, *Klebsiella*, *Enterobacter*, and *Serratia* (Hogan and Smith, 2003). The cow may become exposed to mastitis pathogens through manure, bedding, soil, and water (Hogan and Smith, 2003). Pantoja et al. (2011) found that in-line coliform counts increased 6.3% for every 10% increase in in-line SCC, which could reflect as little as the milk from one mastitic cow being milked into the bulk tank.

Despite there being no federal coliform regulation for raw milk being processed into U.S. Grade "A" dairy products, many states that allow the sale of raw milk for direct human consumption have regulatory limits for coliforms. For example, in California, raw milk "shall contain not more than 15,000 bacteria per milliliter or [not] more than 10 coliform bacteria per milliliter" (California Food and Agriculture Code, 2016). According to a Raw Milk Survey conducted by the National Association of State Departments of Agriculture (NASDA; Ehart) in 2011, 30 states allowed raw milk sales. Five of the thirty states had special regulations for raw milk, including "cow-share" agreements, in which the consumer "owns" all or part of a cow, and therefore, can have access to its milk, or limit raw milk sale to specific markets. Among the 30 states, twelve allow the consumer to access milk at both the farm where the milk is produced and at retail stores that can be separate from the farm. The remaining thirteen states restrict legal sales of raw milk only to the farm where the milk is produced. Of the 30 states that allow sale of raw milk for human consumption, coliform limits of ≤ 10 cfu/mL to ≤ 100 cfu/mL are imposed in 20 states (Table 1; Ehart, 2011).

While the use of coliforms as indicator organisms for the presence of unsanitary conditions in milk handling is increasingly under scrutiny, it is clear that coliforms are not appropriate index organisms for the presence of public health hazards in dairy products. For example, Jackson et al. (2012) examined levels of coliform bacteria in raw silo milk in correlation to the presence and levels of four pathogens of interest

(*Bacillus cereus*, *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp.). The study concluded that there were no significant increases in coliform levels in pathogen-positive samples as opposed to pathogen-negative samples. Similarly, no significant differences existed in coliform counts from samples with zero, one, two, three, or four pathogens detected. These results illustrate that coliform counts are not an index of the presence of these four pathogens, and that coliform testing of raw milk intended for human consumption cannot be used to reliably identify raw milk that presents a public health risk. This is also consistent with other studies (D'Amico et al., 2008) that detected pathogens in raw milk samples that had very high microbiological quality and low coliform counts.

Coliform Contamination in Pasteurized Fluid Milk Leads to High Total Bacteria Counts and Low Sensory Scores

Coliform testing has been used to indicate hygienic condition of dairy products for nearly a century. Coliforms are common contaminants in fluid milk (Carey et al., 2005; Martin et al., 2012), cheeses (Wolfe et al., 2014; Trmčić et al., 2016) and other dairy products. Recent studies have shown post-processing contamination (PPC) with coliforms in 7.6–26.6% of U.S. fluid milk samples tested between 2001 and 2010 (Martin et al., 2012). Pasteurized fluid milk samples that were contaminated with coliforms had significantly higher bacterial counts and significantly lower overall sensory scores (Martin et al., 2012) over shelf-life than samples that tested negative for coliforms. The PMO limits the number of coliforms in pasteurized grade "A" milk to no more than 10 cfu/mL throughout shelf-life (FDA, 2015). In general, due to the heat labile nature of these organisms, the presence of coliforms and other Gram-negative bacteria in pasteurized fluid milk indicates: (i) PPC of the product; or (ii) pasteurization failure. Many coliforms in pasteurized fluid milk products are psychrotolerant, and thus able to grow to high levels at refrigeration temperatures (Carey et al., 2005; Ranieri and Boor, 2009; Martin et al., 2011; Masiello et al., 2016).

A recent study of coliform bacteria in pasteurized fluid milk indicated that species of *Enterobacter*, *Hafnia*, *Citrobacter*, *Serratia*, and *Raoultella* represented the majority of the coliform population (Masiello et al., 2016). Of the coliform isolates collected by Masiello et al. (2016), the majority showed the ability to grow substantially (i.e., > 5 log growth) over 10 days at refrigeration temperatures. This robust growth, accompanied by the ability of many psychrotolerant coliforms to produce lipolytic and proteolytic enzymes (Wessels et al., 1989; Nornberg et al., 2009; Masiello et al., 2016) which are capable of causing flavor, odor and body defects in fluid milk, make the presence of coliforms in fluid milk detrimental to quality and consumer acceptance.

Prevention of PPC with coliforms and other microorganisms remains a major hurdle for some dairy processors in the U.S. (Ralyea et al., 1998; Ranieri et al., 2009; Martin et al.,

TABLE 1 | Summary of coliform standards for raw milk sold for human consumption.

Coliform standard ²	Number of states allowing raw milk sales ¹		
	On-farm sale	Retail milk sale	Cow-share/Other ³
No limit	8	0	2
≤ 10 cfu/mL	4	9	2
≤ 25 cfu/mL	0	1	0
≤ 50 cfu/mL	0	2	0
≤ 100 cfu/mL	1	0	1
Total	13	12	5

¹For details on raw milk regulations by state, see Ehart (2011); as state-level raw milk regulations change frequently, states are not listed here to avoid mis-leading or out-of-date information. ²cfu = colony forming units. ³A cow-share is an agreement entered into by individual(s), who pay a farmer a fee for boarding and milking the cow(s) that they own. After the cows are milked, the individual(s) obtain the milk from the farmer. Technically, these arrangements are not considered "raw milk sales."

2011). In many cases, contamination can be traced back to the presence of biofilms in processing equipment. Many types of bacteria are capable of forming biofilms in equipment, especially in cracks, dead ends and gaskets. Biofilms, which have been described as a functional consortium of microorganisms attached to a surface and embedded in the extracellular polymeric substances produced by the microorganisms (Costerton et al., 1987), allows colonization of populations of microorganisms and provides protection for the microbes from cleaning and sanitization procedures. As the biofilm matures, cells slough off and can contaminate product during processing (Kumar and Anand, 1998). In dairy processing, in particular, the use of clean-in-place (CIP) systems may unintentionally lead to biofilm formation because such systems may fail to remove accumulated microorganisms and organic materials effectively (Kumar and Anand, 1998). The formation of the biofilm begins with a process known as conditioning which begins 5–10 s after milk processing begins (Marchand et al., 2012). In particular in processes where temperatures are high enough to begin to denature whey proteins (i.e., 65°C), adherence of this layer to the surface alters the surface properties and improves the ability of bacterial contaminants to adhere (de Jong, 1997). Continuation of the process of biofilm formation, namely bacterial adhesion, bacterial growth and biofilm expansion (Marchand et al., 2012) leads to biofilms that are resistant to removal, especially using CIP systems. Stringent cleaning and sanitation practices along with attention to sufficient preventative maintenance, hygienic design and employee training are essential to minimize formation of biofilms and prevent PPC.

Coliforms in Cheese Represent a Diverse Group of Organisms

Coliforms are widely found in many cheeses (Khayat et al., 1988; Brooks et al., 2012). However, in contrast to the presence of these microbes in raw and pasteurized fluid milk, and even in some other cultured products (e.g., yogurt), the presence of coliforms in cheese may not necessarily be negative. The vast variety of types of cheese manufactured contributes to the complexity of fully understanding the role of coliforms in cheese quality and safety. Cheese product characteristics, including moisture content, pH, salt content, ripening conditions, age of product, and culture all influence potential levels of and roles for coliforms and other microorganisms in the final product (Wolfe et al., 2014; Trmčić et al., 2016). A survey of raw milk cheeses by Brooks et al. (2012) found that 5 of 41 commercially available raw milk cheese samples had detectable coliforms (i.e., >10 cfu/g). In a similar study, Trmčić et al. (2016) surveyed 273 raw and pasteurized cheeses from the U.S. and other countries and found that 75 of those samples were positive for coliforms in concentrations above 10 cfu/g.

Many individual states in the U.S. have limits of 10 or 100 cfu/g for coliforms in cheese. In the European Union (EU), where microbiological specifications are regulated by the European Commission (EC), there are no regulations

concerning coliforms (EC No 2073/2005) for cheese products. Regulations, instead, are focused on *Salmonella*, coagulase positive *Staphylococci* and *E. coli*. Additionally, regulations set forth by the EC are categorized by type of product (i.e., cheese made from raw milk or from thermized milk, soft cheese, fresh cheese and other cheeses), thus acknowledging the need for a scientific approach to assessing the hygienic conditions and microbial food safety hazards associated with cheeses.

Sources of coliforms in cheese products can vary depending on the product. Due to the nature of raw milk cheeses, the presence of coliforms is not unexpected as coliforms are common in raw milk. However, in pasteurized cheese products, coliforms present in raw milk should have been eliminated by pasteurization, implying that any coliforms present in the finished product resulted from PPC. Recontamination can occur in the processing or aging facility through cheese contact with contaminated water, humans, air, and biofilms on equipment (Lawrence and Lilly, 1972; Dancer et al., 1997; Hughes, 2003; Kilb et al., 2003).

High levels of coliforms in pre-cultured milk intended for cheese making may have deleterious effects on cheese production, specifically if acid development by the lactic acid bacteria (LAB) occurs more slowly than desired. Growth of coliforms early in cheese production may lead to early blowing, or gas production defects in the product (Farkye, 2000; Ledebach and Marshall, 2009). Additional effects and byproducts of coliform growth early in cheese production can be reduction of desirable formation of diacetyl (Ledenbach and Marshall, 2009), lactic acid, acetic acid, formic acid, succinic acid, ethanol, and 2,3-butyleneglycol (Farkye, 2000).

The growth or death of coliforms in cheese products depends on a variety of parameters including cheese pH, age, moisture content, salt content, free fatty acid content and others. Nunez et al. (1985) found that Manchego cheese products made with cooked curd had higher levels of coliforms than those made with uncooked curd. This difference was attributed to lower pH in the uncooked curd (due to superior growth of LAB). Nunez et al. (1985) also found that the temperature of ripening had a significant effect on the reduction of coliforms, concluding that an aging temperature of 15°C was the optimum temperature to achieve reduction in coliforms (and other unwanted bacteria) and also to protect desired sensory attributes. Coliforms are typically inactivated and/or inhibited by the drop in pH during cheesemaking acidification. If pH increases during aging (due to proteolysis, typically in surface ripened cheese), however, conditions may exist to support coliform growth (Ledenbach and Marshall, 2009). Finally, Trmčić et al. (2016) reported that pasteurization, pH, water activity, milk type (e.g., cow milk), and rind type were cheese factors that significantly influenced detection of coliforms in cheese. They also report that water activity is significantly associated with the final concentration of coliforms in cheese; suggesting more than 0.5 log cfu/g higher average final concentration of coliforms for every 0.01-unit increase in water activity.

Proteolytic and lipolytic enzyme production varies greatly in the coliform group (Wessels et al., 1989). Enzyme production is largely dependent on product storage temperature. Proteolytic and lipolytic enzymes can contribute both desirably and undesirably to flavor and texture characteristics of cheese. The proteolytic activities of some strains of coliforms have been studied (Macedo and Malcata, 1997; Nornberg et al., 2009); some are highly proteolytic. To date, studies have primarily focused on the negative impact of enzymatic activity from coliform origin on dairy product quality, but some work has examined possible advantageous impacts that coliform enzymatic activity may have on ripening and flavor development of certain cheeses (Macedo and Malcata, 1997). The notion of coliforms as possible desirable contributors to the complex ecosystem of cheeses, particularly farmstead and artisan cheeses, is supported by studies suggesting that coliforms may be part of the natural microflora of at least some cheeses (Quigley et al., 2011). Further, as different Gram-negative bacteria are being identified as having a high potential for production of aroma compounds during cheese production, new bacterial cultures are being developed to utilize this potential. Some of the species used in these new bacterial cultures (e.g., *Hafnia alvei*) are members of coliforms/Enterobacteriaceae in which case the use of these bacterial groups as indicators would not be appropriate (Morales et al., 2003; Deetae et al., 2009).

In the U.S., testing dairy products for coliforms (beyond fluid milk and cheese) is required by the PMO. Coliform limits in cultured products (e.g., yogurt), ice cream, non-fat dry milk and others are set at ≤ 10 cfu/ml or g (FDA, 2015). Current standard methods recommend testing yogurt for coliforms within 24 h of production to obtain meaningful results (Duncan et al., 2004). However, enumerating *Enterococcus* may provide a more reliable hygiene indicator than coliforms because they are more likely to survive in the low pH environment (Frank and Yousef, 2004). There is little research on the use of Enterococci as indicators in high acid dairy products, however, Birollo et al. (2001) concluded that Enterococci have little industrial use as hygiene indicators in yogurt processing. While the pH of yogurt has long been considered too low to allow survival of coliforms, limited evidence exists to support this conventional wisdom. A recent study by Hervert (2016) evaluated a variety of common coliforms, *Enterobacteriaceae* (EB) and non-EB Gram-negatives (e.g., *Pseudomonas*) for their abilities to survive in commercial yogurt products. The study showed that, in general, coliform and EB organisms were capable of surviving and, sometimes, even growing under conditions encountered in commercial yogurt products, while non-EB Gram-negative bacteria showed rapid die-off. The authors concluded that testing for EB provided the most comprehensive approach for monitoring hygiene indicators in yogurt as opposed to testing for coliform and total Gram-negative bacteria.

Coliform contamination in ice cream has not been widely or recently studied in the U.S., although surveys from other countries indicate that coliform levels range from less than detectable to $>10^4$ cfu/g (Massa et al., 1989; Warke et al., 2000; M-E-Elahi et al., 2002; El-Sharaf et al., 2006). The

storage conditions of ice cream are generally thought to inhibit growth of bacterial contaminants, including coliforms. As a heat-treated product, the presence of coliforms in ice cream and other frozen dairy products is an indicator of PPC. However, because contaminated ingredients (e.g., nuts, fruits, etc) may be added to the product after pasteurization, there is considerable opportunity for bacterial contamination that does not originate from unhygienic conditions, *per se*, in the processing facility (Duncan et al., 2004).

A Century of Coliform Testing – Time to Rethink Our Indicator Organisms in the Dairy Industry?

As the landscape of the global and U.S. food industries changes and responds to new requirements to ensure a safe food supply, there is reason to review traditional methods of evaluating dairy product hygiene and safety. Because of their heat-labile nature, coliforms long have been used in the dairy industry as indicators of PPC. Certainly, in general, coliforms are undesirable in processed dairy products (e.g., fluid milk). However, while coliforms do represent PPC and can cause flavor, odor and body defects in many dairy products, in some dairy products, detection of this group of microbes is insufficient for identifying unhygienic conditions.

Recent work indicates that testing for EB or total Gram-negative bacteria offers a distinct advantage to coliform testing when detecting common PPC organisms in dairy products (Hervert et al., 2016). EB is a taxonomic group of microorganisms that encompasses almost all of the coliform group (Hervert et al., 2016) with the exception of *Aeromonas*, and has been used as a hygiene indicator broadly in Europe (European Communities Regulation, 2010). A benefit of testing for EB over coliforms is increased sensitivity for detecting PPC because of the broader range of contaminants detected (Hervert et al., 2016). Although the EB group includes some pathogenic bacteria (e.g., *Salmonella*), EB are considered indicators as opposed to index organisms. In general, their presence in some food products has no correlation with the presence of pathogens (Johnson, 1996), although this has not been studied specifically in dairy foods. Recent work has identified that the EB group is superior as a hygiene indicator in yogurt products because they are capable of surviving, and even growing, under conditions encountered in that product (Hervert, 2016).

Testing for total Gram-negative bacteria as an indicator of unsanitary conditions in certain dairy products (e.g., fluid milk) offers a distinct advantage over coliform or EB testing (Table 2). *Pseudomonas*, which lacks the ability to ferment lactose and is therefore not a coliform, has been described as the major contributor to PPC in the U.S. fluid milk industry (Ranieri and Boor, 2009; Martin et al., 2012). *Pseudomonas* readily forms biofilms in processing equipment (Ralyea et al., 1998) and, according to a survey of fluid milk across the U.S., accounts for $\sim 70\%$ of fluid milk spoilage from PPC in the U.S. (Ranieri and Boor, 2009). However, coliform tests do not detect *Pseudomonas*

TABLE 2 | Proposed hygiene indicator tests for different dairy products.

Product	Proposed microbial hygiene indicator test ²	Justification	Key references
Fluid milk	Total Gram-negative bacteria	Key hygienic issues in pasteurized fluid milk are (i) PPC and (ii) pasteurization failure. Both can be detected more reliably with a test that detects all GN bacteria (rather than coliform or Enterobacteriaceae [EB] tests).	Ranieri and Boor, 2009; Martin et al., 2012
Fermented dairy products (e.g., yogurt, kefir, etc)	Enterobacteriaceae (EB)	Non-EB Gram-negative bacteria decline rapidly at the pH encountered in fermented dairy products while EB generally survive in these conditions making it possible to detect them as indicators of unhygienic conditions.	Hervert, 2016; Hervert et al., 2016
Aged cheeses	Targeted risk-based pathogen testing ¹	No suitable tests are currently available, specific pathogen tests are recommended based on risks associated with specific cheese characteristics (e.g., pH, a _w , etc).	Schvartzman et al., 2014; Trmčić et al., 2016
Fresh cheeses	EB and/or <i>Escherichia coli</i> (additional research needed) ²	Currently coliforms and EB are commonly used as hygienic indicators in fresh cheeses.	
Dairy powders	EB and/or targeted risk-based pathogen testing (additional research needed) ²	Currently coliforms and EB are commonly used as hygienic indicators, but testing for selected pathogens is typically required for dairy powders that are used in infant formula.	
Ice cream	Total Gram-negative bacteria (additional research needed) ²	Currently coliforms and EB are commonly used as hygienic indicators in ice cream.	
Butter	Total Gram-negative bacteria (additional research needed) ²	Currently coliforms, EB, and proteolytic bacteria are commonly used as hygienic indicators.	

¹Testing for target pathogens of concern may be appropriate for all products (or required under some jurisdictions), even if not specifically mentioned in this Table.

²Proposed indicator tests for these four products (fresh cheese, dairy powders, ice cream, butter) are based on product characteristics, processing parameters and research findings from other dairy products; additional research is needed for these specific products to make more definitive recommendations regarding best practices for microbial hygiene indicator tests.

and other non-coliform Gram-negative bacteria that commonly contaminate fluid milk post-processing. Van Tassell et al. (2012) found that crystal violet tetrazolium agar (CVTA) was the most effective selective medium for detecting a diverse group of *Pseudomonas* commonly associated with PPC in fluid milk, whereas commonly employed coliform media (e.g., violet red bile agar) had limited ability to detect *Pseudomonas*. Therefore, coliform testing is not an effective approach for detecting fluid milk exposed to PPC. As dairy plants strive to reduce PPC, the ability to identify contamination occurrences and to rapidly respond is critical to improving the quality of fluid milk products. Based on the current understanding of the ecology of PPC in fluid milk and the inability of coliform testing to identify the majority of these contaminants, exclusive use of coliform testing for this purpose ironically may prevent the fluid milk industry from detecting and rapidly resolving contamination issues.

Further, in the cheese industry, there is growing concern that coliform testing, especially in raw milk cheeses, provides little in the way of indicating hygienic conditions. Some research suggests that certain members of the coliform group, in fact, may be advantageous microorganisms in certain types of cheese (Macedo and Malcata, 1997; Quigley et al., 2011), and that coliforms serve no scientifically valid function as an index organism (i.e., for suggesting pathogen contamination). At best, coliform testing in cheese may provide insight into potential PPC, depending on the product. At worst, coliform testing may provide a false sense of security when public health

risks from pathogenic contaminants are present. Trmčić et al. (2016) assessed the association between coliform detection in raw and pasteurized cheeses and the presence of *Salmonella*, *Staphylococcus aureus*, Shiga toxin-producing *E. coli*, *Listeria monocytogenes*, and other *Listeria* species. This study found no association between pathogen presence and coliform detection, despite an association between *Listeria monocytogenes* with washed rind style cheeses. Other groups have also found that cheese characteristics (e.g., pH) are associated with the presence of pathogens in the product (Schvartzman et al., 2014). This is not surprising given the association between cheese characteristics and overall microbial diversity in cheese (Wolfe et al., 2014). The lack of association between the presence of pathogens and coliform detection, as well as the evidence that cheese characteristics are associated with pathogen prevalence, suggests that a model whereby products are categorized by their inherent characteristics and tested for organisms that are likely to cause a public health threat in those particular products provides a more effective approach to assuring public health than coliform testing (Table 2).

CONCLUSION

Testing for the presence of coliform bacteria, a method-defined group, has long been practiced in the U.S. dairy industry, from raw milk to processed products. Coliform testing is rapid and

has long been used as a primary indicator test for hygienic conditions associated with dairy products. However, recent advances in taxonomy and understanding of coliforms has led to questions regarding the suitability of testing for this diverse group of organisms as indicators for unhygienic conditions in dairy products. From fluid milk, where coliforms represent a minor proportion of PPC, to cheese products, where coliforms do not accurately represent public health risks, it is time to rethink the relevance of this century-old indicator group as a means for protecting public health. We propose implementation of appropriate pathogen testing (e.g., *Listeria* testing in washed rind cheeses) or testing for a comprehensive group of all organisms linked to PPC (e.g., total Gram-negative testing in fluid milk) to ensure a high quality and safe dairy food supply.

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NM, TH, and AT were primarily responsible for literature review. NM, AT, TH, KB, and MW were responsible for preparing the manuscript.

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***Staphylococcus aureus* Isolates from Goat and Sheep Milk Seem to Be Closely Related and Differ from Isolates Detected from Bovine Milk**

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Dairy goat and sheep farms suffer severe economic losses due to intramammary infections, with *Staphylococcus aureus* representing the main cause of clinical mastitis in small ruminants. In addition, *S. aureus* contamination of goat and sheep milk may cause staphylococcal food poisoning, as many traditional caprine and ovine milk products are not subjected to pasteurization. Data on virulence and antimicrobial resistance genes, as well as on the clonality of *S. aureus* detected in goat and sheep milk is scarce. Therefore, it was the aim of this study to determine (i) spa types and clonal complexes (CC) and (ii) virulence and resistance gene profiles of *S. aureus* isolated from goat and sheep milk. A total of 162 milk samples from sheep and goats presenting signs of an intramammary infection and 104 bulk milk samples were collected. While low prevalence rates of *S. aureus* was detected on single animal level, 46% of the bulk tank milk samples from small ruminants were positive for *S. aureus*. All isolates were spa typed and CC and virulence and resistance gene patterns were determined using a DNA microarray. Data from 49 *S. aureus* isolates was included in the statistical analysis and the construction of a SplitsTree. The analyzed isolates could be assigned to eleven CC, with the large majority of goat and sheep isolates being assigned to CC130 and CC133. The findings of this study suggest that *S. aureus* shows pronounced adaptation to small ruminants in general, but not to sheep or goats in particular. Although some common characteristics among *S. aureus* from caprine, ovine, and bovine milk samples were observed, *S. aureus* from small ruminants seem to form a distinct population. As 67% of the detected *S. aureus* strains exhibited at least one enterotoxin gene, many caprine, or ovine raw milk products may be contaminated with low levels of enterotoxigenic *S. aureus*, stressing the importance of strict maintenance of the cold chain.

Keywords: *Staphylococcus aureus*, sheep, goat, clonality, enterotoxin genes, virulence gene profile, mastitis

INTRODUCTION

Being one of the predominant causes of food poisoning worldwide, *Staphylococcus aureus* is of particular concern to the dairy industry (Oliver et al., 2009). Dairy sheep and goat farms also suffer severe economic losses due to staphylococcal intramammary infections, with *S. aureus* being the main cause of clinical mastitis in small ruminants (Bergonier et al., 2003). However, identification

of affected animals can be challenging, as in contrast to cattle, high somatic cell counts and positive results in the California mastitis test are not necessarily reliable indicators of intramammary infections among small ruminants.

Over the last decade, the production of caprine and ovine milk in Switzerland has been increasing, with 14,000 registered small ruminant farms and a total population of approximately 490,000 heads in 2014 (Swiss Federal Statistical Office). *S. aureus* is one of the most commonly found pathogens in raw caprine and ovine milk (Marogna et al., 2012) and has been detected in over 30% of the examined raw milk of Swiss dairy goat and sheep farms (Muehlherr et al., 2003). As goat and sheep milk are often used for traditional, unpasteurized products such as raw milk cheeses, they represent a potential source of staphylococcal food poisoning (SFP).

The Centers for Disease Control estimate a total number of 240,000 SFP cases per year in the US (Scallan et al., 2011). In the EU, the number of SFP outbreaks is rising, with 386 SFP outbreaks reported in 2014 (Anonymous, 2015). SFP patients present with violent vomiting and diarrhea upon ingestion of staphylococcal enterotoxins pre-formed by *S. aureus* in food (Tranter, 1990). Many different staphylococcal enterotoxins and enterotoxin-like superantigens have been described (Dinges et al., 2000). There is evidence demonstrating emetic activity in humans for all classical enterotoxins SEA-SEE (Dinges et al., 2000) and recently also for some newly described enterotoxins (Jørgensen et al., 2005; Johler et al., 2015).

While the population structure and the genomic characteristics of *S. aureus* from bovine milk are very well described, similar data on *S. aureus* isolated from small ruminants is scarce (Scherrer et al., 2004; Concepción Porrero et al., 2012; Gharsa et al., 2012; Linage et al., 2012; Eriksson et al., 2013; Smith et al., 2014). Data on virulence and antimicrobial resistance genes, as well as on the clonality of *S. aureus* detected in goat and sheep milk is crucial to determine potential routes of transmission, to improve management strategies of affected herds, and to develop effective therapeutic interventions. Therefore, it was the aim of this study to determine clonal complexes (CC) and virulence and resistance gene profiles of *S. aureus* isolated from goat and sheep milk.

MATERIALS AND METHODS

Bacterial Isolation and DNA Extraction

In this study, 162 milk samples of goats ($n = 31$) and sheep ($n = 131$) exhibiting one or several signs of mastitis (increased somatic cell counts, positive California mastitis test, decreased milk yield), as well as 104 raw bulk milk samples were collected from dairy farms in Switzerland (goat farms: $n = 57$; sheep farms: $n = 47$) from March to October 2015. EN ISO 6888-2 was followed for isolation of coagulase-positive staphylococci. One single colony of each different morphology exhibiting an opaque fibrin halo on rabbit plasma fibrinogen agar (Oxoid, Basel, Switzerland) was subcultured. The subcultures were grown on 5% sheep blood agar at 37°C overnight. Chromosomal DNA extraction was performed using the DNeasy Blood and Tissue

Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Staphaurex Latex Agglutination Test

All *S. aureus* isolates were subjected to the Staphaurex latex agglutination test (Oxoid, Basel, Switzerland) following the manufacturer's instructions. This assay targets microbial surface components recognizing adhesive matrix molecules (SpA, ClfA, FnBPA, and FnBPB) and frequently yields false-negative results in bovine *S. aureus* (Stutz et al., 2011; Moser et al., 2013).

DNA Microarray, SplitsTree Analysis, and Comparison to Bovine Isolates

DNA microarray analysis was performed using Staphytype genotyping kit 2.0 (Alere, Jena, Germany) following the manufacturer's instructions. The DNA microarray used in this study determines the presence or absence of over 300 different genes and allelic variants, and allows for assignment of CC (Monecke et al., 2008). All presumptive *S. aureus* isolates were further characterized by DNA microarray profiling, which also served as a tool for species confirmation. The DNA microarray hybridization results of isolates from goats and sheep were compared to those of isolates from an unrelated collection of 78 bovine *S. aureus* strains that were obtained in a comprehensive study investigating mastitis isolates from cows in Switzerland (Moser et al., 2013). The resistance and virulence gene profiles of the caprine, ovine, and bovine isolates were visualized using SplitsTree⁴¹ as previously described (Wattinger et al., 2012).

spa Typing

spa typing, a high resolution single-locus typing technique in *S. aureus*, was performed as previously described (Johler et al., 2011). Briefly, PCR amplicons of the polymorphic X region of the *spa* gene were purified using the GenElute PCR Purification Kit (Sigma-Aldrich, St. Louis, MO, USA), and were subsequently sequenced and assigned to *spa* types².

Inclusion Criteria

Stringent inclusion criteria were employed to avoid bias overrepresentation of strains isolated from both single animals and bulk milk samples of the same dairy farm: only one *S. aureus* isolate was considered for construction of the SplitsTree and statistical analysis, if the analyzed isolates exhibited the same *spa* type and ≤ 3 different hybridization results in the DNA microarray profiling. Two single animal isolates from sheep were therefore excluded from the study, resulting in 49 *S. aureus* isolates taken into consideration for further analyses.

Statistical Analysis

Statistically significant differences in the distribution of virulence and resistance genes between the bovine, caprine, and ovine isolates were assessed by either Chi squared test or Fisher's exact test (in case $n < 5$) using SPSS 23.0 (IBM Corp., Armonk, NY, USA).

¹<http://www.splitstree.org/>

²<http://www.spaserver.ridom.de/>

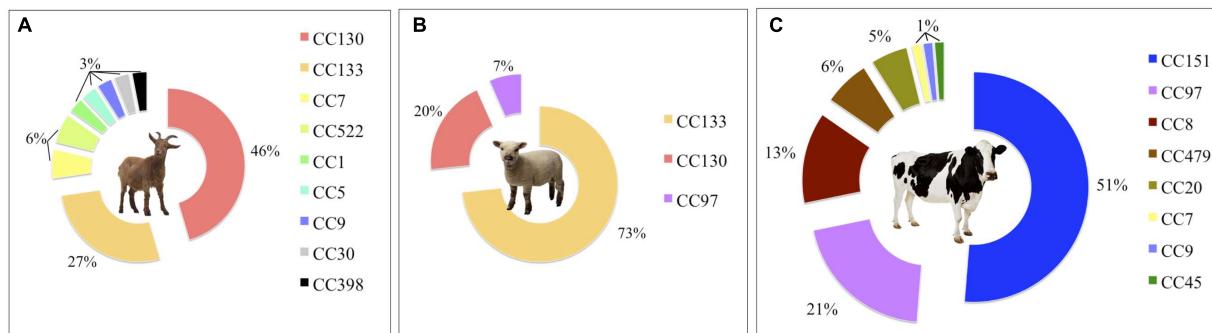


FIGURE 1 | Distribution of clonal complexes (CC) among *Staphylococcus aureus* isolated from the milk of different animal species: (A) goat (*n* = 34), pink; (B) sheep (*n* = 15, blue); (C) cows (*n* = 78, green).

RESULTS

A total of 162 milk samples (goats: *n* = 31; sheep: *n* = 131) of animals presenting signs of an intramammary infection and 104 bulk milk samples (goat farms: *n* = 57; sheep farms: *n* = 47) were collected. On the level of single animals, none of the goat milk samples and 2% (*n* = 3) of the sheep milk samples were positive for *S. aureus*. On the level of bulk milk samples, 60% (*n* = 34) of goat bulk milk

samples and 30% (*n* = 14) of sheep bulk milk samples were positive for *S. aureus*, which equals an overall prevalence of 46% among the examined bulk milk samples of small ruminants.

S. aureus from small ruminants were compared to bovine mastitis isolates from the study of Moser et al. (2013), with results being presented in Figures 1 and 2, as well as in Table 1. The distribution of CC among caprine, ovine, and bovine strains is depicted in Figure 1, and a SplitsTree comparing

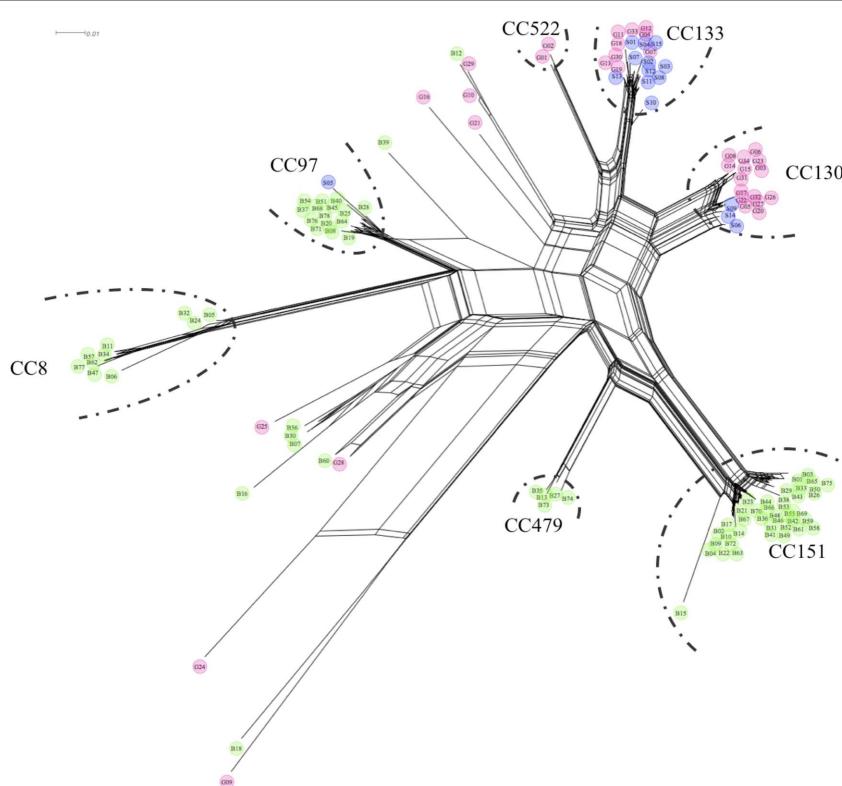


FIGURE 2 | The SplitsTree illustrates similarities between virulence and resistance gene profiles of goat (G), sheep (S), and bovine (B) *S. aureus* isolates. The isolates grouped into clusters mainly based on assignment to CC. Isolates from small ruminants form a distinct population and were mainly found in the clusters of CC130 and CC133. Bovine isolates were predominant in clusters CC8, CC97, CC151, CC479.

TABLE 1 | Prevalence rates of selected virulence and resistance genes detected among *Staphylococcus aureus* strains isolated from goat (G), sheep (S), and bovine (B) milk samples.

Group	Gene/Probe	Function	G (n = 34)	S (n = 15)	B (n = 78)
agr	<i>agrI</i>	Accessory gene regulator, type 1	44*S	80*G,B	41*S
	<i>agrII</i>	Accessory gene regulator, type 2	6*B	0*B	59*G,S
	<i>agrIII</i>	Accessory gene regulator, type 3	50*S,B	20*G,B	0*G,S
Capsule	<i>cap5</i>	Capsule type 5	9*B	7*B	38*G
	<i>cap8</i>	Capsule type 8	91*B	93*B	62*G
Enterotoxins	<i>sea</i>	Enterotoxin A	3	0	10
	<i>sea</i> (320E)	Enterotoxin A, allelic variant 320E	0	0	9
	<i>sea</i> (N315)	Enterotoxin A, allelic variant N315	9	0	1
	<i>seb</i>	Enterotoxin B	0	0	0
	<i>sec</i>	Enterotoxin C	50*B	67*B	15*G
	<i>sed</i>	Enterotoxin D	0*B	0	13*G
	<i>see</i>	Enterotoxin E	0	0	0
	<i>seg</i>	Enterotoxin G	9*B	0*B	65*G,S
	<i>sei</i>	Enterotoxin I	9*B	0*B	65*G,S
	<i>sek</i>	Enterotoxin K	0	0	0
	<i>sel</i>	Enterotoxin L	50*B	67*B	15*G,S
	<i>selm</i>	Enterotoxin-like protein M	9*B	0*B	64*G,S
	<i>seln</i>	Enterotoxin-like protein N	9*B	0*B	65*G,S
	<i>selo</i>	Enterotoxin-like protein O	9*B	0*B	65*G,S
Other superantigens	<i>seq</i>	Enterotoxin Q	0	0	0
	<i>selu</i>	Enterotoxin-like protein U	9*B	0*B	65*G,S
	<i>egc</i>	Enterotoxin Gene Cluster (<i>seg/sei/selm/seln/selo/selu</i>)	9*B	0*B	65*G,S
	<i>tst1</i> ("bovine" allele)	Toxic Shock Syndrome Toxin, allele from strain RF122	35*B	40*B	8*G,S
	<i>etA/B/D</i>	Exfoliative toxins A, B, and D	0	0	0
	<i>pvl</i>	Panton Valentine Leukocidin	0	0	0
	<i>tetK</i>	Tetracycline	12*B	0	0*G
	<i>fosB</i>	Metallothiol Transferase	38*S	73*G	19
Resistance ¹	<i>sdrD</i>	Sialoprotein-binding protein D	97*B	100*B	37*G,S
	<i>spIE</i>	Serine protease E	59*S,B	27*G	35*G
Misc	<i>lukM/lukF-PV</i> (P83)	Bovine leukocidin	21*S,B	93*G	72*G
	<i>Q7A4X2</i>	Hypothetical protein	47*S,B	80*G	86*G
	<i>ssl06/set21</i>	Staphylococcal superantigen-like protein 6	41*S,B	73*G,B	21*G,S
	<i>ssl10/set4</i>	Staphylococcal superantigen-like protein 10	68*S	93*G	73

A comprehensive list of the prevalence of all genes detected by DNA microarray is provided as a supplement. ¹None of the isolates harbored the resistance genes *mecA*, *InuA*, *msrA*, *mefA*, *mphC*, *vatA/B*, *vgaA/B*, *aacA-aphD*, *aadD*, *aphA3*, *sat*, *dfrS1*, *far1*, *Q6GD50*, *mupA*, *cat*, *fexA*, *cfr*, *vanA/Z*, *qacA/C*. *The distribution of the respective gene differed significantly between strains from the stated sources (G, goat, S, sheep, B, bovine) with $p \leq 0.05$.

gene profiles of caprine, ovine and bovine strains is shown in **Figure 2**. In general, bovine isolates and isolates from small ruminants represent distinct populations, with CC130 and CC133 exclusively associated with small ruminants. Six main SplitsTree clusters, corresponding to CC CC8, CC97, CC130, CC133, CC151, and CC479, were identified. Isolates not associated with one of the main SplitsTree clusters were assigned to CC1, CC5, CC7, CC9, CC30, CC101, and CC398.

An overview of all CC and *spa* types detected is provided in **Table 2**. The isolates analyzed could be assigned to eleven different CC, of which only two (CC130 and CC133) were common among both caprine (71%) and ovine (93%) isolates. A total of 22 different *spa* types were detected. The most prevalent *spa* types were t1773 among the caprine and t1166 among the ovine *S. aureus* isolates, to which 26 and 27% of the analyzed isolates could be assigned, respectively. Three new *spa* types

were detected: t15248, t15249, and t15404. While 51% of the bovine *S. aureus* isolates led to false-negative results in the Staphaurex latex agglutination test, all isolates from the milk of small ruminants tested in this study yielded positive results and were thus correctly identified as *S. aureus* by the Staphaurex latex agglutination test.

An overview of the prevalence of the most important virulence and resistance genes detected by DNA microarray is provided in **Table 1**. The supplementary files include a comprehensive list of the prevalence rates of all genes detected (Supplementary Table S1) and a complete overview of all hybridization results (Supplementary Table S2). Overall, 67% of all isolates harbored at least one enterotoxin gene. The most prevalent enterotoxin genes were *sec* and *sel*, which were present in 55% of the isolates from small ruminants. The *sea* gene was found exclusively among caprine isolates. None of the genes encoding exfoliative toxins

TABLE 2 | Clonal complexes (CC) and spa types of the *S. aureus* isolates from goat and sheep milk.

Origin	CC	n	spa type (n)	Isolate ID
Goat (n = 34)	CC1	1	t127 (1)	G16
	CC5	1	t002 (1)	G25
	CC7	2	t091 (2)	G10, G29
	CC9	1	t899 (1)	G28
	CC30	1	t012 (1)	G09
	CC101	1	t056 (1)	G21
	CC130	15	t1773 (9)	G03, G05, G14, G17, G20, G22, G26, G27, G32
			t11826 (2)	G08, G15
			t15248 (4)	G06, G23, G31, G34
	CC133	9	t544 (1)	G18
Sheep (n = 15)			t1166 (2)	G12, G19
			t2678 (3)	G04, G07, G33
			t3583 (1)	G11
			t4735 (1)	G13
			t15249 (1)	G30
	CC398	1	t4475 (1)	G24
	CC522	2	t1534 (1)	G02
			t5428 (1)	G01
	CC97	1	t267 (1)	S05
	CC130	3	t1773 (1)	S09
			t11826 (1)	S14
			t15404 (1)	S06
	CC133	11	t998 (1)	S04
			t1166 (4)	S07, S08, S11, S15
			t2678 (2)	S10, S12
			t3583 (1)	S01
			t4735 (2)	S02, S03
			t6060 (1)	S13

or Panton–Valentine leukocidin were detected. Virulence genes associated with the toxic shock syndrome were found in 27 isolates.

Seven isolates harbored genes conferring penicillin resistance (*blaZ/I/R*). Genes conferring tetracycline resistance were found only among the caprine isolates. All isolates harbored *sdrM*, which encodes a multidrug efflux pump. None of the caprine and ovine isolates harbored genes conferring resistance to methicillin, aminoglycosides, streptogramin A, virginiamycin A, glycopeptides, and vancomycin.

DISCUSSION

The prevalence of *S. aureus* in caprine and ovine bulk tank milk samples varies depending of the country. Muehlherr et al. (2003) detected *S. aureus* in 32% of the caprine and 33% of ovine bulk tank milk samples in Switzerland, while Linage et al. (2012) and Álvarez-Suárez et al. (2015) detected coagulase positive staphylococci in 66% of caprine and 15% of ovine bulk tank

milk samples in Spain. Considering the very low prevalence of *S. aureus* detected among the analyzed milk samples of single animals in this study, the overall detected prevalence of *S. aureus* in the bulk milk samples examined was high. This suggests that the prevalence of *S. aureus* as a subclinical agent of mastitis in small ruminant herds in Switzerland may have been underestimated. This is of particular relevance, as SFP has been associated with raw milk from small ruminants (Giezendanner et al., 2009) and as traditional goat and sheep raw milk cheeses are popular.

Most of the isolates characterized in this study were assigned to CC130 and CC133, suggesting that these lineages may represent the major CC among caprine and ovine *S. aureus* isolates in Switzerland. These results are consistent with the findings of previous studies suggesting that predominance of either CC130/CC133 or CC522 in *S. aureus* isolated from milk of small ruminants is associated with geographical, breed- and infection-related aspects (Concepción Porrero et al., 2012; Eriksson et al., 2013; Shephard et al., 2013; Smith et al., 2014). Only few CC (CC7, CC9, CC97) were detected among strains of both small ruminants and cows.

Even though *S. aureus* isolates originating from caprine and ovine hosts have been *spa* typed in several recent studies (Concepción Porrero et al., 2012; Eriksson et al., 2013; Smith et al., 2014; Bar-Gal et al., 2015), three new *spa* types were detected among the isolates in this study. This suggests that to date, data on the population structure of *S. aureus* isolates originating from small ruminants is still very limited. The *agr* types and *cap* genes detected in this study are consistent with the findings of previous studies investigating *S. aureus* from small ruminants (Alves et al., 2009; Vautour et al., 2009; Bar-Gal et al., 2015).

Most of the isolates analyzed from small ruminants in this study were lacking antibiotic resistance genes. Resistance gene profiles from caprine and ovine strains in this study were not significantly different from those of bovine isolates (Moser et al., 2013). Only the presence of *tetK* in 12% of the caprine isolates was significantly higher compared to ovine and bovine isolates ($p = 0.007$). Overall, the prevalence of *blaZ/I/R* (14%), *tetK* (8%), *tetM* (2%), *ermA/B/C* (2%) detected was lower than the prevalence detected when analyzing *S. aureus* from small ruminants milk or nasal swabs in recent studies from the Middle-East and Africa (Gharsa et al., 2012; Bar-Gal et al., 2015; Jamali et al., 2015). The prevalence of antibiotic resistance genes detected was surprisingly high, considering that herd management differs vastly in small ruminants and cattle, with culling being preferred to antimicrobial treatment in small ruminants.

All isolates harboring *tst1* also harbored the genes *sec* and *sel*, and were assigned exclusively to CC130 and CC133. These genes are located on the ovine pathogenicity island *SaPIov1* (Guinane et al., 2010), and have been previously reported in isolates originating from small ruminants (Smyth et al., 2005; Gharsa et al., 2012). Consistent with findings among *S. aureus* from sheep and goats in Israel (Bar-Gal et al., 2015), in this study, the prevalence of *tst1*, *sec*, and *sel* was significantly higher among small ruminant isolates than among bovine isolates ($p < 0.003$),

which in contrast are more likely to harbor *egc* genes ($p = 0.000$). In this study, the detected overall prevalence of 67% of *S. aureus* carrying at least one enterotoxin gene was similar to 65% reported by Scherrer et al. (2004).

Many genes encoding virulence factors were present at similar rates in caprine, ovine and bovine isolates. This included genes encoding hemolysins (*hla*, *hlb*, *hld*), adhesion factors (*clfA*, *clfB*, *ebps*, *fib*, *fnbA*, *vwb*), hyaluronate lyase (*hysA1/A2*), immunodominant antigen B (*isaB*), transferrin binding protein (*isda*) and serine proteases (*splA*, *sspA*). In several studies, these virulence factors have been reported to play a role in mastitis in cattle (Viana et al., 2010; Ote et al., 2011; Wolf et al., 2011). While many genes were equally distributed among small ruminant and bovine isolates, statistically higher prevalence rates of *cap8*, *sdrD*, *sec*, *sel*, *tst1*, *ssl06*, *edinB*, and *Imrp* (RF122) among *S. aureus* from small ruminants were observed. As for genes associated with biofilm formation (*icaA/C/D*), very high prevalence rates have been previously reported in isolates originating from small ruminants in particular (Bar-Gal et al., 2015) and from ruminants in general (Snel et al., 2014; Prenafeta et al., 2014).

Comparison of goat and sheep isolates tested in this study showed that caprine and ovine *S. aureus* exhibited highly similar virulence and resistance gene patterns. However, some species-specific patterns were observed. Higher prevalence rates of *splE* among the caprine ($p = 0.038$) and of *lukM* ($p = 0.000$) among the ovine isolates was observed. Simultaneous presence of *splE* and *sdrD*, which was detected in four ovine and 19 caprine isolates in this study, has been associated with gangrenous mastitis in small ruminants (Vautor et al., 2009). In contrast, *lukM* was associated with high leukotoxic activity against bovine polymorphonuclear leukocytes (Rainard et al., 2003) and was hypothesized to play a central role in mastitis in ruminants (Barrio et al., 2006). In addition, significant differences in the prevalence of genes *ssl06/set21*, *ssl10/set4*, and *Q7A4X2* in caprine compared to ovine isolates were observed. While genes encoding for superantigen-like proteins (*ssl*), have been associated with immunoevasion by interfering with the toll-like receptor system (Zecconi and Scali, 2013), *Q7A4X2* may be involved in biofilm formation (Snel et al., 2014). These findings suggest that the virulence genes detected, and especially *lukM*, *sdrD*, and *splE*, represent important virulence factors for *S. aureus* strains causing mastitis in small ruminants.

Finally, the performance of the Staphaurex latex agglutination test for identification of *S. aureus* from small ruminants was assessed, as this test was reported to yield false-negative results in 51% of all bovine *S. aureus* strains tested (Moser et al., 2013). The results of this study show that the Staphaurex latex agglutination

test system is a highly reliable diagnostic tool for identification of *S. aureus* isolates from caprine and ovine milk samples.

CONCLUSION

The findings of this study suggest that *S. aureus* shows pronounced adaptation to small ruminants in general, but not to sheep or goats in particular. Comparing *S. aureus* from caprine, ovine and bovine milk samples collected in the same country, some common virulence genes were observed, but the results indicate that *S. aureus* from small ruminants may form a distinct population. Further studies covering an extensive strain collection of *S. aureus* from small ruminants collected at various geographical locations are needed to ensure that this finding can be extrapolated to *S. aureus* in general. Although low prevalence rates of *S. aureus* on the level of single animals exhibiting signs of mastitis was detected, 46% of the bulk tank milk samples from small ruminants were positive for *S. aureus*. This suggests that *S. aureus* may pose problems for animal and consumer health, in particular, as many products made from the milk of small ruminants are consumed raw.

AUTHOR CONTRIBUTIONS

SJ and RS conceived and designed the study. AM carried out the laboratory work. AM and SJ analyzed and interpreted the data. AM and SJ wrote the manuscript. All authors critically revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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***Staphylococcus aureus* Entrance into the Dairy Chain: Tracking *S. aureus* from Dairy Cow to Cheese**

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Staphylococcus aureus is one of the most important contagious mastitis pathogens in dairy cattle. Due to its zoonotic potential, control of *S. aureus* is not only of great economic importance in the dairy industry but also a significant public health concern. The aim of this study was to decipher the potential of bovine udder associated *S. aureus* as reservoir for *S. aureus* contamination in dairy production and processing. From 18 farms, delivering their milk to an alpine dairy plant for the production of smeared semi-hard and hard cheese. one thousand hundred seventy six one thousand hundred seventy six quarter milk (QM) samples of all cows in lactation ($n = 294$) and representative samples form bulk tank milk (BTM) of all farms were surveyed for coagulase positive (CPS) and coagulase negative Staphylococci (CNS). Furthermore, samples from different steps of the cheese manufacturing process were tested for CPS and CNS. As revealed by chemometric-assisted FTIR spectroscopy and molecular subtyping (spa typing and multi locus sequence typing), dairy cattle represent indeed an important, yet underreported, entrance point of *S. aureus* into the dairy chain. Our data clearly show that certain *S. aureus* subtypes are present in primary production as well as in the cheese processing at the dairy plant. However, although a considerable diversity of *S. aureus* subtypes was observed in QM and BTM at the farms, only certain *S. aureus* subtypes were able to enter and persist in the cheese manufacturing at the dairy plant and could be isolated from cheese until day 14 of ripening. Farm strains belonging to the FTIR cluster B1 and B3, which show genetic characteristics (t2953, ST8, enterotoxin profile: sea/sed/sej) of the recently described *S. aureus* genotype B, most successfully contaminated the cheese production at the dairy plant. Thus, our study fosters the hypothesis that genotype B *S. aureus* represent a specific challenge in control of *S. aureus* in the dairy chain that requires effective clearance strategies and hygienic measures already in primary production to avoid a potential transfer of enterotoxic strains or enterotoxins into the dairy processing and the final retail product.

Keywords: *Staphylococcus aureus*, dairy chain, mastitis, FTIR spectroscopy, food safety, subtyping, metabolic fingerprinting

INTRODUCTION

Staphylococcus aureus, a facultative anaerobic Gram-positive coccus, is an important cause of bovine mastitis and one of the most cost-intensive diseases in the dairy industry (Dufour et al., 2012). Furthermore, enterotoxigenic *S. aureus* strains have the potential to induce food borne intoxications in humans transmitted by dairy products (Le Loir et al., 2003; Jørgensen et al., 2005; Schmid et al., 2009; Ostyn et al., 2010). Staphylococcal enterotoxins (SEs), which cause abdominal cramps, nausea, emesis, and eventually diarrhea, withstand pasteurization as well as thermal processes, and are resistant against human gastrointestinal proteases (Balaban and Rasooly, 2000). Thus, once SEs are formed in food production and processing, these highly stable toxins will not be destroyed or inactivated by common hygienic measures and pose a health risk for the consumers. Most staphylococcal food borne intoxications are caused by food-handler to food contamination during food processing (Asao et al., 2003). Several case studies of SE related food borne outbreak revealed food-handler as the most likely contamination source since the same *S. aureus* strains were isolated from food-handlers, foods, and/or patient specimens (Wei and Chiou, 2002; Gallina et al., 2013; Johler et al., 2013). Beside this classical “human to food contamination route,” several other entrance points of *S. aureus* into the dairy chain have been described (for overview see Stessl et al., 2011). Biofilms formation in dairy equipment as well as insufficient acidification during cheese manufacturing opens *S. aureus* niches for multiplication and efficient contamination of the dairy processing lines (Sharma and Anand, 2002). Especially the first hours in cheese processing are important for control of *S. aureus*. Using three model cheeses, produced from cows’ raw milk, maximal levels of *S. aureus* were found after 1 day of ripening and SE production was tightly linked to pH (Delbes et al., 2006).

Furthermore, cows suffering from subclinical mastitis are increasingly discussed as alternative reservoirs of SE producing *S. aureus* contaminating dairy production and processing chains. For instance, a recent food borne outbreak in Austria caused by pasteurized milk products contaminated with SEs could be linked to cows suffering from *S. aureus* mastitis. The cows and not the dairy owner were identified as the most likely reservoir of the enterotoxin producing *S. aureus* (Schmid et al., 2009). Genotype B *S. aureus*, which is usually associated with bovine intermammary infections (IMIs) and known for its high within-herd prevalence of *S. aureus* mastitis (Fournier et al., 2008; Cremonesi et al., 2015), was also reported to be an important source of *S. aureus* contamination of Swiss raw milk cheeses (Hummerjohann et al., 2014). The hypothesis that cows with *S. aureus* IMI infections may indeed represent reservoirs for dairy production chain contamination is further fostered by a recent report from an outbreak at a Swiss boarding school linked to a soft cheese contaminated with *S. aureus*, showing the genetic characteristics of the bovine associated genotype B (Johler et al., 2015). Although the aforementioned reports are pointing toward dairy animals as—so far underestimated—reservoirs for *S. aureus* contaminations of dairy products, a direct proof for the

transmission from cow to dairy plants and products is still lacking.

The aim of this study was therefore to decipher the potential of bovine udder associated *S. aureus* as reservoir for *S. aureus* contamination in dairy production and processing. The whole dairy chain from quarter milk samples of individual cows to the retail dairy products was surveyed. Fourier Transform Infrared (FTIR) Spectroscopy was employed as high throughput method for identification and typing of *S. aureus* and related organisms. FTIR spectroscopy is a vibrational spectroscopic technique that allows the identification and discrimination of microorganisms at different taxonomic levels (Naumann et al., 1991; Ehling-Schulz et al., 2005; Wenning and Scherer, 2013). Very recently, a comparative study, including isolates from animal, human, and food sources, revealed a similar discriminatory power of FTIR spectroscopy and molecular fingerprinting methods for typing of *S. aureus* (Johler et al., 2016a), highlighting the potential of FTIR for tracing and tracking *S. aureus* along the dairy production and processing chain from farm to table.

MATERIALS AND METHODS

Dairy Chain Sampling

To gain a better understanding of the entrance points and contamination routes of *S. aureus* in the dairy production chain, a semi-hard cheese (so called “mountain cheese”) production in west of Austria (district Vorarlberg) was sampled from cow to product. Quarter milk (QM) samples, composite milk samples, and bulk tank milk (BTM) were collected during 2 days (morning and evening milk) from 18 dairy farms delivering their milk to one alpine dairy. After storage of the BTM at the dairy for 18 h at 8°C, the semi-hard cheese made from raw milk was processed within 1 day, soaked for up to 2 days in brine and ripened in the ripening cellar for 4 months. The cheese manufacturing was surveyed by taking samples at several cheese processing stages as outlined below.

Sampling at the Farm Level

At the surveyed farms a total of 1176 quarter milk samples from all cows in lactation were collected in addition to the routinely taken composite milk samples from each cow ($n = 294$). The herd size varied from 3 to 43 lactating animals predominately Brown Swiss breed (see Table 1). The average milk yield per cow ranged from 2.91 to 58.28 liters. The mean amount of milk per herd produced in two milking times ranged from 41.70 to 743.10 liters (median: 249.55 liters). QM samples were collected aseptically from foremilk following the guidelines of the National Mastitis Councils Laboratory Handbook on Bovine Mastitis (Hogan et al., 1999). Each quarter of each cow was screened for cell count abnormalities prior to sample collection by using the California Mastitis Test (CMT). Somatic cell counts (SCC) were determined by a Fossomatic milk cell counter (Foss, Hillerød, Denmark) from composite milk samples and BTM samples. Additionally, QM samples were taken aseptically for microbiological examination. The average sample volume per quarter was 5 ml collected in sterile plastic

TABLE 1 | Farm data comprising Somatic cell counts (SCC), *Staphylococcus* spp. positive cows and bulk tank milk samples.

Farm	Lactating cows (n)	Milk amount (l) ^a	Milk amount/cow (l)	Ø Herd SCC ^b	Ø SCC ^c of SA pos. cows	Individual cow ^d		BTM ^e	
						<i>S. aureus</i>	CNS	<i>S. aureus</i>	CNS
1	43	743,1	17,28	228.976	55.000	1	11	—	+
2	32	93,1	2,91	162.531		0	8	—	+
3	17	83	4,88	134.176	181.909	10	1	+	+
4	10	137,7	13,77	141.700		0	0	+	+
5	21	237,9	11,33	222.857		0	2	—	+
6	16	631,6	39,48	233.187	859.500	2	1	+	—
7	25	525,7	21,03	246.840	381.000	1	0	—	+
8	18	127	7,06	169.000	74.000	1	3	+	+
9	6	41,7	6,95	98.166		0	2	—	+
10	6	44	7,33	236.666		0	1	—	+
11	9	436,4	48,49	747.625		0	1	—	+
12	11	261,2	23,75	301.909		0	0	+	+
13	13	104,8	8,06	125.615	110.300	6	0	+	+
14	29	482,2	16,63	168.642	349.500	2	2	+	+
15	22	292,4	13,29	412.363	132.500	2	3	+	+
16	10	518,7	51,87	173.111		0	0	+	+
17	5	291,4	58,28	77.000	23.000	1	0	—	+
18	3	56,8	18,93	69.333		0	0	+	+

^a Average milk amount per milking.^b SCC: Average somatic cell count = cells/ml determined on herd level including SCC from all cows.^c SCC: Average somatic cell count = cells/ml determined on herd level including SCC from cows tested positive for *S. aureus*.^d Quarter milk samples positive for *S. aureus* and Coagulase Negative Staphylococci (CNS) per individual cow and farm.^e BTM: bulk tank milk samples positive for *S. aureus*.

tubes (Sterilin Limited, Cambridge, UK). Collected samples were stored in cool boxes and transported immediately to the laboratory for further investigation. The sterile QM samples were centrifuged and a loop of sediment (10 µl) was streaked onto on Columbia agar supplemented with 5% sheep blood (Thermofisher Scientific Inc., Oxoid Ltd., Hampshire, UK), and on Baird-Parker agar (Oxoid Ltd.). Plates were incubated at 37°C and examined after 24–48 h for bacterial growth. Furthermore, BTM samples were collected at each farm ($n = 18$) aseptically.

Sampling at Dairy Level

In addition, samples, which are subsumed as “dairy,” were taken during cheese manufacturing at the dairy processing the milk of the aforementioned farms. The sampling of the production of a semi-hard cheese, made from raw milk, included: stored milk in the vat tank, milk before processing, curd, whey, cheese after pressing, brine, cheese during brining, cheese after day 1, 7, 14, 28, 58 of ripening and at retail level. During cheese processing the free water activity dropped from 0.99 (in curd) to 0.92 (end of ripening) while the pH increased from pH 5.1 (first day of ripening) to pH 6.3 (at retail level) (for further details on the cheese manufacturing see Walcher et al., 2014). All samples were transported in cool boxes immediately to the laboratory for further investigation. *Staphylococcus* spp. counts were determined according to ISO 6888-1 (1999). In brief, 25

grams of three subsamples (A, B, C) of each solid sample e.g., curd or cheese at different stages of ripening were diluted 1:10 in 225 ml in sterile Ringer’s solution (Oxoid Ltd.), homogenized in a laboratory blender (Stomacher 400, Seward, Worthing, UK) and 100 µl of each samples were surface plated on Baird Parker agar. Liquid samples, such as milk, brine, and whey, were examined directly and in serial dilutions on Baird Parker agar by spatula method. Thirty seven degree for 24–48 h and analyzed after 48 h. Somatic cell counts (SCC) of BTM were determined by using a Fossomatic milk cell counter (Foss, Hillerød, Denmark).

Bacterial Isolation and Identification

Up to five presumptive coagulase positive *Staphylococci* (CPS) and presumptive coagulase negative *Staphylococci* (CNS) colonies were subcultivated on Tryptone Soya Agar supplemented with 6% yeast (Oxoid Ltd.) for 24–48 h at 37°C. CPS colonies were tested for bound coagulase (clumping factor) by slide coagulase test on sterile microscope slides, and free coagulase by tube coagulase test following ISO 6888-1 (1999) with rabbit plasma (Oxoid, Basingstoke, UK).

The type of hemolysis was recorded on Columbia Sheep Blood agar. Furthermore, the growth potential on BBL CHROMagar MRSA (Becton, Dickinson, and Company, Franklin Lakes, USA) was surveyed. Presumptive *Staphylococcus aureus* colonies were confirmed by nuc PCR (Brakstad et al., 1992) and FTIR spectroscopy as outlined below.

Identification, Subtyping and Capsule Polysaccharide (CP) Serotyping by FTIR Spectroscopy

CPS and CNS isolates were further investigated by chemometric-assisted FTIR spectroscopy. Its high discriminatory power, low costs and high throughput capacity, make FTIR spectroscopy not only to a valuable method for bacterial identification but also an interesting tool for population studies and epidemiological investigations. It has been shown to be a suitable tool for rapid differentiation of *S. aureus* and Coagulase-Negative Staphylococci (CNS) as well as for the identification and discrimination of bovine mastitis associated gram-positive, catalase-negative cocci and for investigation of the population structure of *Bacillus cereus* (Ehling-Schulz et al., 2005; Lamprell et al., 2006; Schabauer et al., 2014). Sample preparation, FTIR spectroscopic measurements and spectral processing was performed as described previously (Fricker et al., 2010; Grunert et al., 2013). In brief, pure cultured strains were spread by a spatula on tryptone soy agar (Oxoid Ltd.) and incubated at 30°C for 24 h. One loopful of the grown confluent lawn of every strain was diluted in 100 µl distilled water and 30 µl bacteria solution was spotted on a ZnSe sample holder and dried at 40°C for 40 min. Infrared spectra were recorded using a HTS-XT microplate adapter coupled to a Tensor 27 spectrometer (Bruker Optics, Ettlingen, Germany). The OPUS software (version 6.5, Bruker Optics) was used for spectral preprocessing and spectral analysis.

For identification of *S. aureus* spectra obtained from presumptive *S. aureus* colonies were compared to existing FTIR reference spectral libraries, which contain over 7000 strains representing more than 800 species (Wenning et al., 2008). For every identified IR spectra, a hit list containing 10 results was shown arranged in descending order of their *d*-values. *D*-values had to be below 1.5 to be selected for positive identification. The identification result with the lowest *d*-value was selected for positive identification (for details see: Kümmel et al., 1998; Fricker et al., 2010).

For *S. aureus* subtyping, the highly discriminatory spectral region between 1200 and 800 cm⁻¹ dominated by vibrations of various oligo- and polysaccharides was used for hierarchical cluster analysis (HCA) (Johler et al., 2016a). Based on normalized 2nd-derivative spectral data, dendograms were generated by using the average linkage algorithm with normalization to repro-level 30. Strains were measured once and considered as distinguishable at a spectral distance value > 0.50.

The expression of capsular polysaccharides (CP serotyping) was determined using the previously established artificial neuronal network (ANN) for differential analysis of CP serotype 5 (CP5), CP serotype 8 (CP8), and the CP non-expressing strains (NT) according to Grunert et al. (2013).

Molecular Subtyping, Enterotoxin Profiling and *mecA* PCR

The *S. aureus* spa typing using the sequence of a polymorphic VNTR in the 3' coding region of the *S. aureus*-specific staphylococcal protein A (*spa*) was determined as described

by Harmsen et al. (2003) (www.spaserver.ridom.de). Spa types were assigned according to the repeat succession with Ridom StaphType TM software (Ridom GmbH, Würzburg, Germany).

Multi-locus sequence typing (MLST) was performed following the protocols of Enright et al. (2000) available on: <http://saureus.mlst.net/misc/info.asp>. Sequence types (STs) and corresponding clonal complex (CC) were assigned through the MLST database (<http://www.mlst.net>). *S. aureus* enterotoxin (SE) profiles were determined by employing a multiplex PCR system that targets *sea*, *seb*, *sec*, *sed*, *seg*, *seh*, *sei*, and *sej* (Gonano et al., 2009). PCR based detection of the *mecA* gene was conducted according to Oliveira and de Lencastre (2002).

RESULTS

Sampling at Farm and Dairy Level

In frame of this study a dairy production chain was sampled from the cow to the retail product (ripened semi-hard cheese made from raw milk).

At 18 dairy farms that are delivering their milk to an alpine dairy plant for the production of smeared semi-hard and hard cheese, quarter milk (QM) samples (*n* = 1176) from all cows in lactation and representative BTM samples were taken and analyzed for the presence of staphylococci. In addition, composite milk samples from each cow (*n* = 294) and bulk tank (*n* = 18) were taken and the SCC, as an indicator for milk quality and potential udder infection, were determined.

The distribution of *S. aureus* and CNS within the tested farms is depicted in Table 1. In total, 9% of the animals were positive for *S. aureus* and 12% for CNS. *S. aureus* was isolated from 4% (49/1176) of the QM samples and CNS were isolated from 3% (41/1176) of the QM samples. *S. aureus* was found in QM samples from 50% (9/18) of the farms. The number of affected cows on these farms ranged from 1 to 10 *S. aureus* positive animals per herd (6–9%). CNS positive animals were found in 61% (11/18) of the herds with a range of 1–11 animals (0.3–3.7%). *S. aureus* was isolated from 56% (10/18) and CNS from 94% (17/18) of the BTM samples, respectively. Only 33% of the farms with *S. aureus*-positive BTM had also *S. aureus*-positive QM samples (farms 3, 6, 7, 8, 15, 17).

Milk samples from 16 out of the 26 cows tested positive for *S. aureus* showed elevated SCC ($\geq 100,000$ cells/ml) but none of these animals showed symptoms of clinical mastitis at the time of sampling. Thus, according to the "NMC Guidelines on Normal and Abnormal Raw Milk Based on SCC and Signs of Clinical mastitis" (Smith et al., 2001), *S. aureus* infected cows were classified as subclinical or latent mastitis cases. No significant differences in SCC of *S. aureus* positive herds and herds tested negative for *S. aureus* were found (as calculated by Student's *t*-test, *P* < 0.05). The BTM SCC was increased ($> 200,000$ cells/ml) in two BTM samples (farms 3 and 16) but represented just in one case (farm 3) an indicator for a herd problem with *S. aureus* (Table 1). Based on their SCC and bacterial count values, the BTM from all other farms could be qualified—according to Austrian milk quality regulation—as so called "S-class-milk" of highest quality (SCC < 250,000 SCC/ml).

The whole collected BTM (5108.70 l) was manufactured into 72 artisan semi-hard cheeses. Samples were taken at defined points throughout the production of these cheeses and analyzed for the presence of *Staphylococci*. *S. aureus* could be isolated throughout the manufacturing process until day 14 of ripening (see **Figure 1**, **Table 2**).

Isolation and Identification of *Staphylococci*

In total, 313 presumptive *Staphylococcus* strains were isolated from the whole dairy chain from cow to cheese. All isolates were cultured and first classified with traditional microbiological approaches. Subsequently, FTIR spectroscopy was employed for identification of isolates as outlined in the material and method section. FTIR spectra were recorded from all isolates and spectral reference libraries were used for species identification. sixty percentage (187/313) of the isolates could be assigned to *S. aureus* while 40% (126/313) of the isolates were assigned to other *Staphylococcus* species. *Staphylococcus chromogenes* (22%) and *Staphylococcus haemolyticus* (18%), followed by *S. pasteuri*, *S. hominis* subsp. *Hominis*, and *S. hominis* subsp. *novobiosepticus*, were the predominant species (data not shown).

From the total of 187 *S. aureus* strains isolated in frame of this study, 64% originate from farm (72 strains from QM and 47 strains from BTM) and 36% from several cheese processing levels at the dairy plant (68 strains). Among the 178 *S. aureus* strains, $\alpha\beta$ -hemolysis was predominately found (53.44%), followed by isolates showing α -hemolysis (35.06%). 5.75% of the isolates showed β - or no hemolysis. For clumping factor, 73.60% of all *S. aureus* strains were positive and 26.40% negative. The distribution of coagulase reaction showed 91.01% positive and 8.99% negative tested isolates. Of all strains, 5.62% were coincidentally clumping factor negative as well as coagulase negative, but revealed either a typical colony morphology and/or $\alpha\beta$ -hemolysis. Among strains isolated from the same sample, differences in hemolysis, clumping factors and coagulase reactions were observed. Species identification of all *S. aureus* strains was further confirmed by *nuc* specific PCR. A screening of all *S. aureus* isolates for MRSA, using the BBL CHROMagar MRSA and *mecA* PCR, showed that all isolates are MSSA.

Population Analysis of *S. aureus* Strains Isolated from the Dairy Production Chain

Chemometrics was used to further analyze FTIR spectral date recorded form all *S. aureus* strains ($n = 187$). Hierarchical cluster analysis (HCA) was employed for gaining insights into the population structure and diversity of strains from the same source and to follow potential transmission routes from the QM samples of individual cows into the cheese processing at the dairy plant. The HCA revealed a high inter farm—and in some cases also intra farm- diversity of *S. aureus* strains (**Table 2**). As shown in **Figure 1**, the *S. aureus* strains can be assigned to 3 main clusters. Cluster A ($n = 42$) is dominated by strains from BTM isolated from 6 different farms and also contains several QM strains derived from the aforementioned farms as well as strains isolated from cheese production at the dairy plant

(designated as “dairy” strains). Cluster B ($n = 139$) is dominated by strains isolated from QM and from BTM of two farms (farm 3 and 13) and strains isolated during cheese production until day 14 of cheese ripening. Cluster C comprises the smallest amount of isolates ($n = 6$). To the latter cluster only strains are assigned, which have been isolated from farms with low *S. aureus* prevalence rates (1/25 animals at farm 7, 2/22 at farm 15, and 1/5 at farm 17) or from farms with *S. aureus* positive BTM but *S. aureus* negative QM samples (farm 12 and 16; see **Table 1**). Notable, the *S. aureus* intra farm diversity was higher at farms with low *S. aureus* prevalence rates than at farms with high prevalence rates. At farm 15, the strains derived from QM of two animals tested positive for *S. aureus*, fall into two different clusters; the isolates from one animal are found in cluster A while the isolate from the second animal is found in cluster C (**Table 2**). In contrast, all strains isolated at farm 3 from QM and BTM were clustering closely together in cluster B1. The prevalence rate of *S. aureus* at the latter farm exceeded 50%. ten out of 17 cows were positive tested for *S. aureus* (**Table 1**). The HCA also revealed that the same *S. aureus* subtypes found on farm level in QM and BTM samples from farm 3, 6, 8, 13, 14, and 18 are also present in samples derived from the dairy plant at various cheese production steps (**Figure 1**). Most of the isolates from the cheese production at the dairy plant (45/65) are clustering together with the isolates from farm 3 in cluster B1. Ten isolates from cheese production are assigned to cluster A2, A3, A4, and A5. One isolate from cheese (ripening day 14) represents cluster B2 as a singleton and the remaining ten isolates from the cheese production fall into cluster B3, together with isolates from farm 13. At the latter farm a second *S. aureus* subtype (B4) was found, which was also detected in BTM samples from three other farms but not in any dairy plant samples. Interestingly, strains belonging to cluster C were only isolated at farm level but not from the dairy plant (Supplementary Table S2).

Since capsular polysaccharide expression was shown to be associated with *S. aureus* persistence, we next investigated if the potential for transmission of *S. aureus* from farm to dairy might be linked to capsule production capacities of strains. The latter was tested by employing a previously established artificial neural network (ANN) for capsule type (CP) determination based on FTIR spectral data (Grunert et al., 2013). All cluster B strains were negative for capsule production (NT type) while all cluster C strains were assigned to CP8 (**Table 2**). A few strains belonging to cluster A were assigned to CP5 but the majority of cluster A strains was negative for capsule production (NT type).

Molecular Subtyping and Enterotoxin Profiling

To gain further insights into the molecular characteristics of the *S. aureus* strains isolated in frame of this work, *spa* types, and ST types of selected strains from the different FTIR clusters were determined following standard procedures as described in material and methods (see **Table 2**). Strains clustering in A1 and A2 were assigned to t524 (ST71) while strains from cluster A3, A4, and A5 belong to t044 (ST97) and strain from cluster A6 and A7 to t337 and t056 (ST101), respectively. All strains, except

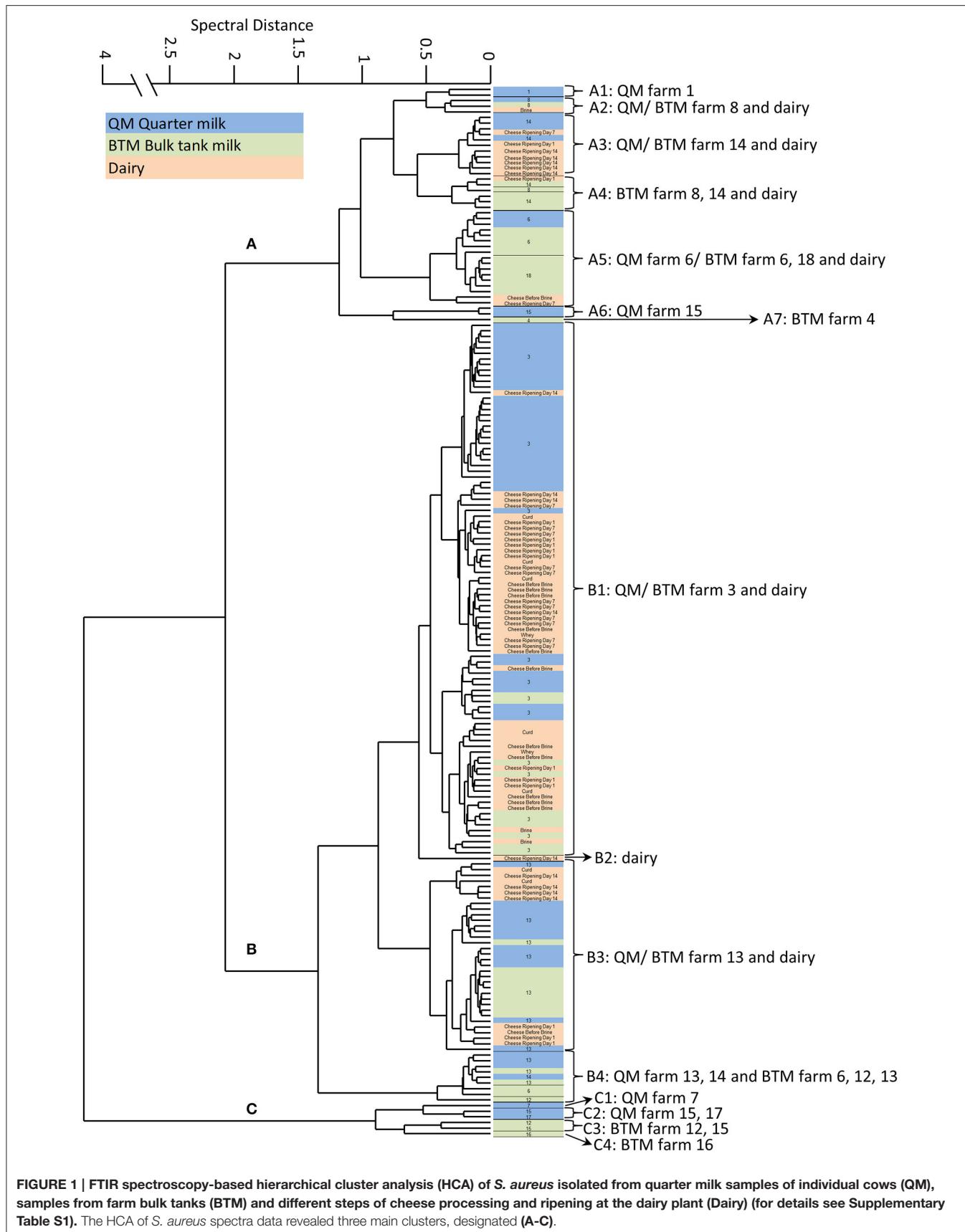


FIGURE 1 | FTIR spectroscopy-based hierarchical cluster analysis (HCA) of *S. aureus* isolated from quarter milk samples of individual cows (QM), samples from farm bulk tanks (BTM) and different steps of cheese processing and ripening at the dairy plant (Dairy) (for details see Supplementary Table S1). The HCA of *S. aureus* spectra data revealed three main clusters, designated (A–C).

TABLE 2 | Strain characteristics of *S. aureus* isolates according to their FTIR biotype.

FTIR biotype	QM/ BTM/ Dairy (n) ^a	spa type ^b	ST type (CC) ^b	CP type	Enterotoxin gene profile ^b	MRSA
A	1 QM1 (2)	t524	71 (97)	NT	neg	neg
	2 QM8 (1)/ BTM8 (1)/ Dairy_B (1)	t524	71 (97)	NT	neg	neg
	3 QM14 (4)/ Dairy_R1 (1); Dairy_R7 (1); Dairy_R14 (5)	t044	97 (97)	NT	neg	neg
	4 BTM 8 (1); BTM14 (4)/ Dairy_R1 (1)	t044	97 (97)	NT	neg	neg
	5 QM6 (3)/ BTM6 (3); BTM18 (6)/ Dairy_BB (1); Dairy_R7 (1)	t044	97 (97)	NT	neg	neg
	BTM6 (2); BTM18 (1)	t044	97 (97)	CP5	neg	neg
	6 QM15 (2)	t337	n.d.	CP5	seg, sei	neg
B	7 BTM4 (1)	t056	101 (Sing.)	NT	neg	neg
	1 QM3 (39)/ BTM3 (10)/ Dairy_C (8); Dairy_W (1); Dairy_BB (11); Dairy_B (1); Dairy_R1 (9); Dairy_R7 (11); Dairy_R14 (4)	t2953	8 (8)	NT	sea, sed, sei	neg
	2 Dairy_R1 (1)	t084	15 (15)	NT	neg	neg
	3 QM13 (14)/ BTM13 (10)/ Dairy_C (2); Dairy_BB (1); Dairy_R1 (3); Dairy_R14 (4)	t2953	8 (8)	NT	sea, sed, sei	neg
	4 QM13 (3)/ BTM13 (2)	t2953	8 (8)	NT	sea, sed, sei	neg
	BTM6 (2); BTM12 (1)	t2953	8 (8)	NT	neg	neg
	QM14 (1)	t2953	8 (8)	NT	neg	neg
C	1 QM7 (1)	n.d.	n.d.	CP8	seg, sei	neg
	2 QM15 (1); QM17 (1)	t529	504 (705)	CP8	seg, sei	neg
	3 BTM12 (1)	t529	504 (705)	CP8	seg, sei	neg
	BTM15 (1)	t529	504 (705)	CP8	sea, sed, sei	neg
	4 BTM16 (1)	n.d.	n.d.	CP8	neg	neg

^aNumber in brackets indicate number of isolates (n); QM (quarter milk) and BTM (bulk tank milk) numbers are referring to the farm number; Dairy_X refer to the respective cheese production steps (X) at the dairy plant: C (curd), W (whey), BB (before brine), B (brine) and R1, 7, 14 (ripening day 1, 7, 14);

^bDetermined for a subset of strains (for details see Supplementary Table S1).

one strain representing cluster B2 as singleton, from cluster B belong to t2953 (ST8). The strain representing cluster B2, which was derived from cheese at ripening day 14, belong to t084 (ST15) while strains from cluster C were assigned to t529 (ST504). Enterotoxin gene profiles of selected strains were determined by multiplex PCR. The vast majority of strains, except two isolates from QM samples of one animal (cluster A6 seg/sei), from cluster A were negative for the tested enterotoxin genes A, B, C, D, E, G, H, I, and J. All strains tested belonging to cluster B show the combination sea/sed/sej, except two strains from a bulk tank milk, which were negative for all enterotoxin genes tested. From the six strains tested belonging to cluster C, four carry the combination seg/sei, one the combination sea/sed/sej and one was negative for all enterotoxin genes tested.

DISCUSSION

This study provides a comprehensive analysis of the potential of primary production as source for *S. aureus* contaminations in the dairy production and processing chain. The dairy chain opens various entrance points for the human pathogen *S. aureus*, including the primary production environment and people involved in the dairy production and processing (Haveri et al., 2008; Johler et al., 2016b). Recent molecular studies as well as reports from food borne dairy associated outbreak linked to *S.*

aureus indicate a potential, yet not sufficiently explored, role of dairy cows as entrance point of *S. aureus* into the dairy production chain (Schmid et al., 2009; Hummerjohann et al., 2014; Walcher et al., 2014; Johler et al., 2015). Thus, in the current study, special emphasis was placed on sampling of dairy cows on quarter milk level to follow *S. aureus* over the complete production and processing chain of a semi-hard raw milk cheese manufactured at an alpine dairy—from cow to product.

Dairy Cow—First Entrance Point of *S. aureus* into the Dairy Production Chain

FTIR analysis of the 187 *S. aureus* strains (119 originating from dairy farms and 68 from cheese production at the dairy plant) isolated in frame of this study, revealed the bovine udder as an important *S. aureus* contamination source for the dairy production chain (Figure 1). Generally, the three major clusters revealed by FTIR are linked to CCs (CC97, CC8, CC705) typically found in bovine *S. aureus* (Smith et al., 2005; Johler et al., 2016b). Only two strains, representing the subclusters A7 and B2 as singeltons, were assigned to the human STs (Table 2). One strain originated from a BTM sample at farm 4 (ST101) and the other from the cheese at day 14 of ripening at the dairy (ST15), suggesting humans as a rather minor *S. aureus* contamination source in the dairy production and processing chain under investigation. *S. aureus* could be isolated and traced

throughout the dairy chain from single animals, farm BTM and dairy processing up to 14 days of cheese ripening. Generally, the diversity of strains from primary production (QM and BTM) was greater compared to the diversity of strains from dairy production. Strains from dairy production were found in cluster A and B together with QM and BTM strains, but not in cluster C. Cluster C was restricted to primary production (QM and BTM), indicating that not all dairy cow associated strain are capable to contaminate and persist in the cheese production chain. Indeed, cluster C contains strains belonging to ST504 (CC705, former CC151), a typical bovine associated CC (Herron-Olson et al., 2007; Ben Zakour et al., 2008). The hypothesis that *S. aureus* farm strains differ in their capacities for transfer and persistence in dairy production is fostered by the results from farm 13 samples. Isolates from QM and BTM of farm 13 are grouped in two clusters, namely B3 and B4. In B3 these isolates are intermingled with dairy isolates, pointing toward a contamination of the dairy production by these isolates while cluster B4 is restricted to strains isolated from farm samples (see Table 2). The vast majority of dairy isolates (84%) and QM isolates (79%) belongs to cluster B while BTM isolates were more evenly distributed among the three clusters. A link between QM and dairy isolates was found for strains from 5 farms, although to a different degree. Generally, cluster A strains originated from farms with low *S. aureus* within herd prevalence (one or two *S. aureus* positive animals) while the majority of cluster B farm strains originated from farm 3 and farm 13, two herds with high *S. aureus* within herd prevalence (59% farm 3 and 46% farm 13). Generally, the *S. aureus* prevalence in dairy cattle herds found in our study (Table 1) is comparable to the prevalence rates from other studies, reporting prevalence rates of 0.7–6% for low prevalence herds (LP) and 28–62% for high prevalence herds (HP) (Cremonesi et al., 2015). We also found in our study all classical bovine associated genotypes (CC complexes, *spa* types) reported recently in a European survey (Boss et al., 2016), including the intercontinental CC 97 (Smith et al., 2005). Thus, it could be assumed that the results from our survey are representative for dairy production and processing. For instance, cluster A strains could be assigned to the genotypes CLF, CLI, and CLR, cluster B strains to the genotype CLB and cluster C strains to the CLC, all genotypes frequently present in milk samples from inframammary infections surveyed in a recent European study, which included 12 different countries (Boss et al., 2016).

Is Specific Bovine Udder Adapted *S. aureus* Subtype Persistent in the Cheese Production Environment?

Although the milk quantity from farm 3 and farm 13 (Table 1) delivered to the dairy plant accounted only for 4% of the total milk volume used for the production of the cheeses, farm 3 and farm 13 *S. aureus* strains achieved the most successful entry of *S. aureus* from farm to dairy. Notable, all farm 3 and farm 13 strains belong to cluster B, a cluster in which almost all strains analyzed were assigned to t2953, regardless of their origin. t2953, is the bovine associated *spa* type of CC8 that is known for its contagiousness and high within herd

prevalence (Cremonesi et al., 2015; Boss et al., 2016) and, as shown in our current study, is also successfully transferred to the cheese production and processing from the bovine udder.

Although we found some dairy strains in cluster A, most of the dairy isolates were assigned together with CC 8 farm strains to cluster B. Thus, it is tempting to speculate that the CC8 farm strains belonging to cluster B are well adapted to the cheese production environment. In particular, their capacity to adhere and form biofilms on the surface of milk processing equipment at dairy plants could contribute to be a source of *S. aureus* contamination of dairy production (Sharma and Anand, 2002; Gutiérrez et al., 2012).

Notable, some of the cluster A and all of the cluster C farm strains—but none of the cluster B strains—showed expression of capsule polysaccharides. All cluster C strains belong to CP8 while the CP positive strains in cluster A were assigned to CP5. It was shown that the encapsulated *S. aureus* strains CP5, CP8, and the non-encapsulated strains can be reliably discriminated by FTIR spectroscopy since the main discriminatory spectral features are primarily based on bacterial surface glycopolymers including capsular polysaccharides (Grunert et al., 2013; Johler et al., 2016a). In particular, our study confirmed the high prevalence of non-encapsulated *S. aureus* strains derived from bovine mastitis, which underscores the importance of losing CP expression to be expected a key *S. aureus* feature associated with persistence (Sordelli et al., 2000; Tuchscherer et al., 2010). The absence of CP expression was shown previously to elevate the exposure of surface-associated adhesins, supporting bacterial adhesion to host cells (Pöhlmann-Dietze et al., 2000; Risley et al., 2007).

None of the dairy strains showed CP expression, which may indicate that similar mechanism allow the successfully entrance and persistence of non-encapsulated strains in the dairy processing. However, further studies systematically addressing the capsule production of dairy *S. aureus* strains will be necessary to elucidate a potential function of capsule production repression in dairy production and processing environments.

Molecular typing and enterotoxin gene profiling revealed that cluster B strains show genetic characteristics (t2953, ST8, and enterotoxin genes *sea/sed/sej*) of the recently described *S. aureus* genotype B (Fournier et al., 2008; Cremonesi et al., 2015). Genotype B has been reported to be linked to high within herd prevalence und frequent intramammary infections (Fournier et al., 2008; Gruber et al., 2009), which is in accordance to the results from our current study. Cluster B strains originated from two farms with high *S. aureus* prevalence (farm 3 56, farm 13 46%). It is therefore tempting to speculate that cluster B strains are indeed representatives of genotype B, which can easily be detected and traced by FTIR spectroscopy as a cost effective high throughput metabolic fingerprinting method.

As shown in our current work, strains with genotype B characteristics are successfully transmitted from cows to the dairy production. Since these strains pose a potential health risk for the consumer, efficient detection and monitoring of this specific *S. aureus* subtypes would not only be important

in the light of cow health, as has been recommended by Fournier et al. (2008), but would also be important from a food safety perspective. For instance, very recently a strain with genotype B characteristics isolated from soft cheese was linked to a food borne outbreak at a boarding school (Johler et al., 2015) and a recent study of Hummerjohann et al. (2014) revealed that genotype B is the predominant *S. aureus* subtype in semi-hard cheeses made from raw milk.

Zoonotic Potential of Bovine *S. aureus* Transmitted into Dairy Chain

The strains belonging to cluster B (ST2945), which have been most successfully transmitted to the dairy, possess the genes for the enterotoxins SEA, SED, and SEJ that are known for their toxicogenic potential for humans. SEA and SED are the major toxins linked to human foodborne outbreaks, including dairy product associated ones (Asao et al., 2003; Schmid et al., 2009; Argudín et al., 2010; Johler et al., 2015). Notable, a SEA and SAD strain from QM showing the same *spa* type (t2953) as our cluster B strains was recently identified as cause of dairy product related outbreak in Lower Austria (Schmid et al., 2009). Generally, strains from cluster A were negative for enterotoxin genes. Only two isolates from one animal of farm 15 were positive for SEG and SEI. However, these strains clustered separately from other strains (subcluster A6) and not together with any dairy strains. Thus, it could be assumed that the zoonotic potential of cluster A strains is rather low and emphasis should be placed on the early detection and prevention of the transmission of cluster B strains to the dairy production.

CONCLUSION

In conclusion, our works highlights the importance of effective hygienic measures on farm level, reemphasizing that food safety starts with the healthy animal. As revealed by FTIR

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spectroscopy, *S. aureus* can effectively enter the dairy production chain via contaminated milk of cows with subclinical *S. aureus* intramammary infections. Certain bovine *S. aureus* subtypes, showing characteristics of the recently described genotype B (Fournier et al., 2008; Gruber et al., 2009), appeared to be better equipped than others for successful transmission into the dairy production and processing. Further studies will be necessary to elucidate the factors allowing these specific *S. aureus* subtypes to conquer the dairy production chain.

AUTHOR CONTRIBUTIONS

ME, MW, and BS have conceptualized and supervised the study. JK, BS, and GW carried out the farm and dairy plant sampling. OB organized and supervised the farm and dairy plant sampling. JK, BS, and MG performed the strain characterization. FTIR analysis and molecular subtyping was done by TG, MF, JK, BS, and MG. Wrote and revised the paper: JK, ME, BS, TG, and MW. ME, acted as overall study director.

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Dose Assessment of Cefquinome by Pharmacokinetic/Pharmacodynamic Modeling in Mouse Model of *Staphylococcus aureus* Mastitis

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This work aimed to characterize the mammary gland pharmacokinetics of cefquinome after an intramammary administration and integrate pharmacokinetic/pharmacodynamic model. The pharmacokinetic profiles of cefquinome in gland tissue were measured using high performance liquid chromatograph. Therapeutic regimens covered various dosages ranging from 25 to 800 µg/gland and multiple dosing intervals of 8, 12, and 24 h. The *in vivo* bacterial killing activity elevated when dosage increased or when dosing intervals were shortened. The best antibacterial effect was demonstrated by a mean 1.5 log₁₀CFU/gland visible count reduction. On the other hand, the results showed that the percentage of time duration of drug concentration exceeding the MIC during a dose interval (%T > MIC) was generally 100% because of the influence of drug distribution caused by the blood-milk barrier. Therefore, pharmacokinetic/pharmacodynamic parameter of the ratio of area under the concentration-time curve over 24 h to the MIC (AUC₀₋₂₄/MIC) was used to describe the efficacy of cefquinome instead of %T > MIC. When the magnitude of AUC₀₋₂₄/MIC exceeding 16571.55 h·mL/g, considerable activity of about 1.5 log₁₀CFU/g gland bacterial count reduction was observed *in vivo*. Based on the Monte Carlo simulation, the clinical recommended regimen of three infusions of 75 mg per quarter every 12 h can achieve a 76.67% cure rate in clinical treatment of bovine mastitis caused by *Staphylococcus aureus* infection.

Keywords: dose assessment, PK/PD, Monte Carlo simulation, cefquinome, mastitis

INTRODUCTION

Staphylococcus aureus is a common Gram-positive bacterium that frequently causes a variety of infections in humans and animals and is the primary pathogen responsible for bovine mastitis. *S. aureus* mastitis can lead to significant economic loss to the dairy industry due to the deterioration of milk quality, veterinary medicine expenses, and prohibitive labor costs (Gruet et al., 2001). According to the clinical features, intramammary infection (IMI) is classified as clinical and subclinical mastitis. Clinical mastitis is acute and severe and may cause cow's death. While

subclinical mastitis is generally not lethal, but can lead to huge financial losses. *S. aureus* can be isolated from the mammary gland (MG) tissue of all forms of mastitis because these organisms are capable of hiding in host phagocytes and mammary epithelial cell to avoid antibiotic effect (Hebert et al., 2000).

Cefquinome is a semisynthetic β -lactam antibiotic and fourth-generation cephalosporin developed for use in veterinary medicine. It is stable to common plasmid- and chromosomally mediated β -lactamases. For example, cefquinome showed higher ability to treat the equine infection than penicillin G and gentamicin (Widmer et al., 2009). When using cefquinome in treatment of IMI diseases, a considerable therapeutic effect is found: the clinical persistence and recurrence of bovine mastitis are reduced during lactation; and the treated cows are less likely to develop clinical mastitis in the dry period (Bradley et al., 2011; Swinkels et al., 2013). Cefquinome can be administered by a parenteral route, intramammary infusion, or parenteral injection combined with intramammary infusion in China. The intramammary treatment may acquire a higher cure rate compared with systemic administration (Shpigel et al., 2006).

With regard to optimization of therapy regimen, pharmacokinetic and pharmacodynamic (PK/PD) model is an advanced approach concurrently analyzing the time course and the antibacterial effectiveness of a drug. The PK/PD analysis may further elucidate an inadequate daily dose or extended dosing interval accompanied under traditional dosing regimen determination. In our previous work, the PK/PD characteristics, especially in the blood, were studied against the *S. aureus* in a mouse mastitis model following an intramammary administration (Yu et al., 2016). However, we wondered how the drug concentrations would be in local MG and if it might be better to use MG PK data to optimize the dosage, given that in some organs (like brain or MG) drug distribution become much more complex due to the special anatomic structures or transport barriers.

The objective of this work was to characterize the PK of cefquinome in MG tissue after an intramammary infusion and integrate PK/PD model of MG tissue. In addition, analysis of surrogate PK/PD indexes required for different levels of antibacterial activity was estimated using the inhibitory sigmoid E_{max} PD model. Furthermore, we aimed to extrapolate the PK/PD profiles to bovine mastitis treatment and assess the clinical therapeutic regimen using Monte Carlo simulation.

MATERIALS AND METHODS

Bacterial Strains, Reagents, and Animals

Staphylococcus aureus isolates from bovine mastitis was the same population reported by our previous work (Yu et al., 2016), of which the MIC₉₀ was 0.5 μ g/ml. A similar sensitivity of these isolates to cefquinome was supported by determining the time-killing curves *in vitro* (Supplementary Figure S1). Therefore, isolate JP41 of MIC equally to 0.5 μ g/ml was chosen randomly for the succeeding trials. The stock solution of cefquinome (Qilu Animal Health Products CO., Ltd, Shandong, China) was

prepared in sterile water at 40,000 μ g/mL and stored at -20°C until use.

Lactating mice (purchased from Vital River Laboratories, Beijing, China) with body weight of 35–45 g, breeding in a special-pathogen-free (SPF) environment with a 12:12 light: dark circle were used in this study. Experiments were conducted on the L4 (fourth on the left) and R4 (fourth on the right) abdominal glands, which have the biggest size among the whole five pairs of mouse glands and can be harvested easily. The animal studies were approved by the Animal Use and Care Committee of South China Agricultural University. During the *in vivo* procedures, guidelines of American Association for Accreditation of Laboratory Animal Care (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, 1996) had been properly respected.

Calculation of PK in MG Tissue

Firstly, three healthy CD-1 lactating mice were employed to evaluate the influence of drug distribution on the concentrations of L4 and R4 glands. In brief, 1–2 h following removal of 10–12 day-old offspring, lactating mice were intramammary administrated to just one abdominal gland (L4 or R4). Through a small cut under a teat, 100 μ L of cefquinome (1000 μ g/mL) was injected into the exposed udder canal using a 32-gage blunt needle. Both the processed and non-treated glands were harvested at time points of 0.08, 0.17, 0.25, 0.5, 0.75, 1, 2, 4, 8, 12, and 24 h after administration. Then drug concentrations in L4 and R4 abdominal glands were measured.

Secondly, MG tissue PK study was performed at a single dose of 25, 50, 100, or 200 μ g/gland intramammary infusion into both the L4 and R4 glands (each gland as an individual), 5 mice a group (i.e., $n = 10$ for glands). The administrative procedure was described above. The R4 and L4 MG samples were harvested at 0.08, 0.17, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, and 24 h after administration.

All the MG samples were processed and analyzed for cefquinome concentrations, and the extracting method and high performance liquid chromatograph (HPLC) condition were described below.

Determination of Cefquinome in MG Tissue

The gland tissues were homogenized and processed based on the previous report with some modification (Sørensen and Snor, 2000). Briefly, a weight of 0.5 g tissue sample was transferred to a 15 mL polypropylene centrifuge tube and a volume of 5 mL acetonitrile was added. The mixture was shaken vigorously for 2 min using Lab dancer machine (IKA, German) and then centrifuged (Thermo Fisher Scientific, USA) at 5,000 g for 10 min. The supernatant was removed and tissue in the bottom was extracted once more again with 2 mL acetonitrile. The supernatant twice extracted was evaporated under a gentle steam of nitrogen (MIULAB, Hangzhou, China) at 38–40°C. The extract was diluted with 5 ml of water and cleaned up by tC₁₈ solid-phase extraction (SPE) cartridge (Waters CO., USA). The analytes were eluted with 2 mL acetonitrile and evaporated under

a stream of nitrogen at 38–40°C. The pellet was redissolved in 1 mL ultrapure water and filtered through a 0.22 µm syringe filter for HPLC analysis (Ultimate 3000, Dionex), equipped with a RP18 column (4.6 mm × 150 mm, 5 µm; Waters Co., USA). The injection volume was 50 µl, and column temperature was maintained at 30°C. The mobile phase consisted of acetonitrile and 5 mM ammonium acetate containing 0.1% formic acid (v/v, 13/87) provided as an isocratic elution with a flow rate of 250 µl/min. The total run time was 7 min.

The extraction recovery (R_E) and coefficient of variation (CV) of intra-assay and inter-assay were calculated. Samples of 10, 20, and 50 µg/gland were prepared by adding the standard work solution directly onto the blank gland tissue. After a 30-min incubation for mixing, samples were homogenized, processed, and tested by HPLC as described above. Triple parallels of each concentration for one trial were performed three times totally. The formulas of R_E and CV were as follows:

$$R_E(\%) = \frac{C}{C_a} \times 100\% \quad (1)$$

$$CV(\%) = \frac{\sqrt{\sum_{i=1}^n (x_i - \bar{x})^2 / (n - 1)}}{\bar{x}} \times 100\% \quad (2)$$

Where C is calculated drug concentration and C_a is added concentration; n represents for the repeater, \bar{x} is average value of concentration.

Design of PD Experiments

Three CD-1 lactating mice were employed for each condition of treatment using the mouse model of *S. aureus* mastitis (Brouillette et al., 2005; Yu et al., 2016). Totally, 21 therapeutic regimens were investigated in this work. The treatment doses ranged from 25 to 800 µg per gland, and the dosing intervals were 8, 12, and 24 h, respectively. An overnight culture of *S. aureus* JP41 isolates in BHI broth was injected in mice MG after an appropriate dilution. When bacterial counts reaching 7 log₁₀CFU/gland in gland tissue (~9 h incubation after inoculation), cefquinome was administrated to L4 and R4 glands simultaneously and at the corresponding dosing intervals during the 24 h experimental circle. After 24 h treatment, three mice in each group were euthanized for colony count determination (i.e., $n = 6$ for glands). The mice in non-treated control group were tested before the intramammary administration and after 24 h.

PK/PD Analysis

The cefquinome PK of gland tissue was analyzed using the non-compartment model and one-compartment with non-absorption model, respectively, by WinNonlin software (version 5.2.1; Pharsight, USA). The surrogate markers of antibacterial efficacy, including the ratio of area under the concentration-time curve over 24 h to the MIC (AUC_{0–24}/MIC), the percentage of time duration of drug concentration exceeding the MIC during a dose interval (%T > MIC) and the ratio of peak concentration divided by the MIC (C_{max}/MIC), were formulated by using *in vitro* MIC₉₀ values in broth and *in vivo* PK parameters obtained after intramammary administration of cefquinome. The units of C_{max}

and AUC in gland tissue were ug/g and hr·ug/g, respectively. The PK/PD parameters of the entire dosing regimens were obtained by extrapolation of the PK profiles determined above.

The antimicrobial effect of cefquinome was analyzed applying the sigmoid E_{max} model of inhibitory effect, as previously reported (Zhao et al., 2014), which is defined as

$$E = E_{max} - \frac{(E_{max} - E_0) \times C_e^N}{EC_{50}^N + C_e^N} \quad (3)$$

where E is the antibacterial effect, measured as the change in the bacterial counts (log₁₀CFU/g gland) in the gland sample after 24 h of treatment compared to the initial colony counts; E_{max} is the Δlog₁₀CFU/g gland in the drug-free control sample; E_0 is the Δlog₁₀CFU_{24 h}/g gland in the test sample containing cefquinome, when the maximum antibacterial effect was achieved; C_e is the PK/PD index (AUC_{0–24}/MIC, C_{max}/MIC or %T > MIC for gland tissue); EC_{50} is the value of PK/PD index of drug producing 50% of the maximum antibacterial effect; and N is the Hill coefficient, which describes the steepness of the concentration-effect curve.

Monte Carlo Simulation

Based on a previous PK study (Li et al., 2014), MIC data (Yu et al., 2016), and the value of PK/PD target magnitude in this work, simulation with Crystal Ball Professional V7.2.2 software was performed for 5,000 sessions. The probability of target attainments (PTAs) of the clinically recommended dose were investigated here. Based on the previous PK study of cefquinome in cows, AUC of milk sample was assumed to be normally distributed in the form of mean values and standard deviation of 4890.19 ± 1906.98 h·µg/mL (Li et al., 2014). The probability density functions (PDF) of MIC was custom defined that frequencies of MIC being 0.25 and 0.5 µg/ml were 0.32 and 0.68, respectively, according to our previous study (Yu et al., 2016). The target values of AUC/MIC were obtained in this work, of which 1- or 1.5-log-unit bacterial reduction can be achieved *in vivo*.

To assess therapeutic effect of cefquinome, the PDF of AUC and MIC were the two main components for Monte Carlo simulation. Random sampling the stochastic variable of specified PDFs, thousands of estimation of AUC/MIC and its range of probability will be attained. Then a target value of AUC/MIC was set to calculate the attainment rate of the corresponding dosing regimen, which is defined as the PTA.

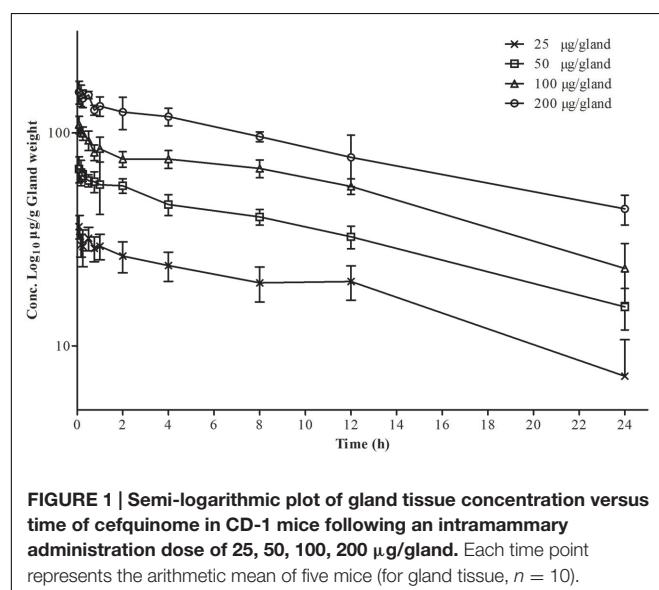
RESULTS

PK Profiles in Gland Tissues after Intramammary Administration

The limit of quantification (LOQ) was 50 ng/mL and the limit of detection (LOD) was 10 ng/mL in MG tissue. The R_{Es} for 10, 20, and 50 µg/gland were 95.43 ± 2.16%, 87.86 ± 4.99% and 73.65 ± 3.22%, respectively. In addition, the CVs of intra-assay and inter-assay are presented in Supplementary Table S1.

No adverse effects, like death of stress, acute death, depression, and abnormal behavior, were observed after intramammary administration. **Supplementary Figure S2** shows the concentration-time curves of both glands, following cefquinome administration into only one MG. The level of cefquinome in the non-treated gland was over 100 times lower than in the administrated one and as low as the LOQ, suggesting that the influence of drug administration to one side of gland on the concentration of the other non-dosed side should be negligible in the same subject. Therefore, we should be able to treat an individual gland as an independent study sample in the study design without concerning the inter-gland drug transfer impact.

Logarithmic concentration-time plots of the MG tissue data after intramammary administration on both sides of the fourth gland are displayed in **Figure 1**. Profiles of PK in gland tissue are presented in **Table 1**, being analyzed via non-compartmental and one-compartmental models, respectively. The median correlation coefficient (R^2) of four concentration-time curves was equal to 0.93 for the non-compartment model and 0.94 for the one-compartment model. Elimination half-life $t_{1/2-MG}$ of 12.44 ± 0.81 and 12.66 ± 0.69 h was calculated, respectively. The AUC_{0-24} , being analyzed via the one-compartment with non-absorption model, was slightly higher than the non-compartment model. The mean residence time (MRT_{MG}) determined by non-compartment model was 9.09 ± 2.31 h. The eliminating pattern of cefquinome and the comparable value of PK parameters obtained by those two Winnonlin models demonstrated that the PK characteristic in glandular tissue was eliminated exponentially, or following first-order kinetics. Therefore, PK features of multiple dosing were extrapolated from the values obtained in the study described above.



PD Evaluation of Various Dosing Regimens

The therapeutic activity of cefquinome was evaluated by bacterial counts ($\log_{10}\text{CFU/gland}$) at $t = 24$ h in mouse model of *S. aureus* mastitis. **Figure 2** shows antibacterial effects against isolate JP41 of 18 therapeutic regimens, with dosages ranged from 25 to 800 $\mu\text{g/gland}$ at three dosing intervals of 8, 12, and 24 h, respectively. The minimal dose amounts to prevent the microbial growth with 24, 12, and 8 h dosing intervals were 100, 50, and 25 $\mu\text{g/gland}$, respectively. Among the single daily dose groups, the greatest antibacterial effect was 1.23 log-unit reduction of bacterial counts when giving the largest dose of 800 $\mu\text{g/gland}$. However, when the dose level was exceeding 400 $\mu\text{g/gland}$ and with 8 or 12 h dosing intervals at the same time, a better antibacterial activity was observed with 1.5 $\log_{10}\text{CFU/gland}$ reductions or more. As the dose increased and the dosing intervals shorten, the antibacterial effectiveness of cefquinome was elevated *in vivo*, exhibiting a declining trend of survival cells by the end of experimental circle (**Figure 2**).

Integration of PK/PD Parameters

Simulation of PK/PD data against isolate JP41 demonstrated a correlation coefficient (R^2) of 0.435, 0.7557 and 0.7413 for $\%T > \text{MIC}$, AUC_{0-24}/MIC , and C_{\max}/MIC respectively (**Figure 3**). However, cefquinome concentrations in gland tissue were maintained above the MIC of 0.5 $\mu\text{g/mL}$ all the time, so the $\%T > \text{MIC}_{90}$ was 100% during the 24 h experimental circle. Therefore, the relationship between gland tissue PK and PD activity was reflected by the PK/PD parameter of $AUC_{0-24}/\text{MIC}_{90}$ ($\text{h} \cdot \text{mL/g}$) instead. According to the sigmoid model, the best killing activity (E_0) was about 1.5 $\log_{10}\text{CFU/gland}$ bacterial count reductions using either AUC_{0-24}/MIC or C_{\max}/MIC analysis. The E_{\max} and EC_{50} were 2.03 $\log_{10}\text{CFU/gland}$ and 2483.88 $\text{h} \cdot \text{mL/g}$ for AUC_{0-24}/MIC . The calculated ratios of AUC_{0-24}/MIC to provide 1 and 1.5 \log_{10} CFU/g gland bacterial load drops were 4714.72 and 16571.55 $\text{h} \cdot \text{mL/g}$ (**Table 2**).

Monte Carlo Simulation

Figure 4 exhibits the AUC/MIC distribution of three regimens of 75 mg once, twice and thrice doses. When the target value of AUC/MIC was set for 1-log-unit decrease, an over 90% PTAs was calculated following different regimens. However, values of PTAs for 1.5-log-unit reduction were much lower, which were 23.12, 60.75, and 76.67% following once, twice and thrice administrations, respectively (**Table 3**).

DISCUSSION

Staphylococcus aureus is usually responsible for contagious mastitis transmitting between cows, as the uninfected quarters are normally exposed to the pathogen during milking (Gruet et al., 2001). Cefquinome is effective against *S. aureus* bovine IMI generally, considering that most pathogens are susceptible to this compound with low MIC value (Supplementary Table S2). The cefquinome MICs in this study are in line with the level

TABLE 1 | Pharmacokinetics of cefquinome in MG tissue after a single intramammary administration at dose of 25, 50, 100, and 200 μg per gland to CD-1 mice and analyzed by non-compartment model and one-compartment model, respectively.

Variable(units)	Intramammary administration dose($\mu\text{g/gland}$) ($n = 10$)				Mean \pm SD
	25	50	100	200	
Non-compartment model					
$T_{1/2-\text{MG}}$ (h)	12.02	11.79	12.34	13.62	12.44 \pm 0.81
$AUC_{0-24-\text{MG}}$ (h· $\mu\text{g/g}$)	439.41	827.80	1334.46	2017.92	
MRT_{MG} (h)	9.08	8.94	9.08	10.00	9.09 \pm 0.14
R^2 (%)	0.8955	0.9793	0.8961	0.9665	0.93 \pm 0.045
One-compartment model					
$T_{1/2-\text{MG}}$ (h)	12.95	11.63	13.06	13	12.66 \pm 0.69
$AUC_{0-24-\text{MG}}$ (h· $\mu\text{g/g}$)	585.88	1064.86	1804.91	2756.41	
$C_{\text{max-MG}}$ ($\mu\text{g/g}$)	31.37	63.49	95.8	146.92	
R^2 (%)	0.9095	0.9763	0.8949	0.9637	0.94 \pm 0.043

$T_{1/2}$, elimination half-life representing the procedure that cefquinome transfers from gland tissue to blood; R^2 , correlation of concentration-time curves, MG, mammary gland. All the parameters are analyzed using five mice (10 glands) average curve data.

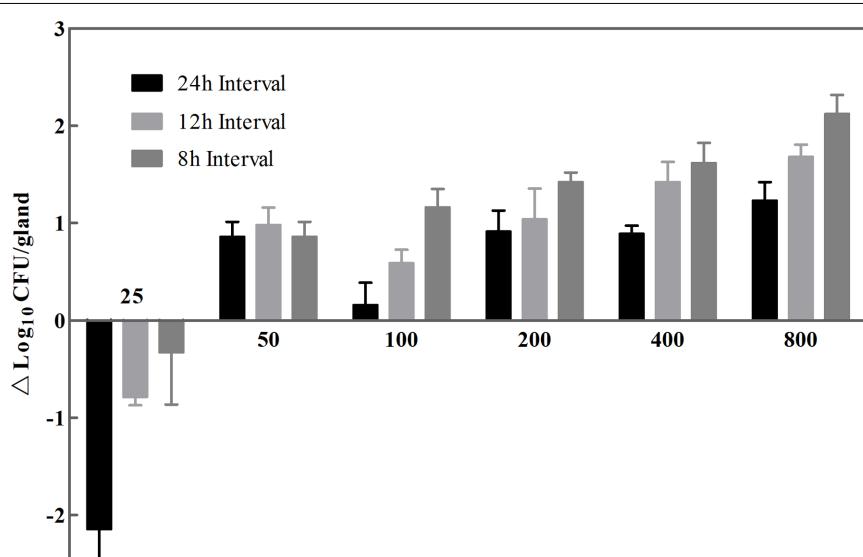


FIGURE 2 | Survived strains size of *S. aureus* wild isolate JP41 after treating with cefquinome at $t = 24$ h. Eighteen dose regimens comprised seven dose levels (25, 50, 100, 200, 400, and 800 $\mu\text{g/gland}$) and three intervals (every 8, 12, and 24 h). A mean value of $7.28 \log_{10} \text{CFU/gland}$ of initial bacterial load was represented as dotted line ($n = 6$ for glands). The limit of detection was shown as full line.

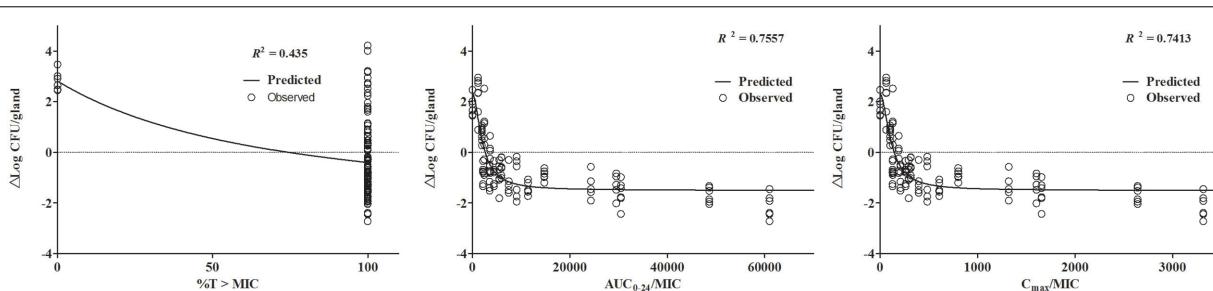


FIGURE 3 | Relationship between PK/PD parameters in gland tissue and drug killing effectiveness ($\Delta \log_{10} \text{CFU/gland}$) of *S. aureus* JP41 analyzing by the sigmoid model. The dots represent the antimicrobial effectiveness of cefquinome (E = final log-unit – initial log-unit) and the lines denoting the predicted value of E which is simulated from the Winnonlin software. The correlation of observed and predicted E value was quite low in $\%T > \text{MIC}$ section because of the distribution of $\%T > \text{MIC}$ (either 100 or 0%), which is not appropriate for PK/PD integration.

TABLE 2 |The AUC_{0–24}/MIC in MG tissue against *S. aureus* JP41 using the inhibitory form E_{\max} sigmoid model after intramammary administration.

Parameter	AUC _{0–24} /MIC
Log E_{\max} (\log_{10} CFU/gland)	2.03 ± 0.23
Log E_0 (\log_{10} CFU/gland)	-1.98 ± 0.20
Log E_{\max} – Log E_0 (\log_{10} CFU/gland)	4.01 ± 0.34
EC ₅₀ (h·mL/g)	2483.88 ± 405.55
For bacteriostatic action	2557.56 ± 49.55
For 1 \log_{10} CFU/gland reduction	4714.72 ± 8.49
For 1.5 \log_{10} CFU/gland reduction	16571.55 ± 49.57
Slope (N)	1.05 ± 0.17

The units of AUC_{0–24}/MIC are and h·mL/g, respectively. PK/PD data for %T > MIC were not available. Bacteriostatic action means no change about bacterial colony counts after 24 h incubation.

of previous reports for bovine (Schmid and Thomas, 2002). Considerable activity of cefquinome has been reported against methicillin resistant *S. aureus* (MRSA) strains isolated from swine, chicken and even human (Aarestrup and Skov, 2010; Wang et al., 2014). MRSA strains were rarely isolated from clinical mastitis cases of bovine (Chin et al., 1992; Murphy et al., 1994). However, for pathogens isolated from human patients, the MICs were much higher ranging from 1 to 16 $\mu\text{g/mL}$ and most of the isolates carried the meca gene. Therefore, susceptibility distribution of cefquinome suggests that this drug has potential to treat the IMI caused even by either methicillin sensitive *S. aureus* (MSSA) or MRSA, which may largely benefit the general public health.

Histologically, the blood-milk barrier, formed by the close link of secretory cells at their apex by tight junctions in lactating udder, is also responsible for the passive transport of drug between both compartments, namely blood and milk (Gruet et al., 2001). These objective factors may explain the situation: when cefquinome is administered to only one gland, the drug concentration in the non-treated gland is as low as the LOQ (Figure 2) or not quantifiable. Comparable findings were reported in bovine in a previous study (Li et al., 2014). Therefore the influence of R4 drug concentration on L4 concentration or vice versa is negligible, and both fourth glands (R4 and L4) are employed and considered as independent study units for intramammary dosing.

After intramuscular or subcutaneous administration, the absorption of cefquinome is quick and complete into the circulation with a high bioavailability (Aarestrup and Skov, 2010; Zonca et al., 2011). Nevertheless, following intramammary infusion drug systemic absorption is limited, and drug concentration in blood is about 0.1 $\mu\text{g/mL}$ in cow (Zonca et al., 2011; Li et al., 2014). Somehow, in our works, we found that drug concentration in blood is higher in mouse than in bovine (Yu et al., 2016). Considering the relative size of MG tissue, drug transportation from gland canal to blood may be easier to happen in mouse than in bovine. Even though, in our mouse study, the concentration in MG tissue is still much higher than in blood (Yu et al., 2016), which is in line with the cow blood/gland distribution pattern (Zonca et al., 2011). The observed very limited systemic absorption from

localized MG dosing is likely because cefquinome, a β -lactam antibiotic and organic acid with pK_a of 2.51 ~ 2.91, has limited lipid solubility to penetrate through membranes, such as the blood-MG barrier, due to its high degree of ionization in both plasma (pH = 7.4) and milk (pH = 6.5 ~ 6.8). The long half-life of elimination ($t_{1/2-\text{MG}}$) of 12.44 ± 0.81 and 12.66 ± 0.69 h calculated respectively using non-compartmental and one-compartmental models indicated that the concentration of cefquinome reached a very high level that was maintained for a very long time in MG tissue. However, the value of $t_{1/2-\text{MG}}$ in mouse model is still somehow longer than that in bovine, which may be attributed to the variation between species and different composition of samples (generally milk samples from bovine). For the consideration of economic reasons, antibiotics should be selected and given with the shortest withdrawal period to make the milk qualified for marketing as soon as possible. Although the elimination of cefquinome from MG tissue was quite slow and the MRT was about 6–10 h, the residue of cefquinome in milk samples cannot be detected after 120 h following intramammary administration (Zonca et al., 2011; Li et al., 2014).

The killing activity of cefquinome in the current study is similar to the previous report, in which the first generation cephalosporins cefalexin, cefalonium, cefapirin, and cefazolin were investigated to treat the mouse *S. aureus* mastitis and a dramatic effectiveness was observed (Demon et al., 2012). Although a 5- \log_{10} CFU count is usually used as the initial inoculum *in vitro* killing trials, in this study a much higher bacterial load of 7.28 \log_{10} CFU/gland is employed in order to simulate an acute and severe IMI. Compared with the previous work (Yu et al., 2016), treatments of wild pathogens infection may call for a larger dose or more frequent dosing intervals, regardless of the fact that the MIC values are the same.

Even given the minimum dose of 25 $\mu\text{g}/\text{gland}$, the concentration of cefquinome in gland tissue during 24 h maintains over the MIC value, which provided a %T > MIC of 100% for all the dosing regimens tested in this study. For time-dependent drugs, antibacterial effectiveness is more closely linked to the exposed duration of bacteria than the concentrations, as long as the drug level is over MIC value. The 100% of %T > MIC means that the time required for killing activity is abundant during the entire observation period. In this situation, the correlation of %T > MIC versus the differences of bacterial counts (\log_{10} CFU/gland) cannot be obtained for modeling purpose, suggesting that if a %T > MIC would be a preferred PK/PD driver, a much lower dose level might be needed. Under the current dosing schedules, AUC_{0–24}/MIC is used instead to fit the data to the PK/PD models.

TABLE 3 |The PTA of AUC/MIC for 1- or 1.5-log-unit decrease after intramammary administration in cows.

Dose Regimen	PTA (1-log decrease)	PTA (1.5-log decrease)
75 mg once	92.80	23.12
75 mg twice	97.14	60.75
75 mg thrice	97.94	76.67

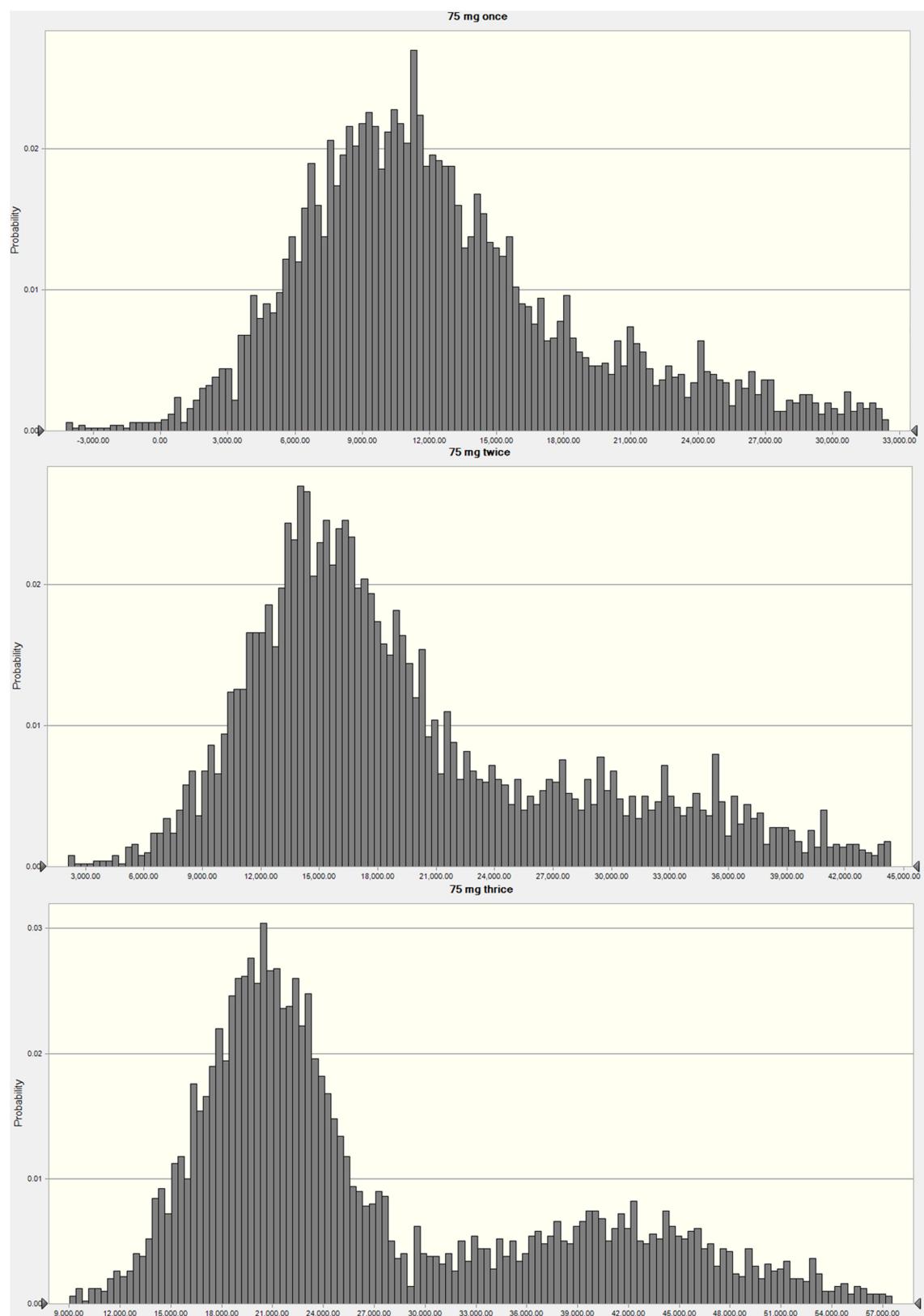


FIGURE 4 | Frequency distribution plots of AUC/MIC analyzed by Monte Carlo simulation mimicking cefquinome dosing regimens of 75 mg intramammary infusion once, twice, and three times.

As indicated in **Figure 2**, the killing activity of cefquinome has elevated only slightly when the drug dose over 200 $\mu\text{g/gland}$, which suggests the regimens of 200, 400, and 800 $\mu\text{g/gland}$ may be over dosed schedules in a mouse model. Similar results are observed in PK/PD sigmoid model (**Figure 3**) that identical decrease of colony counts may require quite different doses on the flat tail of the curve. Therefore, the AUC/MIC indices achieving a 1- and 1.5-log-unit decrease at the knee points were used as the target value in the Monte Carlo simulation, which were 4714.72 and 16571.55 $\text{h}\cdot\text{mL/g}$, respectively. The recommended dose regimen of cefquinome for treatment of bovine mastitis was three infusion of 75 mg per mammary quarter (The European Agency for the Evaluation of Medicinal Products Veterinary Medicines and Inspections, 1998), and the PTAs of 75 mg administration for once, twice, and thrice were estimated. However, narrow frequency distribution of MIC is a considerable limit of the Monte Carlo simulation, as the susceptibilities to cefquinome are mainly concentrated in 0.25–0.5 $\mu\text{g/ml}$ of this population. According to the previous papers, MIC levels of cefquinome or ceftiofur, against *S. aureus* (either mastitis isolates or not), are varied from 0.25 to 1 $\mu\text{g/ml}$ but mainly distribute at 0.5 $\mu\text{g/ml}$ (Zonca et al., 2011; Oliveira et al., 2012; Wang et al., 2014), which are similar to our report. These findings suggested that at most 76.67% infected mammary quarter could be cured, but not bacterially eradicated.

In summary, our study indicates that *in vivo* analysis of antimicrobials is of utmost importance to improve their therapeutic potential. This is the first study ever to assess glandular tissue PK/PD integration for investigating the effectiveness of cefquinome. Additionally, our data highlight the impact of anatomical structure (blood-milk barrier) on the drug distribution and PK characteristics in blood and gland tissue compartments. The glandular tissue PK/PD simulation demonstrates that the value of %T > MIC is generally 100%, the maximum limit in PK/PD principle, following an intramammary infusion administration. Instead, the AUC_{0–24}/MIC serves as substitute parameters under these particular conditions of drug, microbe and local inflammation combination. The magnitude of PK/PD parameters to achieve a remarkable antibacterial efficacy is assessed in this study in relation to treat IMI. The clinical recommended therapeutic regimen can achieve

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approximately 76.67% cure rate as predicted by Monte Carlo simulation.

AUTHOR CONTRIBUTIONS

Y-HL conceived of the study and given the final approval of the version to be published. YY participated in design of the study and drafted the manuscript. Y-FZ carried out the pharmacokinetic studies. XL and M-RC carried out the animal experiments of pharmacodynamic work. JS and X-PL have made substantial contribution to analysis and interpretation of data. G-LQ has been involved in revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.01595>

FIGURE S1 | *In vitro* time-killing curves of cefquinome against *S. aureus* isolates with initial bacterial load of 7-log-unit.

FIGURE S2 | The concentration-time curves of both glands, following cefquinome administration into only one MG. The level of cefquinome in the non-treated gland was over 100 times lower than in the administrated one and as low as the LOQ, suggesting that the influence of drug administration to one gland on the concentration of the other non-dosed side should be negligible in the same subject.

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Molecular Characterization of *Salmonella* Serovars Anatum and Ealing Associated with Two Historical Outbreaks, Linked to Contaminated Powdered Infant Formula

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Powdered infant formula (PIF) is not intended to be produced as a sterile product unless explicitly stated and on occasion may become contaminated during production with pathogens such as *Salmonella enterica*. This retrospective study focused on two historically reported salmonellosis outbreaks associated with PIF from the United Kingdom and France, in 1985 and 1996/1997. In this paper, the molecular characterization of the two outbreaks associated *Salmonella* serovars Anatum and Ealing is reported. Initially the isolates were analyzed using pulsed-field gel electrophoresis (PFGE), which revealed the clonal nature of the two outbreaks. Following from this two representative isolates, one from each serovar was selected for whole genome sequencing (WGS), wherein analysis focused on the *Salmonella* pathogenicity islands. Furthermore, the ability of these isolates to survive the host intercellular environment was determined using an ex vivo gentamicin protection assay. Results suggest a high level of genetic diversity that may have contributed to survival and virulence of isolates from these outbreaks.

Keywords: PFGE, whole genome sequencing, *Salmonella* pathogenicity islands, core genome, infection model

INTRODUCTION

Infant foods, such as baby cereals and powdered infant milk formulae (PIF), can act as vectors for pathogenic microorganisms of importance to human health as technology is currently unavailable to manufacture these foods as sterile products. Thus, despite the implementation of good manufacturing practices PIF may, on occasion, become contaminated with pathogens during production. Those pathogens that present the greatest threat to infant health include *Cronobacter* species (formerly known as *Enterobacter sakazakii*) and *Salmonella enterica*, two bacterial genera designated by the World Health Organisation (WHO) as Class A pathogens. Following the consumption and ingestion of contaminated foods, clinical signs of infection include gastroenteritis which can progress to bacteraemia and meningitis (Cahill et al., 2008). Numerous *Salmonella* outbreaks associated with contaminated infant foods have been documented since the 1950s and 1960s (Brouard et al., 2007).

In this study two *Salmonella enterica* serovars implicated in PIF outbreaks were investigated. The first outbreak occurred in 1985, when *Salmonella* Ealing, was identified and linked to cases of salmonellosis in the United Kingdom. Some 70 individuals were affected, the majority of these being infants. A subsequent investigation showed low numbers of *Salmonella* organisms in the PIF (~ 1.6 CFU/450 g) making their detection challenging during routine testing of the food product (Rowe et al., 1987). A second outbreak was reported between 1996/1997 and this was associated with PIF contaminated with S. Anatum. On this occasion, the outbreak centered in the United Kingdom and France (Threlfall et al., 1998). Public Health England reported an increase in S. Anatum isolations from children less than 1 year old and due to the young age of these patients infant food was suspected to have been responsible for the transmission of the aetiological agent.

This study reports the molecular characterization of *Salmonella enterica* serovars Anatum and Ealing, which were connected to the referenced outbreaks through contaminated PIF. Isolates were initially characterized by macrorestriction-based DNA fingerprinting and later further analyzed by whole genome sequencing (WGS), to observe the genetic diversity of these outbreaks and compare them to other *Salmonella* serovars. Infection assays were performed for two selected isolates from each of these outbreaks. The subsequent analysis focused on *Salmonella* pathogenicity Island (SPI) comparison as well as bacterial survival in *ex-vivo* infection models, to observe potential associations between survival and genetic diversity.

MATERIALS AND METHODS

Bacterial Culture

Thirty seven bacterial isolates were included in this study comprising of 12 S. Anatum and 25 S. Ealing. All were stored on beads at -80°C and sub-cultured on tryptone soya agar (TSA) plates at 37°C when required.

Molecular Macrorestriction Digest by *Xba*1 and *Spe*1

PFGE pulsotypes were obtained for all isolates following digestion with *Xba*1 restriction enzyme as previously described by Ribot et al. (2006). Isolates that produced indistinguishable patterns with this enzyme were subsequently reanalyzed using a second enzyme, *Spe*1 as described by Zheng et al. (2007). Visualization of DNA profiles was carried out using Gel Logic 1500 imaging system. The TIFF files were imported in BioNumerics v.5.1 and dendograms constructed using the UPGMA and DICE algorithms with 1.0% optimization and 1.5% tolerance.

Whole Genome Sequencing and Comparative Phylogenetic Analysis of Core *Salmonella* Genomes

Whole genome sequencing of isolates was performed using the Illumina MiSeq platform. Library preparation was performed using Nextera XT kit (v3 chemistry) according to manufacturer's

instructions producing 300 bp paired end reads. Subsequent raw sequence data was assessed using FastQC. The reads were error corrected using the BFC algorithm before a relaxed quality trim using a sliding window as implemented in Trimmomatic v0.33 (Bolger et al., 2014; Li, 2015). *De novo* genome assemblies were produced using SPAdes assembler v3.6.2 using the default *k-mer* size selection for 300 bp reads with careful mode enabled (used for mismatch error, indel and error correction; Bankevich et al., 2012).

For core genome analysis, comparisons were selected by online BLAST similarity searches of the largest contiguous sequence from S. Anatum CFS0056 and S. Ealing CFS0080 assemblies. Other serovars were included as they have been associated with food-borne outbreaks previously. Protein sequences from all strains identified by annotation with Prokka (version 1.11) were clustered using the pan-genome pipeline Roary (version 3.6.2) (Seemann, 2014; Page et al., 2015). Visualization of the pan-genome was carried out using Anvi'o (version 2.0.2) (Eren et al., 2015).

Comparative Analysis of Selected *Salmonella* Pathogenicity Islands (SPI)

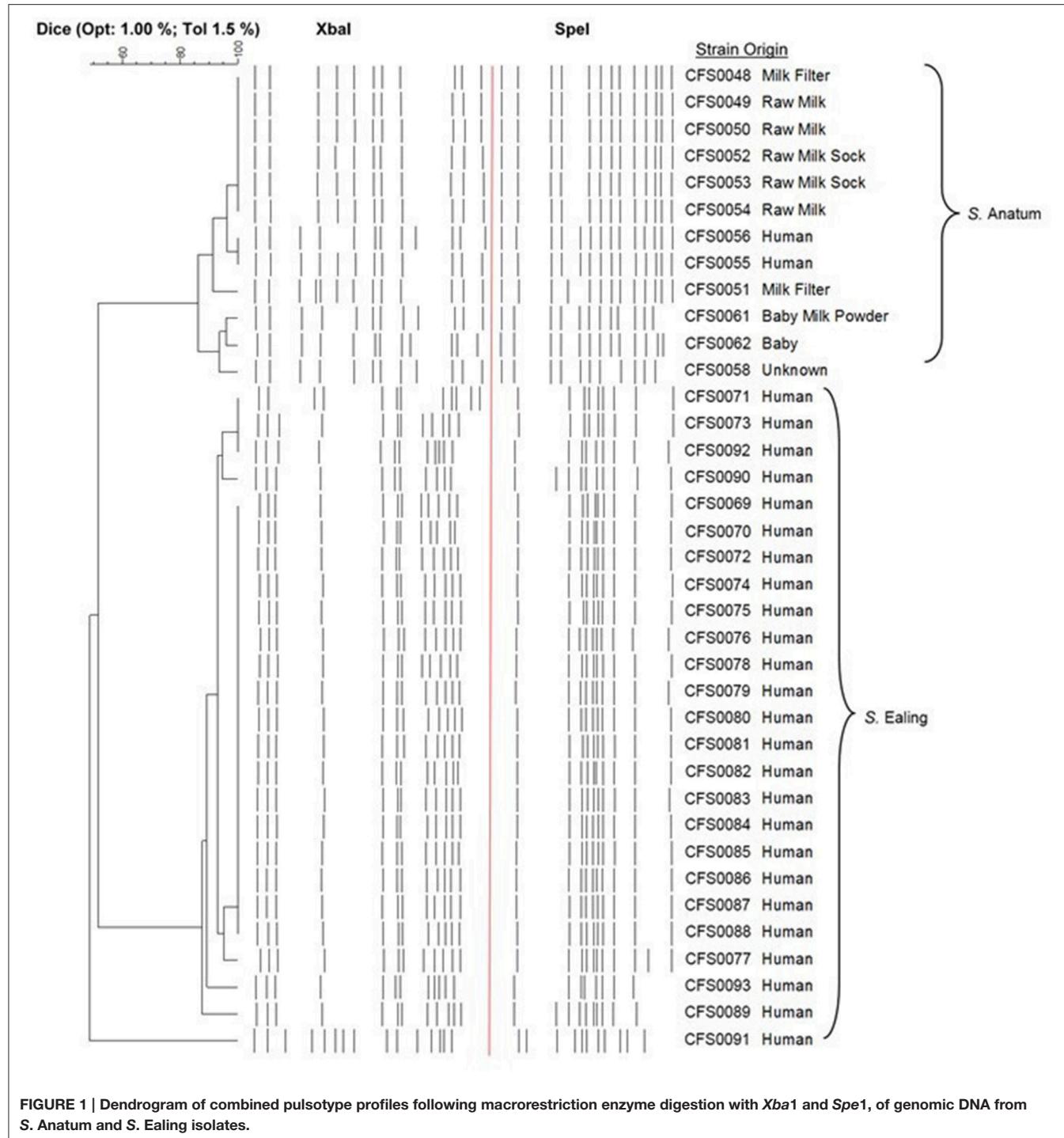
The differences in SPI associated genes from SPI-1 through -5 were investigated as previously described using standalone BLAST+ v2.4 and comparing these against the corresponding loci from S. Typhimurium ST4/74 as the reference genome¹. The resulting nucleic acid sequences were converted to amino acid sequences using Biopython (version 1.66) and the percentage similarity of the SPI proteins from S. Anatum and S. Ealing when compared against the reference were determined using the Needleman-Wunsch alignment with default settings through the EMBOSS analysis software (version 6.6.0) (Needleman and Wunsch, 1970). The raw sequencing data and *de novo* assemblies have been deposited at the Sequence Read Archive/GenBank for S. Anatum CFS0056 (SRP081283/SAMN05560363) and S. Ealing CFS0080 (SRP081283/SAMN05560364).

Ex vivo Gentamicin Protection Assay

A gentamicin protection assay was used to observe whether the isolates could survive phagocytosis using S. Typhimurium ST4/74 as the reference strain adapted from protocols as described previously (Lathrop et al., 2015). Briefly THP-1 monocytes were grown in antibiotic-free RPMI 1640 media (Sigma-Aldrich) supplemented with 10% [v/v] heat inactivated FBS and incubated at 37°C with 5% CO₂. Cells were detached with 1% Triton X-100 and seeded at a density of 1×10^5 cells/mL per well in 24 well-plates. Monocytes were differentiated to adherent macrophages by supplementing media with 20 ng/mL phorbol 12-myristate 13-acetate (PMA) for 5 days.

Prior to infection, bacterial isolates were diluted in complete media to 1×10^6 cells/mL for a MOI of 10:1 and incubated at 37°C for 1 h. Macrophages were washed with 1 mL Hank's Balanced Salt Solution (HBSS), three times, before 1 mL of the bacterial suspension, was added to each well; 1 mL of complete media was added to control wells. The plates were centrifuged

¹danieljhurley/atypical-Salmonella. GitHub Available online at: <https://github.com/danieljhurley/atypical-Salmonella> (Accessed August 10, 2016).



at $300 \times g$ for 5 min and incubated for 1 h to allow for phagocytosis. Following phagocytosis, the cells were washed with 1 mL HBSS three times. To kill exposed bacteria 1 mL complete media supplemented with 100 μ g/mL gentamicin was added to each well before incubation for 1 h. After the wells were washed with 1 mL HBSS. A final 1 mL of complete media supplemented with 20 μ g/mL gentamicin was then added to each well before

incubation at for the desired time points. At each time point cells were washed three times with 1 mL HBSS, after 1 mL 1% [v/v] Triton X-100 Phosphate Buffered Saline (PBS) solution was added to the cells and incubated at room temperature for 10 min. The resulting supernatants were decimaly diluted in PBS, 100 μ L aliquots of the dilutions were plated onto LB agar and incubated for 18 h at 37°C before enumeration.

RESULTS

Molecular Macrorestriction Digest by *Xba*1 and *Spe*1

The pulsotypes of all 37 isolates were investigated to assess the genetic relationship(s) between those *Salmonella* isolates linked with the two outbreaks in this study. A dendrogram based on the combined pulsotypes from *Xba*1 and *Spe*1 digest representing serovars Anatum and Ealing, is shown in **Figure 1**. In the case of *S. Ealing*, the pulsotype of the isolate denoted as CFS0091 a non-outbreak strain was notably different (≤ 7 band difference; when digested with *Xba*1 or *Spe*1) when compared to the other pulsotypes obtained for other members of this serovar (estimated at $\sim 50\%$ similarity). The majority of *S. Ealing* from the outbreak have very similar pulsotypes with the exception of isolates CFS0089, -0093, -0077, -0071, -0073, -0092, and -0090 which displayed more diverse profile (1–3 band differences).

For *S. Anatum* isolates CFS0048-0050 and CFS0052-0056 originally cultured from equipment and clinical cases, these produced very similar pulsotypes, displaying a high degree of similarity ($>95\%$), suggesting a potential epidemiological link. Other isolates from the same serovar showed differences in their restriction patterns (2–4 band difference) with less similarity to clinical isolates (85–90% similarity) making a link less probable.

Determination of the Whole Genome Sequences for *Salmonella Anatum* CFS0056 and *Ealing* CFS0080

Due to the indistinguishable nature of these outbreaks based on their pulsotypes as shown in the PFGE dendrogram (**Figure 1**), two representative isolates from these PIF outbreaks, *S. Anatum* CFS0056, and *S. Ealing* CFS0080 were selected, as both were cultured from clinical sources. The whole genome sequences of *S. Anatum* and *S. Ealing* were obtained using the MiSeq platform. Using these data, comparison of the genomes could provide additional insights into these historical bacterial isolates, the basic statistics of the genome assemblies from this study are shown in **Table 1**; *S. Anatum* CFS0056 and *S. Ealing* CFS0080 displayed the typical *Salmonella* DNA characteristics.

Availing of the Roary pipeline and Anvi'o visualization tool for broad genomic comparison, the core and accessory genomes for both serovars can be determined and then compared to a series of *Salmonella* isolates as shown in **Figure 2**. *Salmonella Anatum* CFS0056 and *S. Ealing* shared $\sim 43\%$ of all unique protein clusters identified (numbering 3544 of 8255) when compared against other selected isolates of this bacterial genus (**Figure 2**). A phylogenetic analysis using a core genome alignment of various *Salmonella* serovars showed that *S. Ealing* CFS0080 and *S. Agona* SL483 displayed a high degree of genetic similarity. *Salmonella Agona* SL483 was isolated in 2008 and epidemiologically linked to cases of food-borne human infections associated with the consumption of contaminated dried cereal foods (Fricke et al., 2011). In contrast *S. Anatum* CFS0056 from this study shared a high degree of similarity with other Anatum serovar isolates, although 260 protein clusters were

TABLE 1 | Comparative features of the genomes of *S. Anatum* and *S. Ealing* with *S. Typhimurium* ST4/74.

Features/ <i>Salmonella</i> serovars	Typhimurium ST4/74	Anatum CFS0056	Ealing CFS0080
Accession number	NC_016857	–	–
SRA number		SRP081283	SRP081283
Assembly contigs	n/a	71	51
Assembly N50	n/a	486,442	408,530
Genome size (Mbp)	4.87	4.87	4.82
%GC	52.18	52.42	52.17
Predicted coding sequences (CDS)	4782	4563	4471
No. of rRNA operons	22	9	10
No. of tRNA operons	85	78	83
No. of tmRNA operons	1	1	1

unique to *S. Anatum* CFS0056 among the isolates included in this analysis. Overall, *S. Ealing* and *S. Anatum* shared seven unique proteins in comparison to the other isolates included in the analysis including TU1 elongation factor 1 along with hypothetical proteins.

Comparative Analysis of Selected *Salmonella* Pathogenicity Islands (SPI) from the Genomes of Serovars Anatum and Ealing

SPIs are horizontally acquired genetic cassettes that play a major role in *Salmonella* survival and virulence. The SPI-containing proteins from *S. Anatum* CFS0056 and *S. Ealing* CFS0080 appear to share high levels of similarity (ranging from <95 to 100%) when compared against the reference *S. Typhimurium* ST4/74 (**Figure 3**). Proteins from SPI-1, -2, -4, and -5 display varying degrees of diversity, in comparison to SPI-3 which appears to be highly conserved. Upon closer inspection, *S. Ealing* CFS0080 demonstrated an overall lower sequence similarity in comparison to that observed for *S. Anatum* CFS0056 from this study. A total of 11 proteins were not detected from this analysis in one or both strains, a feature that should be interpreted with caution.

Intracellular Survival of *S. enterica* Serovars in Human Macrophages

To study the ability of these two isolates to survive phagocytosis, *ex vivo* infections were performed using differentiated human THP-1 macrophages in a gentamicin protection assay. *Salmonella* Typhimurium ST4/74 was included as a reference strain. Infections were carried out at a Multiplicity Of Infection (MOI) of 10:1. Viable internalized bacteria were enumerated at 2, 4, 8, and 24 Hours Post Infection (HPI). Of the two serovars Anatum CFS0056 and Ealing CFS0080 tested in this study, all were found to persist within THP-1 macrophages for a period of up to 24 HPI. Although, the number of *S. Ealing* colonies recovered at 24 HPI was lower in comparison to *S. Anatum* and *S. Typhimurium*, pairwise comparison using ANOVA indicates this difference was statistically significant ($p = 0.02$).

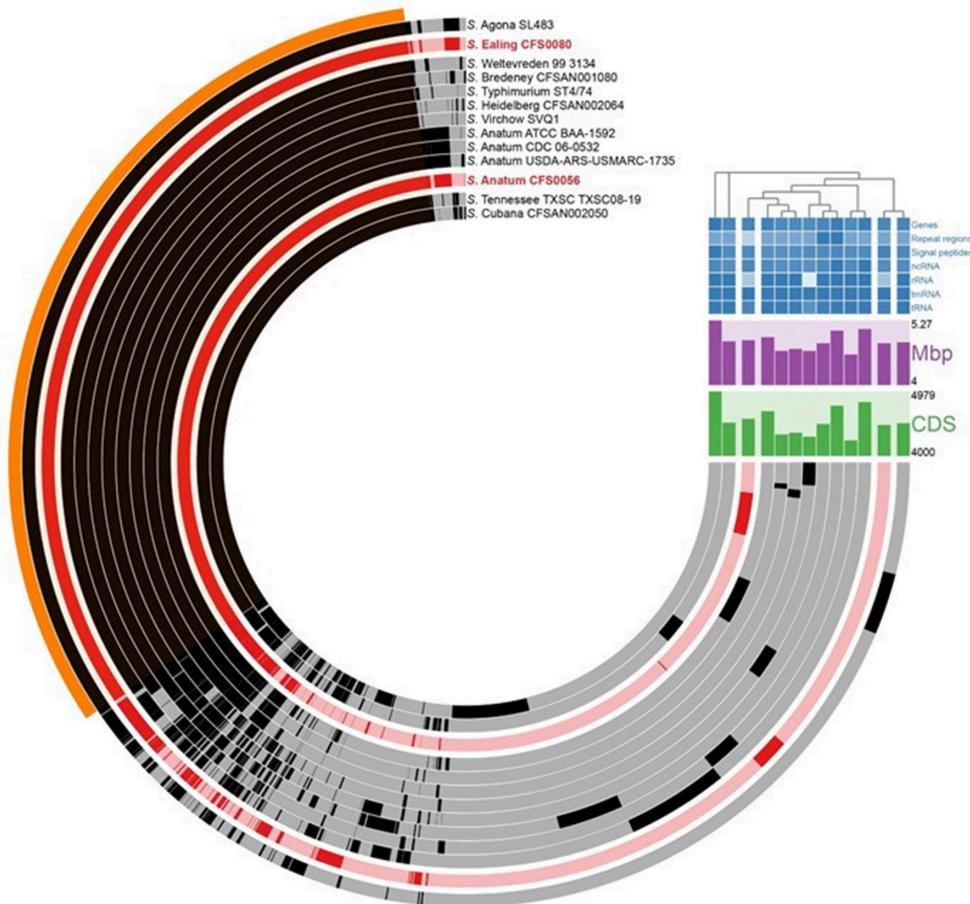


FIGURE 2 | Comparative Anvi'o phylogenetic analysis of core genome from selected *Salmonella* serovars compared against *S. Anatum* CFS0056 and *S. Ealing* CFS0080. Regions of the circular map shown in black denote similar content between isolates. Red colored regions shown in the circular map denote the genomes of the *S. Anatum* CFS0056 and *S. Ealing* CFS0080 study isolates. The region of the map marked in orange represents the core genome across all isolates. The coding sequence content (denoted as CDS; Green); the genome sizes (denoted as Mbp; Purple, see also **Table 1**) and a cluster diagram are also shown.

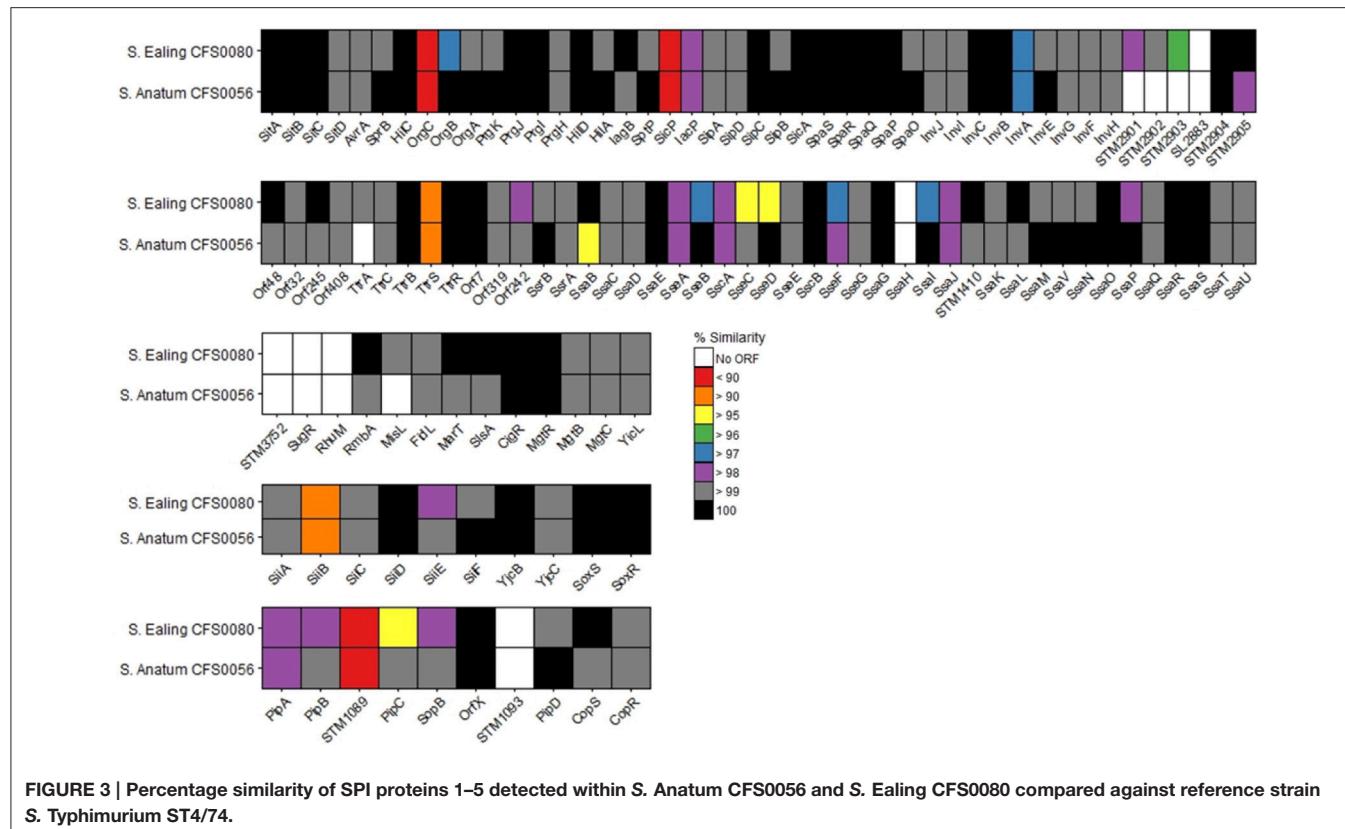
DISCUSSION

PIF can become contaminated with bacteria of importance to human health, some of which may be pathogenic (including *Salmonella* species among others; Mullane et al., 2007). It is a constant challenge for the PIF industry generally, to be able to detect Class A pathogens such as *Salmonella* species in this food matrix prior to distribution due to the low numbers that are present on occasion. Failure to do so, can lead to salmonellosis infection among infants, some of which may be life-threatening (Cahill et al., 2008). Similarly, failures in the food safety management of these associated food production facilities can result in the environment and final product becoming contaminated. This retrospective study investigated *Salmonella* serovars Anatum and Ealing cultured from two historical outbreaks and compared their molecular characteristics.

Molecular fingerprinting identified two clonal clusters consistent with the two serovars, Anatum and Ealing. Overall both serovars displayed a high degree of genetic diversity

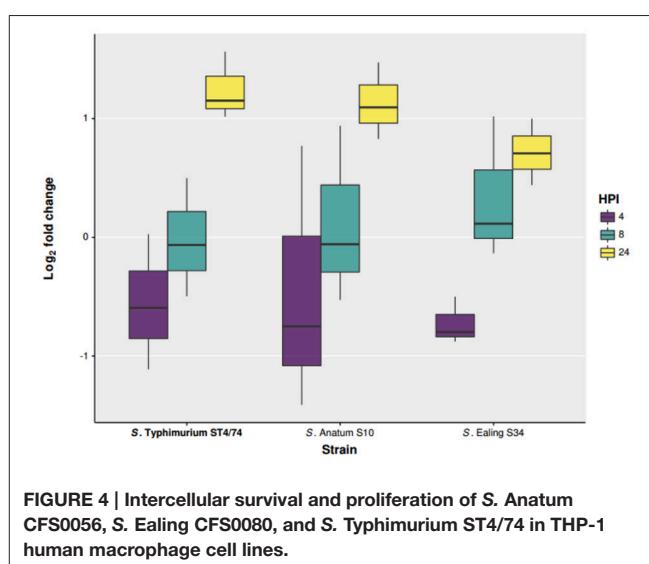
with profiles sharing similarities ranging from 85 to 100%. The majority of *S. Ealing* produced pulsotypes that were indistinguishable using *Xba*I or *Spe*I alone (**Figure 1**), with the exception of isolate CFS0091, which was determined to be un-related to the outbreak. Comparative analysis of the pulsotypes obtained for *S. Anatum* isolates CFS0048-0050 and CFS0052-0056 (**Figure 1**), suggested that these isolates of clinical origin and from the PIF production environment were linked during the 1996/1997 outbreak. Whole genome sequencing was performed on *S. Anatum* CFS0056 and *S. Ealing* CFS0080. These isolates were selected as representatives due to their apparent clonal nature and clinical isolation.

Analysis of the core genome revealed *S. Anatum* CFS0056 and *S. Ealing* CFS0080 share ~43% of the unique protein clusters identified by Prokka annotation. In this study *S. Anatum* was found to be more homologous when compared with other representatives of the same serovar, though with some unique regions being identified (**Figure 2**). These unique genomic loci could be related to the survival phenotype of this isolate in



the PIF production environment and therefore require further exploration. Moreover, *S. Anatum* is not typically associated with milk-related outbreaks but is more commonly encountered in meat-processing settings (Ebuchi et al., 2006; Marasini et al., 2016). In the analysis displayed here *S. Anatum* also demonstrated a distant relationship to *S. Typhimurium* together with the *Salmonella* serovars Virchow and Heidelberg. Both of these latter isolates were previously cultured from outbreaks involving vegetable and meat related products. When analyzed, *Salmonella* Ealing was found to cluster only with *S. Agona* SL483 a pathogen also implicated in a food-related outbreak linked dried cereal food product, suggesting the two isolates have similar genetic elements which could contribute to survival in low moisture environments.

Comparative analysis at the level of the amino acid sequence of SPI-1 through to SPI-5 was performed as these are the most common SPIs in all *Salmonella enterica* serovars sequenced to date. These SPIs code for virulence factors which facilitate internalization of the pathogen and have an impact on the survival of the bacterium in the host intercellular environment. Of specific interest were SPI-1 and -2 that encode genes that function in bacterial virulence including adhesion, and vacuole stabilization. Figure 3 presents the comparison in the form of a heat map that shows a near complete complement of genes was detected across all isolates for SPI-1–SPI-5 together with a high level of amino acid similarity being observed between the outbreak and reference strains. This comparative



approach also identified proteins that are more diverse when compared to the reference, including those with key roles in intercellular survival and virulence (reviewed in McGhie et al., 2009; Fricke et al., 2011; Ruby et al., 2012; Lathrop et al., 2015). Proteins located within SPI-1, -2, -4, and -5 showed the greatest diversity and these in particular included

proteins critical in infection and persistence such as SiiEFG that functions in bacterial adhesion and invasion, SseFG, SopB, which are essential in *Salmonella* containing vacuole stabilization and replication and PipABC which are involved in entropathogenicity (Wood et al., 1998; Morgan et al., 2007; Ibarra and Steele-Mortimer, 2009). SPI-3 showed a high degree of similarity with the reference strain. Of note, three proteins (RhuM, SugR, STM3752), were not detected in this analysis, an observation that does not necessarily mean these genes were absent.

Overall *S. Ealing* CFS0080 displayed a greater degree of sequence diversity across the SPI island proteins in comparison to *S. Anatum* CFS0056, when compared to *S. Typhimurium* ST4/74. The consequences of sequence divergence within the SPI genes is not clear at present but targeted knockout studies of selected genes across SPIs 1–5 suggest that if pseudogenes accumulate in the bacterial genome, an impaired virulence phenotype could result (Rychlik et al., 2009). This particularly relates to SPI-1 and SPI-2 which are essential for host adaption and systemic infection. If these two SPIs become non-functional the virulence and survival of the strains, in the human host could be impaired at the level of the phenotype (Ibarra and Steele-Mortimer, 2009).

Both *S. Anatum* CFS0056 and *S. Ealing* CFS0080 survived for 24 HPI in human THP-1 macrophages (Figure 4). The numbers of bacteria recovered after 4 and 8 HPI were comparable to those of *S. Typhimurium* ST4/74. However, after 24 HPI, the cell count for *S. Ealing* CFS0080 was found to be less than that of *S. Anatum* CFS0056 and *S. Typhimurium* ST4/74. The adaption of *Salmonella* to the stresses experienced in the host-macrophage environment has been associated with the development of viable non-replicating cells (Helaine et al., 2010, 2014). Further, another report has also observed the potential link between reduced virulence and increased SPI variability using cell infection models (McWhorter and Chousalkar, 2015). The variation in sequence conservation across SPI proteins could be

associated with non-functioning proteins leading to attenuated *in-vivo* survival.

CONCLUSION

In this study, the molecular characteristics of *S. Anatum* and *S. Ealing* cultured from powdered infant formula related outbreaks were studied. Pulsotyping with PFGE demonstrated the clonal nature of the outbreak strains and this feature facilitated their distinction from unrelated isolates of the same serovar. WGS and subsequent analysis of selected representatives of these two serovars identified genomic regions that were common, but also showed that a large accessory genome could be identified. Macrophage infection studies confirmed the ability of both serovars to persist in the host cell for similar periods but at different bacterial cell numbers, reflecting differences in sequence conservation among the SPI islands studied.

Overall the results from this study provide a useful foundation to begin to extend the investigation of how these unique bacterial isolates adapted to the PIF-processing environment, a necessary prelude to the outbreaks reported in each case.

AUTHOR CONTRIBUTIONS

LG—drafted the manuscript (MS), collated, and reviewed all results and contributed to the WGS and subsequent bioinformatics analysis. SFi—assembled the collection of isolates and performed the initial geno- and phenotypic-studies whilst contributing to the drafting of the MS. DH—carried out cell culture work; evaluated all of the WGS data and carried to the bioinformatics analysis. LB—performed cell culture work. EW—performed phenotype characterization of the isolates. CI—assembled the collection of isolates and performed the initial geno- and phenotypic-studies whilst contributing to the drafting of the MS. JT—provided bacteria and background information. SFa—co-ordinated the study.

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Longitudinal Study of Two Irish Dairy Herds: Low Numbers of Shiga Toxin-Producing *Escherichia coli* O157 and O26 Super-Shedders Identified

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A 12-month longitudinal study was undertaken on two dairy herds to ascertain the Shiga-toxin producing *Escherichia coli* (STEC) O157 and O26 shedding status of the animals and its impact (if any) on raw milk. Cattle are a recognized reservoir for these organisms with associated public health and environmental implications. Animals shedding *E. coli* O157 at > 10,000 CFU/g of feces have been deemed super-shedders. There is a gap in the knowledge regarding super-shedding of other STEC serogroups. A cohort of 40 lactating cows from herds previously identified as positive for STEC in a national surveillance project were sampled every second month between August, 2013 and July, 2014. Metadata on any potential super-shedders was documented including, e.g., age of the animal, number of lactations and days in lactation, nutritional condition, somatic cell count and content of protein in milk to assess if any were associated with risk factors for super-shedding. Recto-anal mucosal swabs (RAMS), raw milk, milk filters, and water samples were procured for each herd. The swabs were examined for *E. coli* O157 and O26 using a quantitative real time PCR method. Counts (CFU swab⁻¹) were obtained from a standard calibration curve that related real-time PCR cycle threshold (C_t) values against the initial concentration of O157 or O26 in the samples. Results from Farm A: 305 animals were analyzed; 15 *E. coli* O157 (5%) were recovered, 13 were denoted STEC encoding either *stx1* and/or *stx2* virulence genes and 5 (2%) STEC O26 were recovered. One super-shedder was identified shedding STEC O26 (*stx1&2*). Farm B: 224 animals were analyzed; eight *E. coli* O157 (3.5%) were recovered (seven were STEC) and 9 (4%) STEC O26 were recovered. Three super-shedders were identified, one was shedding STEC O157 (*stx2*) and two STEC O26 (*stx2*). Three encoded the adhering and effacement gene (*eae*) and one isolate additionally encoded the haemolysin gene (*hlyA*). All four super-shedders were only super-shedding once during the 1-year sampling period. The results of this study show, low numbers of super-shedders in the herds examined, with high numbers of low and medium shedding. Although four super-shedding animals were identified, no STEC

O157 or O26 were recovered from any of the raw milk, milk filter, or water samples. The authors conclude that this study highlights the need for further surveillance to assess the potential for environmental contamination and food chain security.

Keywords: STEC, super-shedding, raw milk, cattle, recto-anal, swabs

INTRODUCTION

Shiga-toxin producing *Escherichia coli* (STEC) O157 and O26 are well known human pathogens; emerging in the last 20 years as a major cause of illness and a serious public health issue (O'Brien et al., 1983; MacDonald et al., 1988). There were 704 cases of STEC in Ireland in 2014 of those 178 were caused by STEC O157 and 233 by STEC O26 (Health Protection Service Centre [HPSC], 2014). The European Food Safety Authority annual zoonoses reports show that, Ireland had the highest numbers of human cases of STEC in the EU in 2012–2014 (8.99, 12.29, and 12.42/100,000 population, respectively) (European Food Safety Authority [EFSA], 2014, 2015, 2016). The relevance of these pathogens is related to their low infective dose (10–100 bacteria) and the severity of the disease they cause (Karch et al., 2005). Foodborne contamination levels as low as 3–15 viable cells per gram of beef and 3–4 viable cells per 10 g of Salami have been associated with major outbreaks (Rangel et al., 2005). STEC infections in humans can cause three severe syndromes, hemorrhagic colitis (HC), haemolytic-uremic syndrome (HUS), and/or thrombotic thrombocytopenic purpura (TTP) (Mainil and Daube, 2005). Although STEC O157 was shown in a recent study on Irish cattle to be the predominant serotype shed in with bovine feces (2.3%), a range of other STEC serogroups including, O26 (1.5%), O103 (1.0%), O145 (0.7%) were also recovered (Thomas et al., 2012; European Food Safety Authority [EFSA], 2014).

The main virulence characteristic of STEC strains is the production of Shiga-toxins (denoted *stx* 1 and 2) (Sandvig, 2001). It has been reported that *stx2* and its variants are more likely to cause severe human disease (Caprioli et al., 2005; Persson et al., 2007; Bondari et al., 2015). Additionally, some isolates associated with human disease possess an adhering and effacement (*eae*) gene, responsible for the histopathological lesion, located on the pathogenicity island known as the locus of enterocyte effacement (LEE). Some strains may also possess a further virulence determinant haemolysin (*hlyA*) a plasmid-encoded enterohemolysin (Bondari et al., 2015).

It is generally accepted that cattle and other ruminant animals are a major reservoir of transmission with many infections originating either indirectly or directly from exposure to cattle feces (Karmali, 2004). Cattle are asymptomatic carriers of STEC as they lack the Gb3 receptor on their cell surface and the toxins cannot bind (Pruimboom-Brees et al., 2000; Bondari et al., 2015), thus, presenting no clinical symptoms while shedding these bacteria. Intermittent or persistent fecal shedding may occur following repeated exposure to the organism, through contaminated environmental sources or ingestion of contaminated water and feedstuffs (Rice et al., 2003; Carlson et al., 2009).

A small proportion of *E. coli* O157 positive animals shed the organisms at higher levels than others, these animals are denoted 'super-shedders' (shedding $\geq 10,000$ CFU/g of feces) (Matthews et al., 2006a; Chase-Topping et al., 2007, 2008; Cobbold et al., 2007; Stanford et al., 2012; Arthur et al., 2013; Munns et al., 2015). Menrath et al. (2010) studied the phenomenon of super-shedding non-O157 serotypes and reported some significant risk factors for shedding STEC; the month of sampling (prevalence is higher in August, September, and October); the number of lactations (first-time calvers) and days in milk (50–150 days or more than 350 days); the nutritional condition (higher than 3.5); the somatic cell count (lower than 100,000 cells/ml) and the content of protein in milk (higher than 3.0%).

As STEC may be part of the microbiota of the healthy animal, raw milk contamination may occur, inadvertently, during the milking process. Illness associated with the consumption of raw milk is rare in Ireland, as almost all liquid milk consumed is now pasteurized. However, there is documented evidence globally of the risks associated with raw milk consumption due to the possibility of STEC (and other pathogen) contamination in raw milk and raw milk products (Allerberger et al., 2003; Murphy et al., 2005, 2007; Rangel et al., 2005; Schrijver et al., 2008; Lynch et al., 2011; Madic et al., 2011; Pennington, 2014; FSAI Report, 2015). Diverse opportunities for contamination of raw milk exist, e.g., animal to animal contact and environmental sources (fomites, vectors, aerosols) (Ferens and Hovde, 2011). Humans and equipment present on the farm also pose a risk. To maintain the integrity of the production unit, necessary sanitation rules must be enforced this includes the use of disinfectants at key points and the wearing of protective clothing and footwear. Standard bio-security practices including rodent and pest controls together with effective controls on the hygienic quality of feedstuffs and water sources will help to reduce the potential health risk at farm level (Collins and Wall, 2004). However, the presence of super-shedders in a herd may have a disproportionately high impact on the risk of transmission on the farm, in the food chain and for the environment (water catchment areas in particular) (Rangel et al., 2005; Chase-Topping et al., 2008).

Several studies have confirmed that the principal colonization site for these bacteria in cattle is the recto-anal junction (RAJ) (Naylor et al., 2003; Rice et al., 2003; Davis et al., 2006; Cobbold et al., 2007; Nart et al., 2008; Carlson et al., 2009; Arthur et al., 2013). RAMS are deemed to be a sensitive method for the detection of STEC in cattle (Rice et al., 2003; Davis et al., 2006). In this study, RAMS were used as the sample matrix for quantitation, to determine the shedding status of individual animals. Raw milk, milk filter, and water samples were also screened for the pathogens.

Cattle super-shedding STEC increase the risk of transmission of this pathogen in the farm environment and into dairy (unpasteurized milk and farm house cheese) and the beef chain. There is a gap in the knowledge on the frequency of super-shedding and the factors causing it. Identifying such animals will lead to control measures for example; segregation from the food chain or introducing targeted interventions. This study provided preliminary information on super-shedding in Irish Dairy herds, thereby feeding into guidelines on the management of such super-shedding animals. It is hypothesized that the phenomenon of super-shedding may be related to intermittent modulations in the resident micro-flora of the RAJ, allowing *E. coli* O157 (or O26) to flourish and dominate in some animals for a period of time or maybe as a consequence of genetic variations in different STEC strains (Cobbolt et al., 2007; Jeon et al., 2013; Cote et al., 2015). We hypothesize that the presence of STEC super-shedders is directly related to the presence of these pathogens in the milk tank.

The objective of this study was to investigate the presence of active super-shedders in Irish herds and its impact (if any) on raw milk contamination.

MATERIALS AND METHODS

Herd Selection

This study was undertaken on two dairy herds in Ireland over 12 months. The herds were selected out of 18 positive herds that had participated in a National Surveillance Project, investigating the prevalence of STEC O157 and O26 in raw milk and milk filters (FSAI Report, 2015). The design of this study was longitudinal with the intent to determine the shedding status of particular animals in a herd over time, including low-shedders (1–10 CFU/swab), medium-shedders (100–1000 CFU/swab), and particularly the presence (if any) of super-shedders (cows shedding $\geq 10,000$ CFU/swab) and the impact of this phenomenon on potential raw milk contamination.

Animal Selection

The number of animals to be screened was determined using criteria set out by Cannon and Roe (1982). Forty lactating animals from each herd, to be sampled were chosen. The same 40 animals were sampled at each visit to the farm, where possible (herd population size 70; 5% expected proportion of super-shedders in the population; 95% confidence of identifying at least one super-shedder) (Cannon and Roe, 1982). Metadata collected from animals examined for the presence of Shiga-toxin producing-producing *E. coli* in bovine feces were included (Menrath et al., 2010).

Sample Collection

The dairy herd owners participated voluntarily and were assured of confidentiality. At the outset of the study a questionnaire was completed by each herd owner to gather information regarding herd size; animal husbandry; on-farm hygiene practices; feed type; water supply type; family age group and raw milk consumption practices on the premises. The recto-anal swabs

from each individual animal (which was then designated a sample code) were procured by the herd's private veterinary practitioner (PVP) with the assistance of a local authority veterinary surgeon during the milking process. The milk filter used during the milking session was taken for analysis. The raw milk sample was taken, aseptically, from the lower valve of the tank and the water sample was taken as per the European Union (Drinking Water) Regulations (2014) (Statutory Instrument No. 122/2014). The samples were returned to the laboratory within 4 h at 4°C in a temperature controlled container.

Isolation and Characterisation of STEC O157 and O26

Enrichment and DNA Extraction

On return to the laboratory the samples were refrigerated overnight at 4°C, the following morning, the samples were vortexed for 2 min and incubated for 5 h in Tryptone Soya Broth (Oxoid, Basingstoke, Hampshire, England) with novobiocin (Oxoid) [20 µg/ml] (mTSB) at $41.5 \pm 1^\circ\text{C}$. Post-incubation an aliquot of 1 ml was transferred to a 1.5 ml eppendorf for DNA extraction using DNeasy Blood and Tissue Kit, as per manufacturers' instructions (Qiagen GmbH, Hilden, Germany).

Real-Time Polymerase Chain Reaction

Samples were examined for *E. coli* O157 and O26 using a quantitative real time PCR method following an initial enrichment (Lawal et al., 2015). Counts (CFU swab^{-1}) were obtained from a standard calibration curve that related the real-time PCR cycle threshold (C_t) values to the initial concentration of O157 or O26 in the samples. The calibration curve was set up and validated on a Rotor-Gene 6000 instrument using spiked naturally contaminated swabs previously found negative for the presence of STEC ($n = 150$) which were inoculated with EDL933 *E. coli* O157 and NFC361 *E. coli* O26 at 10^1 – 10^7 CFU/swab.

Culture Methods

In the event of a positive result from the real time PCR the samples were culturally examined to obtain an isolate. Immuno-magnetic separation (IMS) was performed using Dynabeads anti-*E. coli* O157 or O26 as per manufacturers' instructions (life technologies, Oslo, Norway). After IMS the bead-bacteria complex was resuspended in 50 µl of phosphate buffered saline (PBS, Fannins-LIP, Galway, Ireland) and plated onto Cefixime-Tellurite Sorbitol MacConkey Agar (CT-SMAC) and ChromAgar O157 (Fannins-LIP) for *E. coli* O157 detection and in duplicate onto Cefixime-Tellurite Rhamnose MacConkey Agar (CT-RMAC, Lab M, Lancashire, UK) for *E. coli* O26 detection. Plates were incubated at $37 \pm 1^\circ\text{C}$ for 18 to 24 h. When present, up to five typical colonies from CT-SMAC and CT-RMAC were carried forward for confirmation tests. Typical colonies were subjected to a slide agglutination test conducted with single antisera (O157 and/or O26; Statens Serum Institut, Copenhagen, Denmark) and screened for the presence of indole production (ProLab Diagnostics, Bromborough, UK) (ISO 16654, 2001). All work was performed in a Category 3 facility within the Cork County Council campus, protocols and standard operating procedures were strictly adhered to for the duration of the study.

All positive strains were stocked at both -20 and -80°C and are held at the Veterinary Food Safety Laboratory.

Virulence Determination

DNA extraction from the cultures was performed using a DNeasy Blood and Tissue Kit (Qiagen, Crawley, West Sussex, UK) as per manufacturers' instructions. Nucleic acid amplification tests using real-time PCR were applied to the extracted DNA to determine the virulence status. The assay targets were the four common virulence genes of STEC O157 and O26 (*stx1*, *stx2*, *eae*, and *hlyA*). Two duplex real-time PCR's were employed, one to amplify *stx1* and *eae* (ISO/TS 13136, 2012) and the other to amplify *stx2* and *hlyA* (This Study; Accession No: AB779751.1 and Accession No: AY278115.1, respectively). Sequence searches were carried out using the BLAST program available at the NCBI BLAST home page¹ (Altschul et al., 1997). Probes, primers, associated fluorescent and quencher dyes are shown on Table 1. Additional reagents and final concentrations were as follows: *Taq* JumpStart mix [1X] (Sigma-Aldrich, St. Louis, MO, USA); MgCl₂ [25 mM] (Sigma-Aldrich); Bovine Serum Albumin [50 ug/ml] (Thermo Fisher Inc., Waltham, MA, USA); Exogenous Internal Positive Control (IPC) mix [10X] and IPC DNA were used as per manufacturers' instructions (Life technologies). All probes (*stx1*, *stx2*, *eae*, and *hlyA*) were at a concentration of 5 pM. The *stx1* primers were used at 20 pM and *eae* primers at 10 pM (ISO/TS 13136, 2012), the *stx2* and *hlyA* primers at 10 pM (this study). Molecular grade DNase and RNase free water (Roche GmbH, Mannheim, Germany) was added to bring the volume to 24 and 1 μl of template DNA [10 ng/ μl] to a final volume of 25 $\mu\text{l}/\text{tube}$. RT-PCR conditions were as follows: hold at 94°C for 2 min; cycling 95°C for 15 s; 60°C for 60 s repeat 30 times. Probes and primers developed for this study were designed using an online bioinformatic tool, GenScript Real-time PCR (*Taqman*) Probe/Primer Design². All primers and probes were generated by Eurofins Genomics, Regensberg, Germany³.

¹<http://www.ncbi.nlm.nih.gov/BLAST>

²<http://www.genscript.com>

³<http://www.eurofinsgenomics.com>

RESULTS

A total of 529 RAMS were analyzed for the presence of STEC O157 and O26 over a 12-month period. Farm A was visited eight times and Farm B was visited six times between August, 2013 and July, 2014 (Tables 2 and 3). Three hundred and five individual swabs were sampled from Farm A and 224 from Farm B.

Farm A: 15 of the 305 (5%) recto-anal swabs were *E. coli* O157 positive, 13 of those were confirmed as STEC as they encoded *stx1* and/or *stx2* (Table 2). All 13 isolates were positive for the adhering and effacement (*eae*) gene. Two of the isolates were *stx* negative but positive for *eae* (VFSL473 & 434). Five STEC O26 were recovered, all were *stx2* and *eae* positive. Two STEC O26 strains that were additionally positive for haemolysin gene (*hlyA*) were recovered from the same animal (VFSL731) (Table 2). One super-shredder (VFSL537) was identified shedding STEC O26 at 10,000 CFU/swab (Table 2). In addition, one animal was found to be shedding STEC O157 over three consecutive months (VFSL887).

One animal was colonized with *E. coli* O157 in January, 2014 and STEC O26 in July, 2014 (VFSL473).

One animal was intermittently shedding STEC O157 in November, 2013 and again in May, 2014 (VFSL531) and one STEC O26 in November, 2013 and July, 2014 (VFSL731) (Table 2). Additional data on the animals being sampled showed that the age range of positive animals was 2 years 6 months to 12 years 7 months (three were first time calvers) body condition between 3 and 4. A 12-year-old animal (VFSL165) was on her 11th lactation, with the remaining animals between 1 and 6 lactations; the number of days in milk is recorded as between 126 and 291. The super-shredder identified in the herd (VFSL537) was 3 years 3 months old on her second lactation, with a body score of 3 and was 184 days in milk, a SCC of 44,000 and a percentage protein of 3.53 (Table 2).

Farm B: eight of the 224 (3.5%) recto-anal swabs were *E. coli* O157 positive, seven being denoted STEC. Five strains were *stx2* and *eae* positive (VFSL578; 777; 783; 724; 646). The *stx* negative strain was *eae* and *hlyA* positive (VFSL770) and two strains were *stx2* (only; VFSL633 & 700) (Table 3). Nine STEC O26 were recovered, all nine were *stx2* and *eae* positive with five of these

TABLE 1 | Probes and primers used for amplification of virulence genes in real-time PCR assays (ISO/TS 13136, 2012 and this study).

Oligo name	5' -Label	Primer sequence <5' → 3'>	3'-Label	Reference
stx1- Probe	FAM	CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA A	BHQ1	ISO/TS 13136, 2012
stx1- Forward		TTT GTT ACT GTG ACA GCT GAA GCT TTA CG		
stx1- Reverse		CCC CAG TTC AAT GTA AGA TCA ACA TC		
ee- Probe	ROX	ATA GTC TCG CCA GTA TTC GCC ACC AAT ACC	BHQ2	
ee- Forward		CAT TGA TCA GGA TTT TTC TGG TGA TA		
ee- Reverse		CTC ATG CGG AAA TAG CCG TTA		
stx2- Probe	FAM	CTG TCT GAA ACT GCT CCT GTG	BHQ1	This study
stx2- Forward		CCA GTT CAG AGT GAG GTC CA		
stx2- Reverse		TCA GTT CGA TAC CCG CTG CAG C		
hlyA-Probe	ROX	TCT CCG GAA TTC TTT CTG CT	BHQ2	
hlyA-Forward		GCG AAA CAG CTT TAC CAA CA		
hlyA-Reverse		CGTC TCC CGG CGTC ATC GTA		

additionally *stx1* positive. One strain encoded all four virulence factors (VFSL700) interestingly this was one of the super-shedders. Three super-shedders were identified (VFSL832; 700 and 633) two shedding STEC O26 and one shedding STEC O157 at 10,000 CFU/swab (**Table 3**). In addition, one of the super-shedders was intermittently shedding STEC O26 in December, 2013 but was shedding very low numbers of STEC O26 in May, 2014 (VFSL832). One animal colonized both STEC O157 and STEC O26 and found to be super-shedding the STEC O26 (VFSL700). The age range of the animals on Farm B was 3 years 6 months to 7 years 8 months, no first time calvers; lactation's ranged from 2 to 6. Days in milk ranged from 132 to 229. The ages of the three super-shedders was 3 years 8 months (two lactations); 5 years 8 months (four lactations) and 6 years 8 months (five lactations) all had body condition scores of 3 (**Table 3**).

No STEC was recovered from any of the water, milk filter, or raw milk samples from either farm.

DISCUSSION

To our knowledge this is the first study investigating active super-shedding in dairy cattle in Ireland and the first internationally to examine for super-shedding of O26. This study addressed the carriage and shedding of STEC O157 and O26 in two dairy

herds and particularly their level of excretion, i.e., low (<10 – 100 CFU/swab); medium (100 – 1000 CFU/swab), or super-shedding (\geq 10,000 CFU/swab). The results of this study showed low numbers of super-shedders among the animals screened. The majority of the positive animals were low-shedders; two were medium-shedders with evidence of persistent shedding in some animals tested. There was no STEC O157 or O26 recovered from any of the raw milk, water, or milk filter samples analyzed.

The study identified four super-shedders over the 12-months, in December, 2013; January, 2014; April, 2014 and June, 2014 (**Tables 2 and 3**). Only a single super-shedder was identified in Farm A in January, 2014. The other three animals from Farm B were found to be super-shedding in December, 2013; April, 2014 and June, 2014. Each animal was identified as super-shedding only once during the study. For all animals regardless of shedding status, no seasonality could be determined as recovery of these pathogens occurred over every month of the year (**Tables 2 and 3**). No STEC was recovered from any of the water, milk filter or raw milk samples during the study.

The frequency of super-shedding in a herd is not well understood; a study on super-shedding in 60 heifers in the US reported 3.8% prevalence (Cobbolt et al., 2007). A Canadian study on feed-lot cattle recorded 25% (Cernicchiaro et al., 2010) and a German study on dairy herds found 10% were super-shedders (Menrath et al., 2010). Our study identified four

TABLE 2 | Farm A: Month of sampling, numbers of animals sampled, animal code, number of lactations, age of animal, *E. coli* serogroup isolated, shedding status, and virulence characteristics.

Month of sampling	No. Lactating animals sampled	Animal code	No. of lactations	Age of animal	<i>E. coli</i> serogroup isolated	Shedding status CFU/swab	Virulence status of recovered isolates			
							<i>stx1</i>	<i>stx2</i>	<i>eae</i>	<i>hlyA</i>
August, 2013	38	VFSL434	3	4y7m	O157	10	–	+	+	–
November, 2013	37	VFSL364	5	6y7m	O26	10	–	+	+	–
		VFSL731*	2	3y5m	O26	10	–	+	+	+
		VFSL432	3	4y7m	O157	<10	–	+	+	–
		VFSL531*	2	3y1m	O157	<10	–	+	+	–
		VFSL331	6	7y7m	O157	<10	–	+	+	–
		VFSL435	3	4y7m	O157	<10	–	+	+	–
January, 2014	35	VFSL887*	1	2y6m	O157	<10	–	+	+	–
		VFSL537	2	3y3m	O26	10,000	–	+	+	–
		VFSL473*	4	6y8m	O157	<10	–	–	+	–
February, 2014	39	VFSL703	2	3y3m	O157	<10	+	+	+	–
		VFSL887*	1	2y7m	O157	<10	–	+	+	–
March, 2014	40	VFSL165	11	12y7m	O157	<10	–	+	+	–
		VFSL434	3	5y2m	O157	<10	–	–	+	–
		VFSL887*	1	2y8m	O157	10	–	+	+	–
April, 2014	40	No STEC Detected								
May, 2014	38	VFSL326	6	7y7m	O157	<10	–	+	+	–
		VFSL405	3	5y7m	O157	<10	–	+	+	–
		VFSL531*	2	3y7m	O157	<10	–	+	+	–
July, 2014	38	VFSL731*	2	4y1m	O26	<10	–	+	+	+
		VFSL473*	4	7y2m	O26	<10	–	+	+	–

*animals positive for STEC more than once during the 1-year sampling program.

super-shedders from 529 animals tested (0.8%). To further our understanding on these results our collaborators in the project (Teagasc) will examine the entire micro-flora of the RAJ from the STEC super-shedding animals identified along with the animals shedding STEC (but not super-shedders) and a control group (non shedders) using a novel 16 s gene-based compositional metagenomic approach to assess the composition and proportion of microbes present at that time. These data should provide knowledge and a broader understanding of this phenomenon.

The presence of these pathogens in a herd even at low numbers may give rise to increased animal to animal and animal to environment transmission. In this present study, the two different serogroups examined colonized one single animal at the same time, and this has been previously reported in a study that showed, three different serogroups colonized one animal (Blanco et al., 1996). Interestingly, the animal which was colonized by both STEC O157 and O26 was super-shedding STEC O26. To our knowledge this is the first report of STEC O26 super-shedding in cattle, thus it is interesting to note that *E. coli* O26 is now the most common STEC serogroup in human illness in Ireland (Health Protection Service Centre [HPSC], 2014).

Virulence screening of the recovered isolates showed that all isolates contained, between one and four of the virulence genes that are commonly associated with human disease (Madic et al., 2011; Bondari et al., 2015). It is essential to monitor ruminants to evaluate the risk associated with STEC infections in humans. Although, this study did not recover any STEC from the raw milk samples, other studies have recovered these pathogens from raw milk and raw milk products (Allerberger et al., 2003; Rangel

et al., 2005; Schrijver et al., 2008; Madic et al., 2011) these raw products are associated with higher risk of STEC infection due to the survival of the organism during the manufacturing process. Elhadidy and Álvarez-Ordóñez (2016) applied a total of seven different stresses including, starvation, freeze-thaw, acid, heat, cold, osmotic and oxidative, to two different genotypes of *E. coli* O157:H7, the authors found multi stress resistance in the strains most frequently associated with human disease cases.

The results from the questionnaire showed that neither farm families consumed raw milk; one had a private water supply (Farm A) and one a public water supply (Farm B). Animals on both farms were housed between November and March each year and fed a diet of concentrates during the housed period. Increased awareness of the potential public health implications of this pathogen and the methods for its control, particularly at farm level, were discussed with each farmer (and some farm family members) and the PVPs during the visits. In the event of a positive result, advice was given verbally on personal hygiene and best practice on the farm to prevent the spread of STEC. As outlined in Food Research Ireland: Department of Agriculture (2011) the Government strategy links the sustainability of Ireland's food sector to its food safety performance and thereby protecting the consumer from serious pathogens (Food Harvest, in press).

This study, although confined to two herds links directly with current strategies by providing knowledge on the risk posed by cattle shedding STEC in large numbers. Eliminating high-level fecal excretion of STEC at farm level may reduce the prevalence

TABLE 3 | Farm B: Month of sampling, numbers of animals sampled, animal code, number of lactations, age of animal, *E. coli* serogroup isolated, shedding status, and virulence characteristics.

Month of Sampling	No. Lactating animals sampled	Animal code	No. of lactations	Age of animal	<i>E. coli</i> serogroup isolated	Shedding status CFU/swab	Virulence status of recovered isolates			
							stx1	stx2	eae	hlyA
September, 2013	39	No STEC detected								
December, 2013	37	VFSL868	2	3y6m	O26	100	-	+	+	-
		VFSL858	2	3y6m	O26	<10	-	+	+	-
		VFSL832*	2	3y8m	O26	10,000	+	+	+	-
February, 2014	37	No STEC detected								
April, 2014	37	VFSL578	6	7y8m	O157	<10	-	+	+	-
		VFSL700*	4	5y8m	O157	<10	-	+	-	-
		VFSL700*	4	5y8m	O26	10,000	+	+	+	+
		VFSL777	3	4y8m	O157	<10	-	+	+	-
		VFSL783	3	4y7m	O157	<10	-	+	+	-
		VFSL770	3	4y8m	O157	<10	-	-	+	+
May, 2014	37	VFSL724	4	5y7m	O157	<10	-	+	+	-
		VFSL780	3	4y7m	O26	100	+	+	+	-
		VFSL832*	2	4y1m	O26	<10	+	+	+	-
		VFSL840	2	3y7m	O26	<10	-	+	+	-
June, 2014	37	VFSL763	3	4y8m	O26	<10	+	+	+	-
		VFSL788	3	4y7m	O26	<10	-	+	+	-
		VFSL646	5	6y8m	O157	10	-	+	+	-
		VFSL633	5	6y8m	O157	10,000	-	+	-	-

*animals positive for STEC more than once during the 1-year sampling program.

of the organism in the host and in-turn reduces the risk of human infection. The study used a robust method to identify super-shedders, once detected a possible strategy may be to remove the super-shedder from the herd prior to movement of animals or slaughter, thus, protecting public health. In addition, measures should be considered for slurry treatment prior to spreading, in tandem with a review of intensive grazing systems, stocking densities and management of the grazing platforms.

Internationally recognized experts strongly advocate a multi-hurdle approach toward minimizing the risk presented by STEC O157 and other Shiga-toxin producing organisms (Collins and Wall, 2004; Matthews et al., 2006b). A further broader study on super-shedding of significantly more farms with a larger number of animals has now been completed and a publication is in preparation (Murphy et al., manuscript in preparation). This study highlights the need for further surveillance to assess the potential for environmental contamination and food chain security.

ETHICAL STATEMENT

Standard practices of animal care and use were applied to animals sampled in this project. Research protocols were approved by the Veterinary Department, Cork County Council, Cork, Ireland.

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AUTHOR CONTRIBUTIONS

GD, MM, and JB conceived and designed the study. BM, JB, and DC selected the herds and collected samples. BM and EM carried out the laboratory work. BM, EM, and SF analyzed and interpreted the data. BM and MM wrote the manuscript. All authors critically revised and approved the final manuscript

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Comparative Genomic Analysis of Two Serotype 1/2b *Listeria monocytogenes* Isolates from Analogous Environmental Niches Demonstrates the Influence of Hypervariable Hotspots in Defining Pathogenesis

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The vast majority of clinical human listeriosis cases are caused by serotype 1/2a, 1/2b, 1/2c, and 4b isolates of *Listeria monocytogenes*. The ability of *L. monocytogenes* to establish a systemic listeriosis infection within a host organism relies on a combination of genes that are involved in cell recognition, internalization, evasion of host defenses, and *in vitro* survival and growth. Recently, whole genome sequencing and comparative genomic analysis have proven to be powerful tools for the identification of these virulence-associated genes in *L. monocytogenes*. In this study, two serotype 1/2b strains of *L. monocytogenes* with analogous isolation sources, but differing infection abilities, were subjected to comparative genomic analysis. The results from this comparison highlight the importance of accessory genes (genes that are not part of the conserved core genome) in *L. monocytogenes* pathogenesis. In addition, a number of factors, which may account for the perceived inability of one of the strains to establish a systemic infection within its host, have been identified. These factors include the notable absence of the *Listeria* pathogenicity island 3 and the stress survival islet, of which the latter has been demonstrated to enhance the survival ability of *L. monocytogenes* during its passage through the host intestinal tract, leading to a higher infection rate. The findings from this research demonstrate the influence of hypervariable hotspots in defining the physiological characteristics of a *L. monocytogenes* strain and indicate that the emergence of a non-pathogenic isolate of *L. monocytogenes* may result from a cumulative loss of functionality rather than by a single isolated genetic event.

Keywords: comparative genomic analysis, *Listeria monocytogenes*, pathogenesis, hypervariable hotspots, attenuated virulence, stress survival islet, LIPI-3, DPC6895, serotype 1/2b

INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultatively anaerobic food-borne pathogen, and is the causative agent of the bacterial disease listeriosis in humans and animals. Recent figures demonstrate that approximately 99% of all human listeriosis cases arise due to the consumption of contaminated food produce (1), with serotypes 1/2a, 1/2b, 1/2c, and 4b implicated as the source of infection in

95% of these cases (2). Its psychrotrophic nature coupled with its tolerance of low pH and high salt concentrations (3) allows the bacterium to survive in refrigerated foods and reach levels required for human infection, if the food can support growth. *L. monocytogenes* is also commonly found in farm environments and silage in particular (4), and as such contaminated feeds represent a similar vector for food-borne transmission of the bacterium to animals used in food production. Its ability to cause a systemic infection in humans and animals alike is reliant on a combination of physical attributes, including resistance to environmental stresses and a capacity for virulence and survival within the host.

Traditionally, genetic relationships between *L. monocytogenes* strains are elucidated either by pulsed-field gel electrophoresis (PFGE) involving macrorestriction of genomic DNA to generate an associated DNA fingerprint (5) or by multilocus sequence typing (MLST) where specific sequences from a number of housekeeping genes are analyzed (6). These approaches are limited, however, in that they provide little insight into the pan genome of *L. monocytogenes* isolates. Comparative genome analysis has emerged as a robust tool for evaluating underlying genetic properties of bacterial strains, such as their evolutionary relationships, pathogenic potential, antibiotic resistances, and niche adaptation capabilities. With regard to *Listeria*, comparative genomics has proven to be particularly effective in determining the basis behind a number of observed phenotypic characteristics of *L. monocytogenes*, including the putative identification of many virulence genes responsible for *L. monocytogenes* pathogenesis on the basis of their relative absence in strains of the non-pathogenic *Listeria innocua* (7–9). Also, recent comparative analysis of two persistent *L. monocytogenes* strains that were isolated from separate fish processing plants almost 6 years apart (10) identified an extremely close relationship between their genomes. As such, it was proposed that strains with specific genetic traits may be selected for within a given environmental niche, providing a potential insight into the mechanisms of persistence of *L. monocytogenes*. Persistence is defined as the regular re-isolation of a given strain from the same environment over the course of several months or years. Comparative genomics has also been used to analyze *L. monocytogenes* isolates associated with listeriosis outbreaks (11, 12), to understand the unique genomic properties harbored by these strains contributing to systemic infection, and to determine the most efficient manner in which to analyze the epidemiological traits of future outbreak strains (13).

From an evolutionary perspective, one particular study involving a range of *L. monocytogenes* genomes of differing lineage and serotype demonstrated that this bacterial species, like others, has a highly conserved set of genes shared by all sequenced strains known as the “core genome” (14). While this core genome is common to all strains, subtyping methods (such as PFGE, MLST, and ribotyping) have demonstrated that examined *L. monocytogenes* isolates form a structured population consisting of a number of different evolutionary lineages (15). The majority of tested strains of serotypes 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4b, and 4e cluster to evolutionary lineages I and II. Flagellar type “a” isolates such as serotypes 1/2a and 3a cluster to

lineage II along with serotype 1/2c and 3c isolates, while flagellar type “b” isolates such as the 1/2b, 3b, and 4b serotypes all cluster within lineage I along with serotype 4d and 4e isolates (15). Two other evolutionary lineages have also been discovered and characterized. Lineage III contains serotype 4a, 4c, and a small number of 4b isolates (16) and represents a sister group to lineage I (15). Lineage IV, which was originally thought to represent a subgroup of lineage III (IIIB), is the most recently discovered (17), though only a limited number of isolates belonging to this lineage have been characterized to date. In general, the genomes of lineage I strains of *L. monocytogenes* (serotypes 1/2b, 3b, 4b, and 7) share a much higher degree of sequence similarity and exhibit a much lower degree of recombination than their lineage II and III counterparts (18, 19). Indeed, lineage I strains of *L. monocytogenes* predominantly differ from one another only in terms of their serotype, sequence type, prophage compositions, and a small fraction of chromosomal genes (12–23% of the total genome) that are collectively known as the accessory genome (14). Accessory genes are not as highly conserved as the core genes and in many cases can be strain-specific. Furthermore, while these accessory genes are located throughout the *L. monocytogenes* genome, their distribution is not entirely random. In certain chromosomal regions, accessory gene accumulations occur as a result of prophage acquisition (14), while other regions exhibit a non-random accumulation of these genes and are therefore denoted “hypervariable hotspots,” with nine such genomic regions recently defined in *L. monocytogenes* (19).

In this study, the genomes of two serotype 1/2b isolates of *L. monocytogenes* were subjected to comparative analysis in order to determine if there is a link between their core and accessory genome contents and their phenotypic characteristics. The two strains differed in their infection abilities. One of the isolates, strain DPC6895, was incapable of establishing a systemic infection within its animal host, despite it representing one of the four *L. monocytogenes* serotypes responsible for the vast majority of listeriosis cases (2, 20). Instead, this isolate caused a subclinical infection (21), and such subclinical infections generally go undetected, resulting in a potential public health hazard. On the other hand, strain FSL J2-064 did cause a systemic infection within its animal host. The aim of this research was to focus on a broad comparison of genes responsible for infection, intracellular survival, and proliferation within the host, in an attempt to discover a genomic basis for the perceived attenuation of pathogenesis in strain DPC6895 compared to strain FSL J2-064, and to evaluate the importance of the accessory genome in *L. monocytogenes* virulence and disease manifestation.

MATERIALS AND METHODS

Input Strains for Comparative Analysis

The two *L. monocytogenes* strains examined in this study were of the 1/2b serotype. Strain DPC6895 was originally isolated from raw milk expressed by a cow with subclinical bovine mastitis (20, 21), while strain FSL J2-064 is a bovine clinical isolate (22, 23), but of a ribotype (or restriction digest fingerprint) that is also commonly found among food isolates (DUP-1052), and is

associated with human disease (24). The genomes of both strains are available from public databases. The Whole Genome Shotgun project for *L. monocytogenes* strain DPC6895 was deposited at DDBJ/EMBL/GenBank and is available for download under the accession number LABG00000000. The version described in this paper is version LABG01000000. The genome sequence of *L. monocytogenes* strain FSL J2-064 is available from GenBank under the accession number NC_021824.

Identification of Strain-Specific Genes in Each of the Input Genomes

Whole genome comparisons were undertaken using BLAST Ring Image Generator (25) and Mauve (26), in order to visually identify unique genomic regions belonging to each of the strains. Genes within these regions were then confirmed to be strain-specific to each particular isolate through BLAST comparisons of their translated protein sequences against the genome of the other isolate, using RAST (27, 28).

Detection of Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-Associated (Cas) Systems and Prophage Identification

Clustered regularly interspaced short palindromic repeat clusters in each genome were identified using CRISPRFinder (29), with flanking sequences of these clusters subsequently scanned for the presence Cas gene sequences. Viable and cryptic prophages within each of these genomes were detected using the PHAST tool (30).

Linear Comparisons and Identification of Hypervariable Hotspots

Linear comparisons of genes and gene clusters were prepared with Artemis (31) and subsequently visualized using the EasyFig software (32). Hypervariable hotspot locations in each genome were determined via BLAST analysis using conserved core gene identifiers and previously defined hypervariable hotspot locations for *L. monocytogenes* strain SLCC2755, which was used as a reference (19).

RESULTS AND DISCUSSION

General Features of the *L. monocytogenes* Serotype 1/2b Strains

The main features of both strains used in this study (Table 1), as well as the locations of their respective hypervariable hotspots (Table S1 in Supplementary Material) are shown. Both genomes were similar in length (2.9–3.0 Mb) and had a G + C content of 37.8–38%, which is within the range typically observed for strains of *L. monocytogenes*. Neither of these strains contained any plasmids.

Comparative Genomic Analysis of Strains DPC6895 and FSL J2-064

Strains DPC6895 and FSL J2-064 were both obtained from bovine sources. While strain FSL J2-064 was originally isolated from a

TABLE 1 | General features of the *Listeria monocytogenes* input strains.

	DPC6895	FSL J2-064
Origin of isolate	Bovine	Bovine
Genome length	2,919,539	2,943,218
Contigs	9	1
G + C content	37.80	38.00
No. of coding sequences (CDS)	2,874	2,828
No. of tRNA genes	54	58
No. of plasmids	0	0

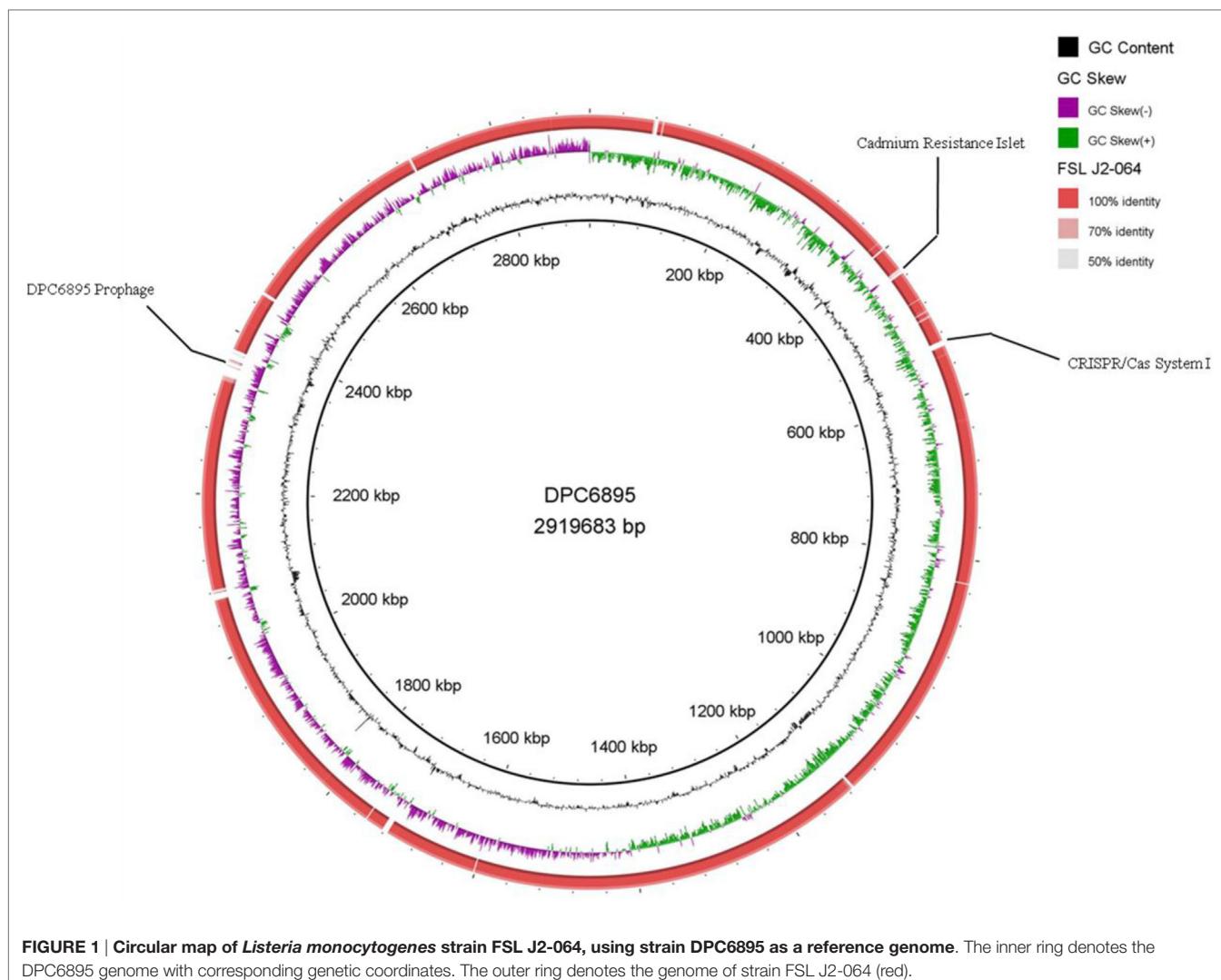
Nucleotide sequence blast (BLASTn) between input isolates (% query coverage, % identity, E-value)		
	DPC6895 ^a (subject)	FSL J2-064 (subject)
DPC6895 ^a (query)		(96%, 99%, 0.0)
FSL J2-064 (query)		(95%, 99%, 0.0)

^aGenome is not closed.

diseased animal (22, 23), strain DPC6895 was notably unable to establish a systemic clinical listeriosis infection in its respective host (20), though this strain did survive numerous antibiotic treatments and continued to be detected in milk expressed from the host for a prolonged period of time. While the host's immune system may have been a contributing factor, comparative analysis of these two strains was undertaken in order to determine the extent of genetic diversity between them and to identify genomic characteristics, which may account for the observed physiological differences. *L. monocytogenes* strain DPC6895 was determined to contain a total of 123 genes that were not present in the genome of FSL J2-064 (Figure 1; Table S2 in Supplementary Material), while strain FSL J2-064 contained a total of 121 genes that were absent from the DPC6895 genome (Figure 2; Table S3 in Supplementary Material).

Strain-Specific Genes in *L. monocytogenes* Strain DPC6895

The strain-specific genes in DPC6895 predominantly had functions, which contributed to an enhanced survival ability of this strain in a number of unfavorable environmental conditions. First, a number of these strain-specific genes had annotated functions in bacteriophage resistance. *L. monocytogenes* utilizes a number of biological systems in order to achieve resistance to bacteriophage infection. Foremost among these are the CRISPR sequences together with adjacent Cas genes and the restriction modification (RM) systems, which are widely distributed among prokaryotes (33). CRISPR/Cas genes comprise the adaptive immune system in many bacterial species including *L. monocytogenes* and have a role in defense of the bacterial cell against invading bacteriophages or plasmid-derived elements (34). Immunity against foreign invasion in bacteria is achieved first by integration of a small piece of viral or plasmid DNA (known as a spacer sequence) into the CRISPR locus. During infection, CRISPR-RNAs are transcribed, which guide the Cas proteins to target DNA that matches these spacer sequences that are then cleaved (35). RM systems are also used by bacteria in order to protect themselves from foreign invading DNA, of which there are three distinct classical types in addition to



several atypical systems, which differ from one another in terms of their composition and cofactor requirements (36). Each of the different classical RM system types have been previously observed in *L. monocytogenes* (12, 37–39). Strain DPC6895 contained one CRISPR cluster, which consisted of 22 highly conserved direct repeat (DR) regions interspersed with 21 spacer sequences (Table 2). Flanking this CRISPR cluster were a total of seven open reading frames (ORFs) with annotated functions such as Cas genes. BLASTn analyses of the spacer sequences identified homologies to a number of different temperate serovar 1/2-specific *L. monocytogenes* phages including A006, A118, and LP-101, suggesting that this system has a functional role in resistance to infection from these particular siphoviruses. These homologies indicate a role for this particular system in expanding the immunity of these two particular strains to cover a range of both lytic and temperate *L. monocytogenes* phages. The presence of this CRISPR/Cas system may enhance the capacity of strain DPC6895 to withstand a wider array of extracellular threats posed by bacteriophages. No definitive CRISPR/Cas systems were detected in strain FSL J2-064; however, this strain

contained all three subunits of a type I RM system, suggesting a difference between these strains in terms of their mechanisms of phage resistance. Strain DPC6895 contains two genes with 100% nucleotide identity to the R and M subunits of this system in FSL J2-064 but does not harbor the third S subunit, and as such this system is presumed to be non-functional in strain DPC6895.

Second, a number of the strain-specific genes in DPC6895 had annotated functions associated with antibiotic and heavy metal resistance. *L. monocytogenes* has previously been demonstrated to have quite a broad spectrum of resistance to numerous antibiotics and antimicrobial agents (2, 40) in addition to exhibiting an elevated tolerance to heavy metals (41). The two strains in this study were analyzed for the presence of antibiotic and antimicrobial resistance genes and for heavy metal transporters. The results of this analysis (Table S4 in Supplementary Material) demonstrated that each of the genomes harbor specific resistance genes to a number of antibiotics, including the β-lactams, quinolone, fosfomycin, lincomycin, vancomycin, and tetracycline. Additionally, these strains also contained a number of antimicrobial and

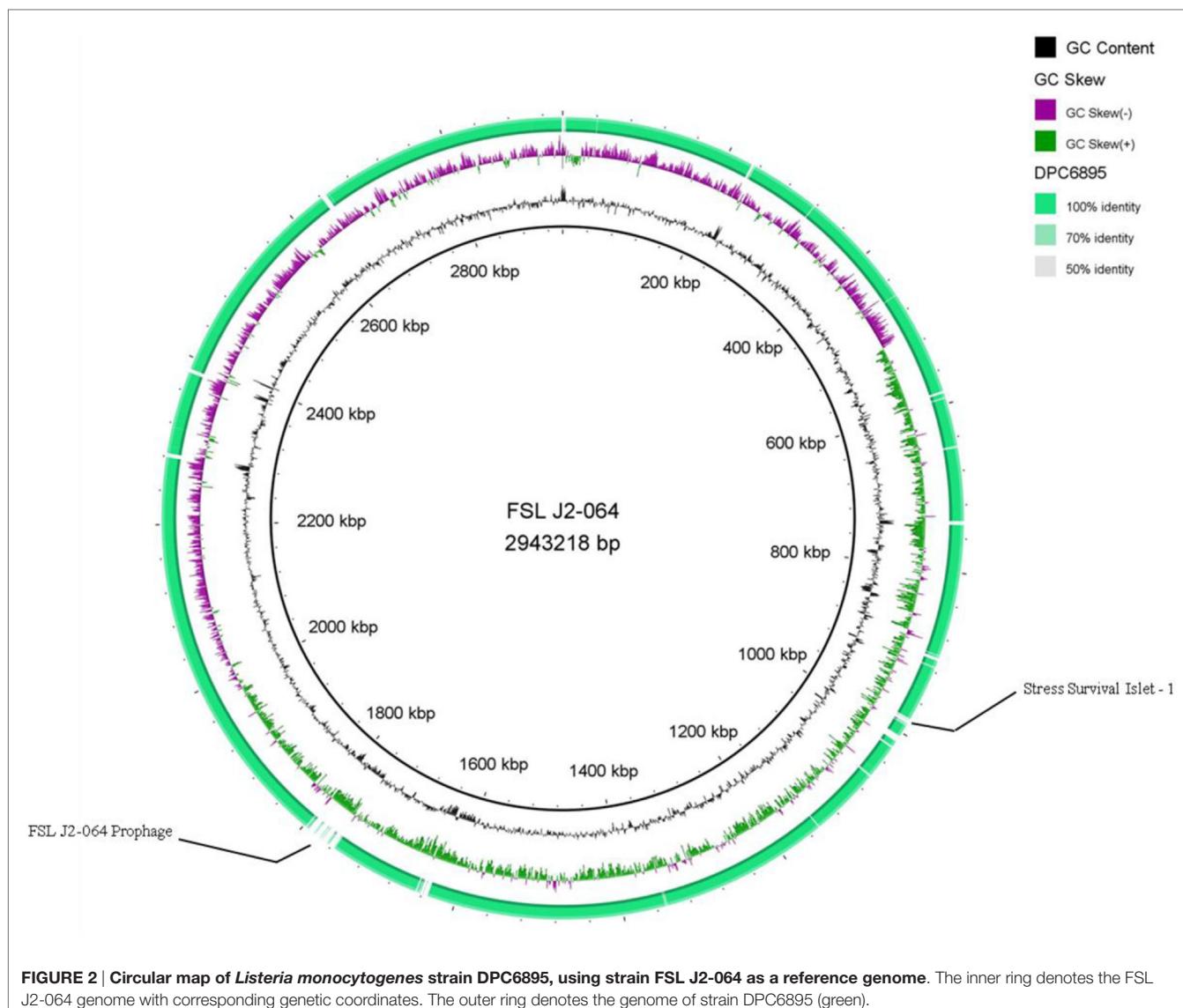


TABLE 2 | Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) systems in each of the *Listeria monocytogenes* isolates.

Strain	No. of CRISPR clusters	Location(s) on genome	(F/R)	Conserved region [direct repeat (DR)] consensus sequence	DR length	No. of spacer sequences	No. of flanking Cas genes
DPC6895	1	49,584–50,975 (contig 3)	F	GTTTTAACTACTTATTATGAAATGTAAAT	29	21	7
FSL J2-064	–	–	–	–	–	–	–

quaternary ammonium compound resistance genes including *mdrL* and *lde*, which are believed to be associated with increased tolerance of *L. monocytogenes* to benzalkonium chloride (42). Furthermore, *lde* is also thought to function in resistance of *L. monocytogenes* to fluoroquinolone (43). While both strains encoded many non-specific multidrug resistance transporters, strain DPC6895 harbored one additional multidrug transporter (locus tag *TZ05_2661c*), which was absent from strain FSL J2-064. The exact function of this particular transporter has not yet been

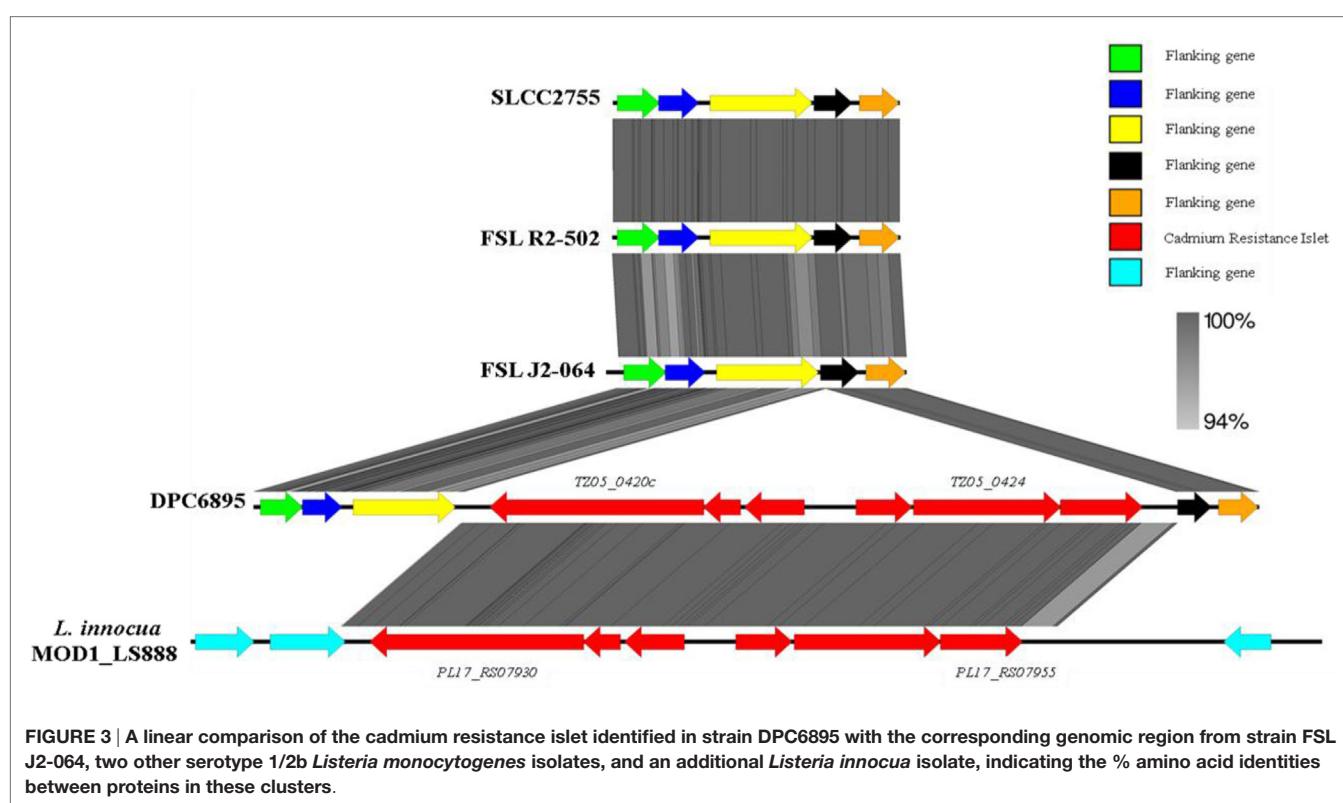
fully elucidated, but subsequent BLASTp analysis identified a conserved domain within the translated protein product of this gene, which has a putative function in resistance to the lantibiotic gallidermin (44), suggesting a potentially similar role for this gene in each of these strains. As previously stated, *L. monocytogenes* strain DPC6895 was originally isolated from raw milk expressed by a cow with subclinical bovine mastitis. Following the confirmation of a subclinical infection, the infected cow was medically treated with subsequent intramammary injections of

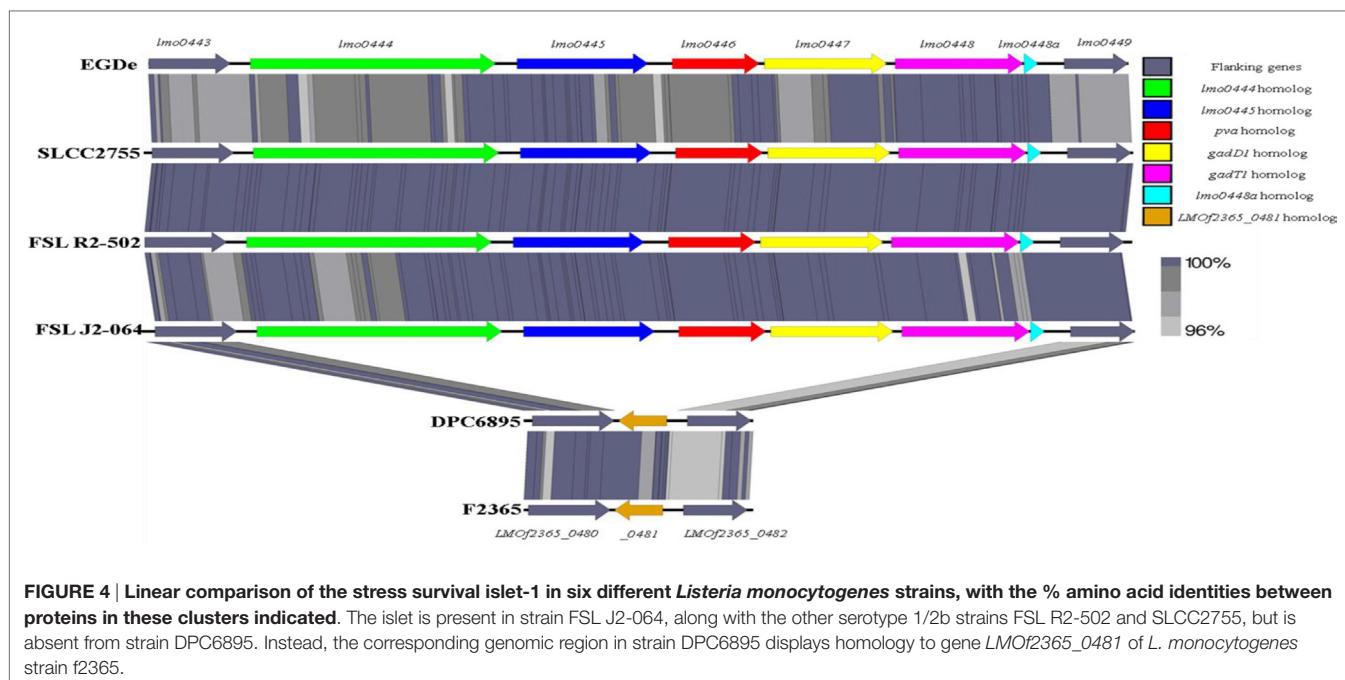
Synulox LC, Tylosin, and oxytetracycline. However, the infected animal's milk continued to test positive for *L. monocytogenes* despite the intervention with antibiotic treatments (20). Synulox LC contains the antibiotics clavulanic acid and amoxicillin, which are both β -lactams, while oxytetracycline is an antibiotic that is related to tetracycline, and tylosin is a macrolide antibiotic. Analysis of the DPC6895 genome identified a total of eight genes encoding proteins with associated functions in resistance to β -lactams, while a gene associated with tetracycline resistance was also identified. The presence of the aforementioned *mdrL* gene in strain DPC6895 may account for its perceived resistance to Tylosin, given that previous research has demonstrated that disruption of this particular gene resulted in a higher susceptibility to macrolides (45).

In terms of heavy metal resistance, both of the strains contained a number of non-specific heavy metal transporters and specific lead/cadmium/zinc resistance genes. Interestingly, strain DPC6895 was also found to contain a novel 6.5 kb "islet" consisting of six genes, which included a heavy metal transporting ATPase, and a cadmium efflux system accessory protein (Table S5 in Supplementary Material). The G + C content of this islet was 35.9%, indicating that it is possibly of plasmid origin. This six-gene islet has not previously been observed in *L. monocytogenes*, with the only known *Listeria* homologs to these genes found in the recently sequenced *L. innocua* strain MOD1_LS888 (46). Linear alignments between these genomic regions identified a shared 99% nucleotide sequence identity, while also demonstrating that this islet is absent in strain FSL J2-064 and two other *L. monocytogenes* serotype 1/2b genomes that were available

on Genbank (Figure 3). The product of one of the genes in this cluster, namely *TZ05_0424*, shares 100% amino acid sequence identity with the *Staphylococcus aureus* transposase Tn552 (47), suggesting strain DPC6895 may have acquired this cadmium resistance islet through a horizontal gene transfer event.

Finally, a number of the strain-specific genes in DPC6895 had annotated functions associated with peptide transport. The oligopeptide permease (*opp*) operon in *L. monocytogenes* consists of five genes (*oppA*, *oppB*, *oppC*, *oppD*, and *oppF*) that are essential for growth at low temperatures and contribute to intracellular growth of this bacterium (48). Comparative analysis of each of the isolates in this study identified the presence of this operon in both of the genomes (data not shown). However, in addition to the oligopeptide transporter operon, strain DPC6895 also contained a unique 5 kb cluster of genes (*TZ05_2018–2022*) within hypervariable hotspot 9, which BLASTp analysis indicated as a dipeptide transport system (*dppABCF*) (Table S2 in Supplementary Material). The role of this system in strain DPC6895 is unclear. However, previous research has indicated that the presence of dipeptide transporters may confer a selective advantage on *L. monocytogenes*, given the fact that unlike numerous competing bacteria within an environment, it would not need to expend vast amounts of energy on protease synthesis (49). In addition, the presence of a dipeptide transporter would allow the organism to thrive in food samples, which may be deficient in free amino acids but rich in peptides. The presence of this system in strain DPC6895, therefore, could allow it to proliferate in what would be otherwise considered unfavorable environmental conditions.





Strain-Specific Genes in *L. monocytogenes* Strain FSL J2-064

The strain-specific genes in FSL J2-064 predominantly had functions, which contribute to enhancing the pathogenicity of this isolate. First, a number of the strain-specific genes in FSL J2-064 had annotated functions associated with virulence. *L. monocytogenes* requires a wide array of genes in order to successfully establish a systemic infection within a host organism. These genes, termed “virulence factors,” have functions in a number of different biological processes throughout the infection cycle, including host interaction, internalization, host defense evasion, and *in vitro* proliferation. A large family of leucine-rich proteins, known as the internalins, are important virulence factors involved in host interaction and internalization of pathogenic strains (50). The internalins are classified into four general types on the basis of their specific surface binding domains (51). Type I are known as the LPXTG internalins due to the presence of this sorting signal motif and are covalently anchored to the bacterial cell surface by another virulence factor known as Sortase A. Type II are the GW and WxL internalins, of which just two members (including *inLB*) have been classified to date. Both members of this subfamily display a C-terminal domain that directs a non-covalent association with the *L. monocytogenes* cell surface (51). Type III internalins lack a cell wall-anchoring domain and are secreted by the bacterium. They are thought to promote the cell-to-cell spread of *L. monocytogenes* by relieving the cortical tension of the host cell and enhancing the ability of the bacterium to protrude into the plasma membrane (52). A fourth type of internalin, which contains an atypical leucine-rich repeat region, has also been recently described (53) with *lmo0460* as the sole representative member. The genomes of both strains in this study were examined for the presence of internalin and internalin-like

genes. A similar complement of internalins was observed in each input strain (Table S6 in Supplementary Material). Strain FSL J2-064 contained 20 type I internalins, while strain DPC6895 contains 21 (*TZ05_2026c* is novel to this strain). In addition, both isolates contained a virtually identical set of type II and type III internalins. Five homologs of the *L. monocytogenes* strain EGDe type IV internalin *lmo0460* were identified in strain FSL J2-064, localized within hypervariable hotspots 7 and 9. Though the precise function of these type IV internalin proteins has yet to be fully established, they are known to be present in many strains of *L. monocytogenes*, but absent from non-pathogenic *Listeria* species such as *L. innocua*, and thus may have a role in *L. monocytogenes* virulence. Interestingly, strain DPC6895 lacked any identifiable homolog to the recently described type IV internalin of *L. monocytogenes*, and as such, the absence of a type IV internalin in strain DPC6895 may be a contributing factor to its perceived attenuated virulence. Further research, however, is necessary to fully establish their functional role in infection. Additionally, two homologs of the internalin-like gene *lmo0463* of *L. monocytogenes* strain EGDe (hypervariable hotspots 7 and 9, respectively) were identified in strain FSL J2-064 but were absent once again from strain DPC6895. Likewise, their precise role in *L. monocytogenes* virulence remains unclear.

Second, the stress survival islet (SSI-1) of *L. monocytogenes* was identified to be present in the genome of strain FSL J2-064 but is absent from that of strain DPC6895 (Figure 4). This islet is an 8.7 kb region of DNA located between *lmo0443* and *lmo0449* of strain EGDe and contains five genes that have been previously implicated to assist in the survival of the bacterium under suboptimal environmental conditions (54). Included within this cluster are the *pva* gene, which has a role in resistance of *L. monocytogenes* to acute toxicity from bile and bile salts (55),

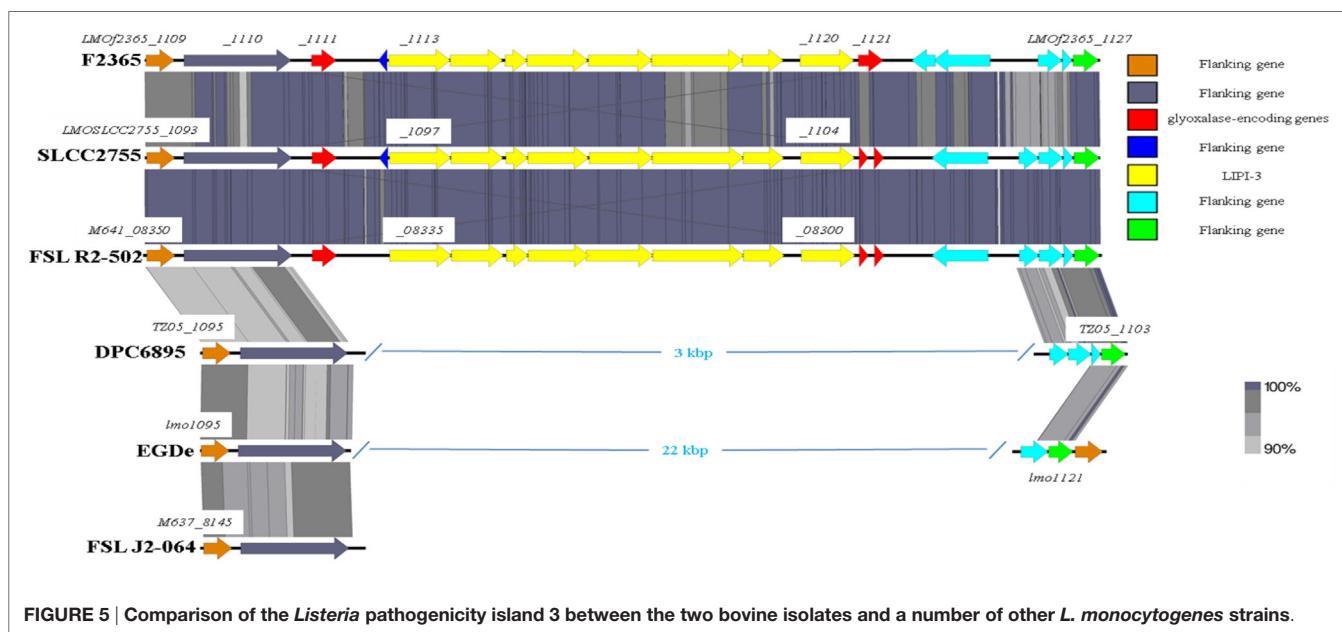
and the *gadD1* and *gadT1* genes, which contribute to the efficient growth of the bacterium at low pH (56). The corresponding region in strain DPC6895 was identified to contain a single gene encoding a protein that is 100% identical to LMOf2365_0481 of *L. monocytogenes* serotype 4b strain F2365. The presence of this 182 amino acid protein in strain DPC6895 represents a common feature of islet-negative strains of *L. monocytogenes* (54). Although this particular gene has been observed to be highly conserved within islet-negative strains of *L. monocytogenes*, the function of its hypothetical protein product has not yet been established. Prior research has shown that strains, which do not harbor SSI-1 were observed to grow less efficiently under acidic stresses (12), suggesting that the presence of this particular islet within the genome is beneficial in promoting survival in unfavorable acidic environments posed by the stomach and intestinal tract of a host organism. Additionally, the observed differences in growth efficiency between islet-positive and islet-negative strains of *L. monocytogenes* indicate that the presence of this islet could confer an enhanced ability to proliferate within an organism and lead to an overall higher rate of infection. Therefore, the absence of SSI-1 in DPC6895 may have been a contributing factor in the observed inability of this isolate to establish a clinical infection in its host (20). A number of the strain-specific genes in FSL J2-064 also had annotated functions associated with iron uptake that were absent in strain DPC6895, including the twin-arginine translocase system (57). Given the known association between iron uptake and *L. monocytogenes* virulence (58), the absence of these genes in strain DPC6895 provides another potential insight into its inability to cause a systemic infection.

Finally, the genome of strain FSL J2-064 contained an intact copy of the *comK* gene, while a prophage insertion (contig 7, position 257959–311605) interrupted the *comK* gene in strain DPC6895. The entire *comK* gene sequence in DPC6895 is instead represented by two separate ORFs; TZ05_2272 and

TZ05_2336, which together share 100% nucleotide sequence identity with the N- and C-terminal regions of the *comK* gene in FSL J2-064, respectively. A prophage insertion into the *comK* gene of *L. monocytogenes* is a common observation, as this gene represents a “hotspot” for integration of the serotype 1/2-specific bacteriophage A118 and other related phages (11, 13, 59). The phage insertion into this gene may hold downstream consequences for the pathogenic potential of strain DPC6895, as the *comK* gene has recently been shown to have an important role in phagosomal escape of *L. monocytogenes* during infection (60). As such, this interruption to the *comK* gene may be a contributing factor to the attenuated virulence of strain DPC6895. Interestingly however, the same research demonstrated that the *comK* prophage in *L. monocytogenes* strain 10403S excises during bacterial phagocytosis resulting in a reactivation of this gene and the production of a functional ComK protein product, and such an occurrence, therefore, must also be considered a possibility in strain DPC6895. Further investigation is required in order to fully understand the consequences of this prophage insertion.

Influence of Hypervariable Hotspots on the Virulence of Strains DPC6895 and FSL J2-064

As previously mentioned, the absence of type IV internalins (all of which are located within hypervariable hotspots in the *L. monocytogenes* genome) may be a contributory factor to the inability of strain DPC6895 to establish a systemic infection in the host. In addition, *Listeria* pathogenicity island 3 (LIPI-3) is a relatively recently discovered pathogenicity island, which has been identified in a subset of atypical *L. innocua* isolates (61) and a number of lineage I strains of *L. monocytogenes*. LIPI-3 contributes to virulence and intracellular survival of the pathogen (62) and is located within hypervariable hotspot 8.



The main function of this island is the production of a second *L. monocytogenes* hemolysin, namely listeriolysin S, which is induced under oxidative stress conditions (62). LIPI-3 consists of eight *lls* genes flanked on either side of the island by two related glyoxalase-encoding genes. Comparative analyses with other serotype 1/2b strains of *L. monocytogenes* identified that LIPI-3 was not found in either of the bovine isolates DPC6895 or FSL J2-064 (**Figure 5**), though homologs of the flanking glyoxalase-encoding genes were identified. The high variability generally observed within hypervariable hotspots of the *L. monocytogenes* genome may account for the absence of this island in these strains. While the presence of LIPI-3 does not appear to be essential in order to establish a systemic infection, the absence of this island may hinder the ability of a particular strain in the establishment of a systemic infection within the host.

CONCLUSION

The results of this study demonstrate the high degree of variability that exists between the accessory genomes of closely related *L. monocytogenes* isolates. The hypervariable hotspots found in various areas of the genome may be crucial in defining the physiological characteristics of a particular strain, as evidenced by the presence of important gene clusters such as the type IV internalins and the LIPI-3 within these regions. *L. monocytogenes* strain DPC6895 was shown to be missing some of the key factors that are associated with *in vivo* survival and virulence, including SSI-1 and LIPI-3, providing insights into the inability of this strain

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to establish a systemic infection in its host. The results highlight a number of potentially crucial factors for *L. monocytogenes* virulence within the accessory genome and suggest that bacterial pathogenesis in *L. monocytogenes* relies on the cumulative effect of a number of genetic factors rather than any single attribute alone. From a regulatory perspective, differentiation of virulent from non-virulent strains is crucially important. As used in this study, whole genome sequencing can be employed as a tool to explore this differentiation.

AUTHOR CONTRIBUTIONS

ACasey carried out the laboratory work; KJ, OM, ACoffey, ACasey, and EF were involved in obtaining funding, designing the experiments, interpreting the results, and writing the manuscript.

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Shortening of the *Lactobacillus paracasei* subsp. *paracasei* BGNJ1-64 AggLb Protein Switches Its Activity from Auto-aggregation to Biofilm Formation

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AggLb is the largest (318.6 kDa) aggregation-promoting protein of *Lactobacillus paracasei* subsp. *paracasei* BGNJ1-64 responsible for forming large cell aggregates, which causes auto-aggregation, collagen binding and pathogen exclusion *in vitro*. It contains an N-terminus leader peptide, followed by six successive collagen binding domains, 20 successive repeats (CnAB-like domains) and an LPXTG sorting signal at the C-terminus for cell wall anchoring. Experimental information about the roles of the domains of AggLb is currently unknown. To define the domain that confers cell aggregation and the key domains for interactions of specific affinity between AggLb and components of the extracellular matrix, we constructed a series of variants of the aggLb gene and expressed them in *Lactococcus lactis* subsp. *lactis* BGKP1-20 using a lactococcal promoter. All of the variants contained a leader peptide, an inter collagen binding-CnAB domain region (used to raise an anti-AggLb antibody), an anchor domain and a different number of collagen binding and CnAB-like domains. The role of the collagen binding repeats of the N-terminus in auto-aggregation and binding to collagen and fibronectin was confirmed. Deletion of the collagen binding repeats II, III, and IV resulted in a loss of the strong auto-aggregation, collagen and fibronectin binding abilities whereas the biofilm formation capability was increased. The strong auto-aggregation, collagen and fibronectin binding abilities of AggLb were negatively correlated to biofilm formation.

Keywords: AggLb, collagen binding domains, CnAB-like domains, auto-aggregation, biofilm formation

INTRODUCTION

Lactobacillus strains could exhibit probiotic characteristics, which confer a variety of beneficial health effects on the host and they have a number of features that make it particularly suitable for dairy applications (Salminen et al., 1998; Lebeer et al., 2008; Sisto and Lavermicocca, 2012; Giraffa, 2014). *Lactobacillus* effector molecules that contribute to the health-promoting interactions with the host (intestinal) system are likely located in the bacterial cell envelope (Bron et al., 2004;

Kleerebezem et al., 2010; Hymes et al., 2016). It was found that adhesion of lactobacilli to components of the extracellular matrix (ECM) such as mucin, fibronectin, collagen, laminin, or fibrinogen may thus have a direct impact on their probiotic function, e.g., in preventing the adhesion to and the colonization of damaged intestinal tissue sites by invading pathogens (Lorca et al., 2002). It has been reported that damage of the mucosal layer of the ECM can result in its colonization by pathogens, resulting in subsequent infection (Styriak et al., 2003).

The ability of pathogenic bacteria to adhere to distinct components of the ECM, such as collagen and fibronectin, is enabled or facilitated by the expression of ECM-binding proteins, termed adhesins. Adhesins are important virulence factors of pathogens, as they are involved in the initiation of infection (Flock, 1999). Group A streptococci (GAS, *Streptococcus pyogenes*) have evolved a number of surface-bound and secreted virulence factors, of which the M proteins are probably the best characterized. Binding of GAS to epithelial cells involves an interaction between M protein and fibronectin (Oehmcke et al., 2010). Epithelial cell invasion by Group B *Streptococcus* (GBS) is associated with expression of alpha C protein (Bolduc and Madoff, 2007). Aggregation protein encoded by *asp1* gene of enterococci, characterized as a virulence factor of 142 kDa plays a crucial role in adherence to eukaryotic cells (Galli et al., 1990). In the skin abscess model, a sortase-deficient *Staphylococcus aureus* strain lacking all of its cell-wall anchored proteins was less virulent than its wild-type strain. Also, strains specifically lacking protein A, fibronectin binding proteins, clumping factor A or surface protein SasF were impaired in their virulence (Josefsson et al., 2008; Kwiecinski et al., 2014). In addition some biofilm factors related to aggregation ability, for example, Bap protein of *S. aureus* facilitates the persistence in the mammary gland by enhancing adhesion to epithelial cells and prevents cellular internalization through the binding to GP96 host receptor (Taglaleagna et al., 2016).

Since systematic analysis of efficacy of probiotic therapy demonstrated that probiotic activities are strain-specific (Hungin et al., 2013; Sanders et al., 2013) the paradigm of probiotic research is rightfully shifting toward understanding the mechanistic action of each specific strain (Johnson and Klaenhammer, 2014). It has been demonstrated that the purified collagen binding protein (Cbp) from *L. plantarum* 91 possess anti-adhesion activity against the enteric pathogen *Escherichia coli* O157:H7 on immobilized collagen (Yadava et al., 2013). Surface fibronectin binding protein from *L. casei* BL23 participates in cell attachment to immobilized fibronectin (Muñoz-Provencio et al., 2010). Also, binding of immobilized collagen and fibronectin by *L. acidophilus* CRL 639 depends on cell-surface proteins (Lorca et al., 2002). The S-layer proteins of *L. crispatus* ZJ001 also inhibited the adhesion of *Salmonella typhimurium* and *E. coli* O157:H7 to HeLa cells (Chen et al., 2007). In addition, the S-layer protein associated with moonlighting proteins acted as an adherence factor, which has been evidenced by the high capability of adhesion,

auto- and co-aggregation of *L. helveticus* T159 (Waśko et al., 2014).

The ability of lactobacilli to form multicellular aggregates is an important property for colonization of the oral cavity, human gut or urogenital tract. The underlying mechanisms and the functionality of surface aggregation factors are not fully understood; on the one hand aggregation ability may not be the only components responsible for adhesion, and some of the criteria may be part of a complex mechanism that enables the microorganisms to interact with the host and to exert their beneficial effects (García-Cayuela et al., 2014). On the other hand, important mechanisms involved in this process are thought to include adherence as well as colonization of the GIT (Nazzaro et al., 2012; Skrzypczak et al., 2015). The expression of adhesins on the cell surface could induce cell aggregation visible as auto-aggregation. Aggregation promoting factors of lactobacilli differ in size, from 2 kDa in the strain *Lactobacillus gasseri* 2459–318.6 kDa in *L. paracasei* subsp. *paracasei* BGNJ1-64 (Boris et al., 1997; Miljkovic et al., 2015). Interestingly we have reported a new group of aggregation promoting factors of a high molecular mass, recently discovered in LAB (Kojic et al., 2011; Miljkovic et al., 2015). They differ in size and primary structure; however, they share similar structural organization and functions because they are composed of a large number of collagen-binding and CnaB-like domains (Miljkovic et al., 2015). Currently, no experimental evidence exists concerning the role of these domains in aggregation except for predictions that are based on a *S. aureus* collagen-binding Cna protein that mediates bacterial adherence to collagen. The major differences between the aggregation factors of the LAB and the Cna protein of *S. aureus* are that the primary structure of Cna has a non-repetitive collagen binding A region, followed by a repetitive B region (one–four 23 kDa repeating units B1–B4, depending on the strain). It has been suggested that the A region is involved in collagen binding, while the B region acts as a “stalk” that projects the A region from the bacterial surface, facilitating its adherence to collagen (Deivanayagam et al., 2000).

As mentioned above, the AggLb protein is the largest (318.6 kDa) aggregation factor of lactobacilli responsible for auto-aggregation, collagen binding and pathogen exclusion *in vitro*. AggLb consists of six diverse collagen binding domains (from 13202–15256 Da repeating units) and 20 almost identical CnaB-like domains (a 9916 Da repeating unit). The aim of this study was to investigate the roles of the different domains of the AggLb protein involved in probiotic function; this information might prove useful for its potential application. A series of variants of *aggLb* gene/protein were constructed, and their capability to induce auto-aggregation, binding to collagen and fibronectin, and biofilm formation was analyzed. It was concluded that AggLb could provide all of these functions: aggregation and binding to collagen and fibronectin as well as biofilm formation. Interestingly, strong auto-aggregation, collagen and fibronectin binding capacities of AggLb are negatively correlated with the ability of biofilm formation.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The strains, their derivatives and plasmids used in this study are listed in **Table 1**. *L. paracasei* was grown in De Man-Rogosa-Sharpe (MRS; Merck GmbH, Darmstadt, Germany) medium at 30°C. *Lactococcus lactis* subsp. *lactis* was grown at 30°C in M17 medium (Merck) supplemented with 0.5% glucose (GM17). *Pseudomonas aeruginosa* PAO1 and *E. coli* DH5α and M15 used for cloning and propagation of constructs were routinely grown in Luria-Bertani medium (LB) at 37°C with aeration. To obtain solid medium, agar (15 g/l; Torlak, Belgrade, Serbia) was added. Erythromycin was added to a final concentration of 10 µg/ml and 300 µg/ml for LAB and *E. coli*, respectively. Ampicillin and kanamycin were added to a final concentration of 100 µg/ml for *E. coli*. When necessary, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; Fermentas, Vilnius, Lithuania) was added to LB medium plates at a final concentration of 40 µg/ml for blue/white color selection of colonies.

DNA Manipulations

Electrocompetent *Lc. lactis* subsp. *lactis* BGKP1-20 cells were prepared as described by Holo and Nes (1989). Transformations were done by electroporation using an Eppendorf Electroporator (Eppendorf, Hamburg, Germany), except *E. coli* DH5α and M15, which was transformed by heat shock (Hanahan, 1983). Appropriate agar plates with antibiotics were used for the selection of transformants.

Plasmid DNA from *E. coli* DH5α was isolated by QIAprep Spin Miniprep kit (Qiagen GmbH, Hilden, Germany). Digestion with restriction enzymes was conducted according to the supplier's instructions (Fermentas). DNA fragments were purified from agarose gels using a QIAquick Gel extraction kit as described by the manufacturer (Qiagen). DNA was ligated with T4 DNA ligase (Agilent technologies, USA) according to the manufacturer's recommendations.

Specific primers used in this study are listed in section: Construction of the *aggLb* gene variants. KapaTaq DNA polymerase (Kapa Biosystems, Inc., Boston, MA, USA) was used to amplify DNA fragments by PCR using a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR products were purified with a QiaQuick PCR purification kit (Qiagen) according to the protocol of the supplier and sequenced by the Macrogen Sequencing Service (Macrogen, Netherlands). The DNA Strider program was used for open reading frame (ORF) prediction. Commercial pGEM-T-Easy (Promega, Madison, WI, USA), pCR2.1-TOPO (Thermo Scientific) and pCRII (Thermo Scientific) vectors were used for cloning of PCR products.

Construction of the *aggLb* Gene Variants

From construct pALb35 (Miljkovic et al., 2015) using *Xba*I-*Sall* restriction enzymes we made shorter construct pAggLbXS carrying only *aggLb* gene, in pAZIL vector (Supplementary Figure 1A). *Pst*I restriction site is located in *aggLb* gene

at position to divide it into two regions: first containing leader peptide sequence and six collagen binding domains and second containing 20 CnAB-like domains and anchor domain (**Figure 1**). In order to facilitate the construction of a large number of variants, *aggLb* gene was subcloned from pAggLbXS in two parts into pBScript vector (Agilent technologies): first part as *Xba*I-*Pst*I (construct pBS-XP) and second as *Pst*I-*Sall* fragments (construct pBS-PS; Supplementary Figure 1A). Bioinformatic analysis showed that *Hind*III (in both fragments; Supplementary Figures 1B,D) and *Ssp*I (only in *Xba*I-*Pst*I fragment; Supplementary Figure 1C) restriction enzymes dividing AggLb protein to distinct portions that contain the exact number of codons without free base except one in *Xba*I-*Pst*I fragment, so that they can be deleted or combined because they provide in frame junction. Constructs pBS-XP [consisting of three *Hind*III fragments of 820 bp, 821 bp (this two cannot be deleted separately since deletion of each fragment changed frame and introduce frameshift mutation) and 1461 bp] and pBS-PS (consisting of four *Hind*III fragments of 846 bp, 1266 bp, and two of 1410 bp) were partially digested with *Hind*III restriction enzyme and ligated. We successfully constructed pBS-XP-1, pBS-XP-4, pBS-PS-A, pBS-PS-B, pBS-PS-C, pBS-PS-D, and pBS-PS-E (for details see **Table 1** and Supplementary Figure 1). From construct pBS-XP fragment carrying *Xba*I/*Pst*I was recloned into pCR2.1-TOPO (since does not contain *Ssp*I restriction site; Thermo Scientific, Lithuania) giving construct pCR-XP, which was additionally partially digested with *Ssp*I restriction enzyme and ligated (constructs pCR-XP-2 and pCR-XP-3; Supplementary Figure 1). In next step, different constructs containing deletion in first part (pBS-XP-1, pBS-XP-4, pCR-XP-2, and pCR-XP-3) were combined with constructs containing deletion in second part (pBS-PS-A, pBS-PS-B, pBS-PS-C, pBS-PS-D, and pBS-PS-E) in pBScript vector (for details see **Table 1** and **Figure 1**). In order to obtain expression in lactococci, lactococcal promoter *PlsbB* (Uzelac et al., 2015) was cloned into pAZIL vector together with leader sequence of *aggLb* gene as *Sac*I-*Eag*I fragment (construct pAZIL-pSE). After that different combinations of variants from pBScript vector were cloned as *Eag*I-*Sall* fragments into pAZIL-pSE (for details see **Table 1** and **Figure 1**). *Lc. lactis* subsp. *lactis* BGKP1-20 was transformed with chosen constructs and expression of different AggLb variants were confirmed by Dot blot analysis using anti-AggLb antibody.

In addition, using template clone KPPvScI (Kojic et al., 2011) and specific set of primers: KPFw (5'GCAAAGCGCCATTCGCC3'), KPPstIRev (5'CGTTCCCTCTGCAGTTCCAC3'), after PCR amplification, we obtained clone pCRII-KPI. *Bam*HI-*Pst*I fragment containing first part of AggL (aggregation factor from *Lc. lactis* subsp. *lactis* BGKP1) was recloned from pCRII-KPI into pBS-PS, from which entire hybrid molecule as *Bam*HI/*Xho*I was transferred to pAZIL vector (digested with *Bam*HI/*Sal*I) and finally obtained clone was named as pKP-Lb (**Table 1**).

Auto-aggregation Assay

The first step of screening strains was visual auto-aggregation assay. The aggregation phenotype was scored as positive when clearly visible snowflakes-like particles, formed by aggregated

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

Strain	General characteristics	Source or reference
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>		
BGNJ1-64	Natural isolate; Agg ⁺	Miljkovic et al., 2015
BGNJ1-641	Derivative BGNJ1-64; Agg ⁻	Miljkovic et al., 2015
<i>Lactococcus lactis</i> subsp. <i>Lactis</i>		
BGKP1	Natural isolate; Agg ⁺	Kojic et al., 2011
BGKP1-20	Derivative BGKP1; Agg ⁻	Kojic et al., 2011
BGKP1-20/pAZIL-pPIAggLb	Derivative BGKP1-20 carrying pPIAggLb	This study
BGKP1-20/pPI4E	Derivative BGKP1-20 carrying pPI4E	This study
BGKP1-20/pPI3C	Derivative BGKP1-20 carrying pPI3C	This study
BGKP1-20/pPI3D	Derivative BGKP1-20 carrying pPI3D	This study
BGKP1-20/pPI3E	Derivative BGKP1-20 carrying pPI3E	This study
BGKP1-20/pPI2B	Derivative BGKP1-20 carrying pPI2B	This study
BGKP1-20/pPI2D	Derivative BGKP1-20 carrying pPI2D	This study
BGKP1-20/pPI2E	Derivative BGKP1-20 carrying pPI2E	This study
BGKP1-20/pPI1A	Derivative BGKP1-20 carrying pPI1A	This study
BGKP1-20/pPI1D	Derivative BGKP1-20 carrying pPI1D	This study
BGKP1-20/pPI1E	Derivative BGKP1-20 carrying pPI1E	This study
BGKP1-20/pkP-Lb	Derivative BGKP1-20 carrying pkP-Lb	This study
<i>Lc. lactis</i> subsp. <i>cremoris</i>		
MG7284	Pr ⁻ , Lac ⁻ , Bac ^r , Fus ^r , Spc ^r	Gasson, 1983
<i>Escherichia coli</i>		
DH5 α	supE44 Δ lacU169 (ϕ 80 lacZ Δ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Hanahan, 1983
M15	Nal ^s , Str ^s , Rif ^s , Thi ⁻ , Lac ⁻ , Ara ⁺ , Gal ⁺ , Mtl ⁻ , F ⁻ , RecA ⁺ , Uvr ⁺ , Lon ⁺	Qiagen
<i>Pseudomonas aeruginosa</i>		
PAO1		Laboratory collection
Plasmids and constructs		
pGEM-T Easy Vector	3015 bp, Amp ^r , bacterial, non-viral, transient, constitutive, high expression level, cloning vector	Promega
pBScript vector	2958 bp, Amp ^r , cloning vector	Agilent technologies
pCR2.1-TOPO	3908 bp, Amp ^r , Kan ^r , cloning vector	Thermo Scientific
pCRII	3971 bp, Amp ^r , Kan ^r , cloning vector	Thermo Scientific
pQE30	Amp ^r , ColE1 replicon, HIS6 expression vector	Qiagen
pAZIL	Em ^r , shuttle cloning vector	LMBP 9596
pALb35	pAZIL SJ derivative carrying 11377 bp SacI fragment of pNJ1 plasmid from BGNJ1-64	Miljkovic et al., 2015
pAggLbXS	XbaI-Sall fragment from pALb35 cloned in pAZIL vector	This study
pBS-XP	First part of aggLb cloned as XbaI-PstI into pBluescript vector	This study
pCR-XP	First part of aggLb cloned as XbaI-PstI into pCR2.1-TOPO vector	This study
pBS-PS	Second part of aggLb cloned as PstI-Sall into pBluescript vector	This study
pBS-XP-1	pBS-SP were partially digested with HindIII restriction enzyme and ligated (without 1461, 820, and 821 bp)	This study
pBS-XP-4	The same as pBS-XP	This study
pCR-XP-2	pCR-XP were partially digested with SspI restriction enzyme and ligated (without 630 and 1611 bp)	This study
pCR-XP-3	pCR-XP were partially digested with SspI restriction enzyme and ligated (without 1611 bp)	This study
pBS-PS-A	The same as pBS-PS (aforementioned)	This study
pBS-PS-B	pBS-PS were partially digested with HindIII restriction enzyme and ligated (without both fragments of 1410 bp)	This study
pBS-PS-C	pBS-PS were partially digested with HindIII restriction enzyme and ligated (without 846 and both fragments of 1410 bp)	This study
pBS-PS-D	pBS-PS were partially digested with HindIII restriction enzyme and ligated (without both fragments of 1410 and 1266 bp)	This study
pBS-PS-E	pBS-PS were partially digested with HindIII restriction enzyme and ligated (without 846, both fragments of 1410 and 1266 bp)	This study
pBS-PI4E	XbaI/PstI fragment from pBS-XP-4 pooled with PstI-Sall fragment from pBS-PS-E, used pBScript vector	This study

(Continued)

TABLE 1 | Continued

Strain	General characteristics	Source or reference
pPBS-PI3C	XbaI/PstI fragment from pCR-XP-3 pooled with PstI-SalI fragment from pBS-PS-C, used pBScript vector	This study
pPBS-PI3D	XbaI/PstI fragment from pCR-XP-3 pooled with PstI-SalI fragment from pBS-PS-D, used pBScript vector	This study
pPBS-PI3E	XbaI/PstI fragment from pCR-XP-3 pooled with PstI-SalI fragment from pBS-PS-E, used pBScript vector	This study
pPBS-PI2B	XbaI/PstI fragment from pCR-XP-2 pooled with PstI-SalI fragment from pBS-PS-B, used pBScript vector	This study
pPBS-PI2D	XbaI/PstI fragment from pCR-XP-2 pooled with PstI-SalI fragment from pBS-PS-D, used pBScript vector	This study
pPBS-PI2E	XbaI/PstI fragment from pCR-XP-2 pooled with PstI-SalI fragment from pBS-PS-E, used pBScript vector	This study
pPBS-PI1A	XbaI/PstI fragment from pBS-XP-1 pooled with PstI-SalI fragment from pBS-PS-A, used pBScript vector	This study
pPBS-PI1D	XbaI/PstI fragment from pBS-XP-1 pooled with PstI-SalI fragment from pBS-PS-D, used pBScript vector	This study
pPBS-PI1E	XbaI/PstI fragment from pBS-XP-1 pooled with PstI-SalI fragment from pBS-PS-E, used pBScript vector	This study
pAZIL-pSE	Lactococcal promoter <i>PlsbB</i> was cloned into pAZIL vector together with leader sequence of <i>aggLb</i> gene as SalI-EagI fragment	This study
pPIAggLb	EagI-SalI fragment cloned from pALb35 into pAZIL-pSE construct	This study
pPI4E	EagI-SalI fragment cloned from pBS-PI4E into pAZIL-pSE construct	This study
pPI3C	EagI-SalI fragment cloned from pBS-PI3C into pAZIL-pSE construct	This study
pPI3D	EagI-SalI fragment cloned from pBS-PI3D into pAZIL-pSE construct	This study
pPI3E	EagI-SalI fragment cloned from pBS-PI3E into pAZIL-pSE construct	This study
pPI2B	EagI-SalI fragment cloned from pBS-PI2B into pAZIL-pSE construct	This study
pPI2D	EagI-SalI fragment cloned from pBS-PI2D into pAZIL-pSE construct	This study
pPI2E	EagI-SalI fragment cloned from pBS-PI2E into pAZIL-pSE construct	This study
pPI1A	EagI-SalI fragment cloned from pBS-PI1A into pAZIL-pSE construct	This study
pPI1D	EagI-SalI fragment cloned from pBS-PI1D into pAZIL-pSE construct	This study
pPI1E	EagI-SalI fragment cloned from pBS-PI1E into pAZIL-pSE construct	This study
pCRII-KPI	First part of KPPvScl cloned as PCR fragment into pCRII vector	This study
pKP-Lb	Hybrid clone; consisting of first part of <i>aggL</i> gene as Pvul-PstI fragment and second part of <i>aggLb</i> gene as PstI-SalI fragment into pAZIL vector	This study
pQE ₃₀ -AggB5	Fusion His-tagged part of AggLb protein into pQE ₃₀ expression vector; in order to production of polyclonal antibody	This study

cells, gravitated to the bottom of the tube, forming a precipitate and leaving clear supernatant.

The auto-aggregation ability of the selected strains and derivatives was tested according to García-Cayuela et al. (2014) with minor modifications. Briefly, cells of overnight culture were harvested by centrifugation (5000 × g, 10 min, 4°C), washed twice with phosphate-buffered saline – PBS (10 mM Na₂HPO₄, 1 mM KH₂PO₄, 140 mM NaCl, 3 mM KCl, pH 7.1) and resuspended in the same buffer. The mixture was vortexed and incubated at 30°C for a period of 5 h. Absorbance (OD₆₀₀) was measured at different time points. Percentage of auto-aggregation was determined using the equation: [1 – (A_t/A₀) × 100] where A_t represents the absorbance at different time points (1, 2, 3, 4 and 5 h) and A₀ is absorbance at time 0. Auto-aggregation assay was done in three independent experiments. Data are presented as average of absorbance values from three independent experiments per each strain. The significance was determined by Student's *t*-test.

Biofilm Formation Assay

The ability of selected strains and derivatives to form biofilm was assayed in microtiter plates as previously described by Peter et al. (2013). *P. aeruginosa* PAO1 and *E. coli* DH5α were used as positive and negative control strains, respectively. Additionally, PBS buffer was included to ensure that the influence on biofilm formation by strains (resuspended in the same buffer) not

attributed to a non-specific binding effect to crystal violet. The results are presented as average of absorbance values from three independent experiments per each strain. The significance was determined by Student's *t*-test.

Collagen and Fibronectin Binding Assays

The wells of Maxisorb plates (Nunc, Roskilde, Denmark) were coated with type I collagen (from rat tail, BD Bioscience, Franklin Lakes, NJ, United States; 100 µg/ml) and human fibronectin (Serva, Heidelberg, Germany; 100 µg/ml) for 16 h at 4°C. The collagen binding ability of the selected strains and derivatives was tested according to Miljkovic et al. (2015), while the ability of tested strains and derivatives to bind to fibronectin was assayed as previously described by Ahmed et al. (2001). After immobilization, wells were washed with PBS and blocked with 2% BSA in PBS. Upon removal of BSA solution and washing wells with PBS, the test cultures (100 µl, 10⁸ CFU/ml) were added and plates were incubated on an orbital platform shaker for 2 h at 37°C. Non-adherent cells were removed by washing the wells three times with 200 µl of PBS. The adhered cells were fixed at 60°C for 20 min and stained with crystal violet (100 µl/well, 0.1% solution) for 45 min. Wells were subsequently washed three times with PBS to remove the excess stain. The stain bound to the cells was dissolved by 100 µl of citrate buffer (pH 4.3). The absorbance was measured at 570 nm, after 45 min, using the microtiter plate reader. Collagen and fibronectin binding

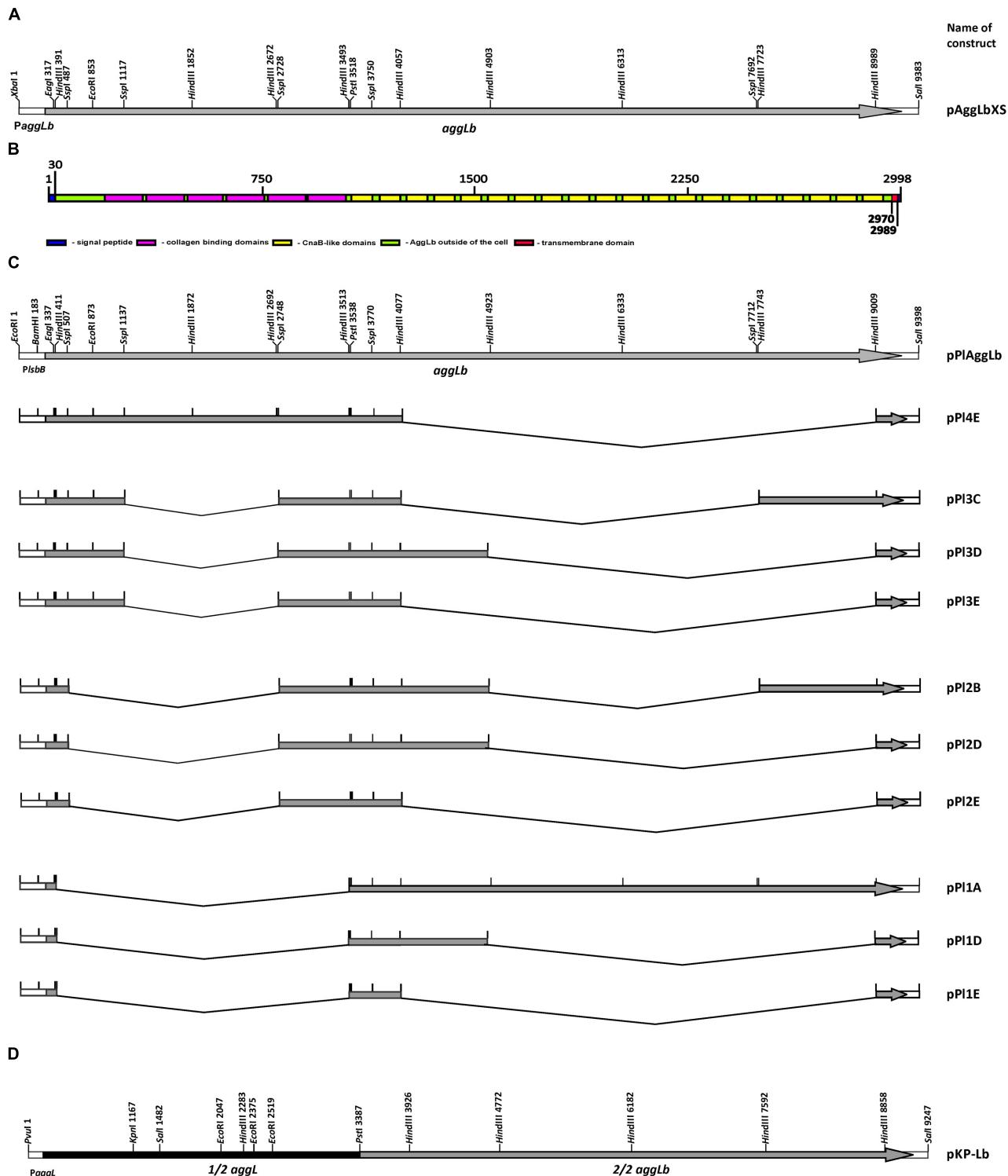


FIGURE 1 | Schematic representation of strategy for construction variants of *aggLb* gene and hybrid construct. (A) *aggLb*; **(B)** AggLb domain organization (boxes indicate domains of protein); **(C)** series of variants expressed using the lactococcal promoter *PaggLb*; **(D)** hybrid clone pKP-Lb.

was assayed as described above and the average of absorbance values from three independent experiments per each strain was presented. The significance was determined by Student's *t*-test.

Determination of Relationships between Auto-Aggregation, Collagen/Fibronectin Binding, and Biofilm Ability of Transformants Carrying Different Variants of the *aggLb* Gene

Plots of correlation were produced using Python 2.7.8 and *scipy* library (version 0.14.0).

Production of Polyclonal Antibody

Since whole AggLb protein was not able to be expressed in *E. coli* the part of AggLb protein containing the inter region of 190 amino acids between collagen binding and Cnab-like domains (from 1096 aa to 1286 aa) present in all variants was expressed using pQE₃₀ vector with 6 × His tag (Qiagen) for production of anti-AggLb polyclonal antibody. Using clone pALb35 (Miljkovic et al., 2015), *Hind*III fragment of 560 bp containing *Pst*I restriction site was cloned into pBScript. This fragment was recloned from pBScript vector as *Bam*HI/*Sal*I in frame into expression vector pQE₃₀ with 6 × His tag (pQE₃₀-AggBS). Fusion His-tagged protein was expressed in *E. coli* M15 cells. His-tag affinity purification of part of AggLb protein was conducted under denaturing conditions: the refolding method using urea to disrupt non-covalent bonds and increase protein solubility was used to solubilise and make the His-tagged AggLb more accessible to the nickel-nitrilotriacetic acid (Ni-NTA) resin. Purification of the fusion protein was applied according to protocol recommended by The QIAexpressionist. The eluted protein was dialyzed by ultrafiltration (Centrifugal Filter Units, Amicon Ultra-15 Centrifugal Filter Devices, 3K, Millipore). Polyclonal antibodies were produced by immunization of mice with the synthetic or purified fusion proteins in animal house of ICGBE, Trieste, Italy.

Dot Blotting

Samples (2 μl of serial dilutions of total proteins dissolved in buffer which contains: 100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 8.0) were loaded into a PVDF membrane (Merck Millipore, Darmstadt, Germany) by directly spotted on membrane as described by Niedergang et al. (2000). The same quantity of non-diluted samples was loaded on PAGE-SDS gel stained with Coomassie brilliant blue (Supplementary Figure 2). Membrane was incubated with 10% skim milk diluted in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) over night at 4°C in order to block non-specific reactions. Following blocking, the membrane was incubated 1 h at room temperature with gentle agitation in dilutions of primary antibody (mouse polyclonal antibody anti-AggLb-Ab). Primary antibodies were diluted in 5% skim milk diluted in TBS-T. After washing three times in TBS-T for 15 min, membrane was incubated for 1 h with horseradish peroxidase-labeled anti-mouse IgG (A9044 anti-mouse; Sigma, Germany) at a 1:10000

dilution in 5% skim milk diluted in TBS-T. The blots were washed three times in TBS-T for 15 min. Spots were detected using EMD Millipore Immobilon™ Western Chemiluminescent HRP Substrate (ECL; Fisher Scientific, USA) following the manufacturer's instructions.

RESULTS

Construction of the AggLb Variants

We performed functional studies of the various domains of the AggLb protein. To produce many different domain variants of the AggLb protein, the *aggLb* gene was subcloned into two parts *Sac*I-*Pst*I and *Pst*I-*Sal*I fragments, using the pBscript vector. Both cloned fragments first partially digested using the *Hind*III restriction enzyme, and the first part of the gene was also digested using *Ssp*I; importantly, both of these enzymes leave the residual *aggLb* gene in frame. After obtaining different variants of both fragments they were combined to obtain constructs with different numbers of collagen binding and Cnab-like domains. The construct pPI1E did not contain any collagen binding domains and contained only two Cnab-like domains, whereas pPIAggLb contained the complete *aggLb* gene. For details of all the constructs see **Figure 1** and **Table 1**. All the different combinations were recloned into the pAZIL vector using the lactococcal promoter *PlsB* to provide identical transcription activity of all the constructs (Uzelac et al., 2015). The constructs (**Figure 1**; **Table 1**) were transformed into *Lc. lactis* subsp. *lactis* BGKP1-20 (the lactococcal derivative BGKP1-20 was used because the original lactobacilli strains had an extremely low efficiency of transformation) and expression was analyzed by Dot blot (**Figure 2**) using an anti-AggLb antibody raised against the transitional region covering the last part of the first region and the beginning of the second subclone of AggLb because this part is present in all of the constructs. Similar expression was obtained for all of the constructs regardless of the length of the protein (34.2 kDa pPI1E, 63.9 kDa pPI1D, 65.0 kDa pPI2E, 87.6 kDa pPI3E, 94.8 kDa pPI2D, 117.3 kDa pPI3D, 132.0 kDa pPI3C, 139.3 kDa pPI2B, 145.5 kDa pPI4E, 207.3 kDa pPI1A, and 318.6 kDa pPIAggLb). In addition, the hybrid molecule pKP-Lb (314.2 kDa), consisting of the first part of the lactococcal *aggL* gene from *Lc. lactis* subsp. *lactis* BGKP1 (Kojic et al., 2011) as a *Pvu*I-*Pst*I fragment and a second part of the lactobacilli *aggLb* gene from *L. paracasei* subsp. *paracasei* BGNJ1-64 as a *Pst*I-*Sal*I fragment, was constructed (**Figure 1D**; **Table 1**). All of the variants constructed were used for functional assays in order to determine the role of various domains of the AggLb aggregation protein. The correct in-frame joining of all the fragments was confirmed by DNA sequencing and expression analysis using a Dot blot (**Figure 2**; Supplementary Figure 2).

Auto-Aggregation Ability of Transformants Carrying Different Variants of the *aggLb* Gene

The auto-aggregation ability of the wild-type strain and of the derivatives harboring the different variants of *aggLb* in the *Lc.*

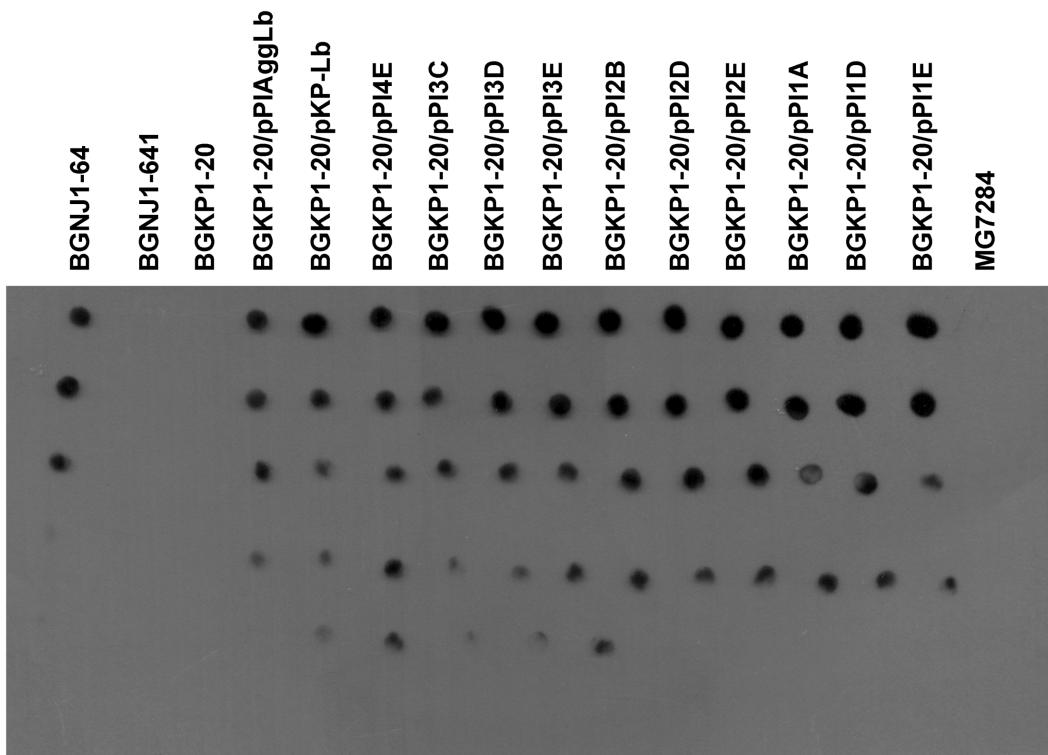


FIGURE 2 | Dot blot using anti AggLb antibody. Total proteins of the wild-type strain and of derivatives harboring the different *aggLb* variants in *Lc. lactis* subsp. *lactis* BGKP1-20 strain.

lactis subsp. *lactis* BGKP1-20 (see above) was measured for a period of 5 h, and the results are presented in Supplementary Table 1. We concluded that only the constructs carrying all six collagen binding domains and the first two CnaB-like domains were able to strongly auto-aggregate (BGKP1-20/pPI4E; **Figure 3**; Supplementary Table 1). Alternatively, the absence of the other CnaB-like domains, did not cause a significant effect on auto-aggregation (BGKP1-20/pPI3C, BGKP1-20/pPI3D, BGKP1-20/pPI3E, BGKP1-20/pPI2B, BGKP1-20/pPI2D, BGKP1-20/pPI2E, BGKP1-20/pPI1A, BGKP1-20/pPI1D, and BGKP1-20/pPI1E; **Figure 3**; Supplementary Table 1). It is also interesting to note that an additive effect dependent on the number of collagen binding domains on auto-aggregation was not linear, indicating that individual collagen binding domains do not have the same contribution. Careful observation revealed that the derivatives BGKP1-20/pPI2E, BGKP1-20/pPI1A, and BGKP1-20/pPI1E formed small aggregates (resembling sand or dust) that did not contribute to the rapid aggregation of the cells. Nevertheless, a negligible level of aggregation that was visible after overnight growth in a test tube was often observed in our collection of LAB. This observation may indicate a relationship between the type and number of collagen binding domains and/or CnaB-like domains within the aggregation factor(s) and the level or types of auto-aggregation. It was, therefore, concluded that the auto-aggregation ability of strains/derivatives was directly dependent on the collagen binding domains, while the 18 C-terminal CnaB-like domains were not required for

auto-aggregation. Transformants of *Lc. lactis* subsp. *lactis* BGKP1-20 carrying the hybrid construct pKP-Lb composed of the first part of the *aggL* gene (carrying three collagen binding domains originating from the *Lc. lactis* subsp. *lactis* BGKP1) and the second part of the *aggLb* gene were unable to form big aggregates, which indicated that the resulting hybrid molecule was not functional in strong auto-aggregation, collagen, or fibronectin binding (BGKP1-20/pKP-Lb; **Figures 3–5**) as wild-type strains (*L. paracasei* subsp. *paracasei* BGNJ1-64 and/or *Lc. lactis* subsp. *lactis* BGKP1).

Collagen and Fibronectin Binding Ability of the Transformants Carrying Different Variants of the *aggLb* Gene

In our previous studies, we found that isolates carrying the *aggL* or *aggLb* genes exhibited a direct correlation between auto-aggregation and their collagen binding ability (Miljkovic et al., 2015). All domain variants of the *aggLb* gene constructed in this study were tested for the ability to bind to collagen and fibronectin. Transformants carrying the different constructs adhered to immobilized collagen (**Figure 4**) and fibronectin (**Figure 5**) to different extents. Significant differences in the adherence to immobilized collagen and fibronectin were apparent between aggregation-positive strains (*L. paracasei* subsp. *paracasei* BGNJ1-64 and *Lc. lactis* subsp. *lactis* BGKP1) and their aggregation-negative derivatives (*L. paracasei* subsp.

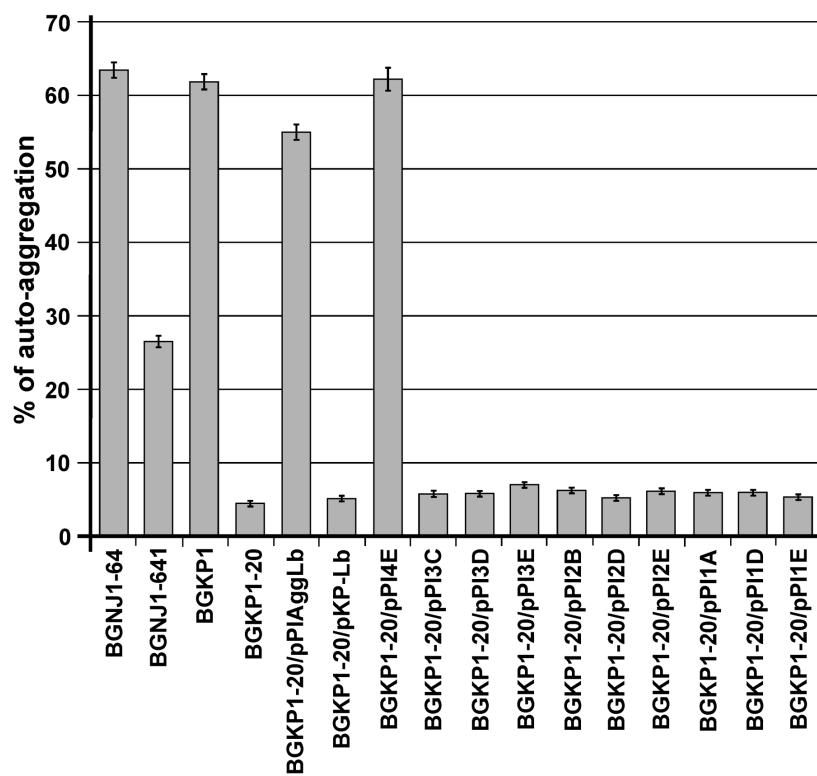


FIGURE 3 | Comparison of the auto-aggregation ability of the wild-type strain and of derivatives harboring the different *aggLb* variants in *Lc. lactis* subsp. *lactis* BGKP1-20 strain after 5 h incubation at 30°C. Auto-aggregation ability is expressed as percentages. The error bars represent standard deviations of three independent observations.

paracasei BGNJ1-641 and *Lc. lactis* subsp. *lactis* BGKP1-20) and also between strains carrying the first part of the *aggLb* gene (consisting of six collagen binding domains and the first two CnAB-like domains; BGKP1-20/pPIAggLb, BGKP1-20/pPI4E) and those variants that had only two or fewer collagen binding domains; these results indicate a role of the collagen binding domains in the interaction with collagen and fibronectin, but the last 18 CnAB-like domains are not indispensable (Figures 4 and 5). As observed in other experiments reported in this study (see above), we noticed that the additive effect dependent on the number of collagen binding domains was much lower than the impact of the specific collagen binding domains (II, III, and IV). The specific binding of AggLb to collagen and fibronectin was dependent on the collagen binding domains in a manner similar to the auto-aggregation ability. It appears that all the three phenotypes (auto-aggregation, collagen and fibronectin binding) are determined by the presence of the same structures of the AggLb protein such as the collagen binding domains.

Biofilm Formation of the Transformants Carrying Different Variants of the *aggLb* Gene

We determined the role of the AggLb in biofilm formation. Its ability to form biofilms was tested in the wild-type strain, aggregation deficient derivatives and transformants carrying

different variants of *aggLb* using the adherence of the cells to the surfaces of microtiter plates. The strongest biofilm formation was observed for the transformant carrying the construct pPI2D, followed by pPI3C, pPI3D, and finally, pPI3E (Figure 6). A comparative analysis of the variants led to the conclusion that the biofilm formation ability has a negative correlation with auto-aggregation, collagen, and fibronectin binding. It appears that the presence of collagen binding domains determines the formation of certain structures on AggLb that play a role in the interaction with collagen and fibronectin, but simultaneously enable the cells to auto-aggregate (pPI4E). Most likely, the absence of the collagen-binding domain (especially II, III, and IV) allows other structures to come to the fore (i.e., they are unmasked) which promotes biofilm formation. The difference between pPI1D and pPI2D is limited to the presence of a sixth collagen binding domain of AggLb in pPI2D (Figure 1; Table 2); thus, this result indicates that this domain is probably required in combination with the other domain(s) to allow biofilm formation.

Relationships between Collagen/Fibronectin Binding and Biofilm Ability of Transformants Carrying Different Variants of the *aggLb* Gene

We established correlations between auto-aggregation, collagen/fibronectin binding and biofilm formation ability

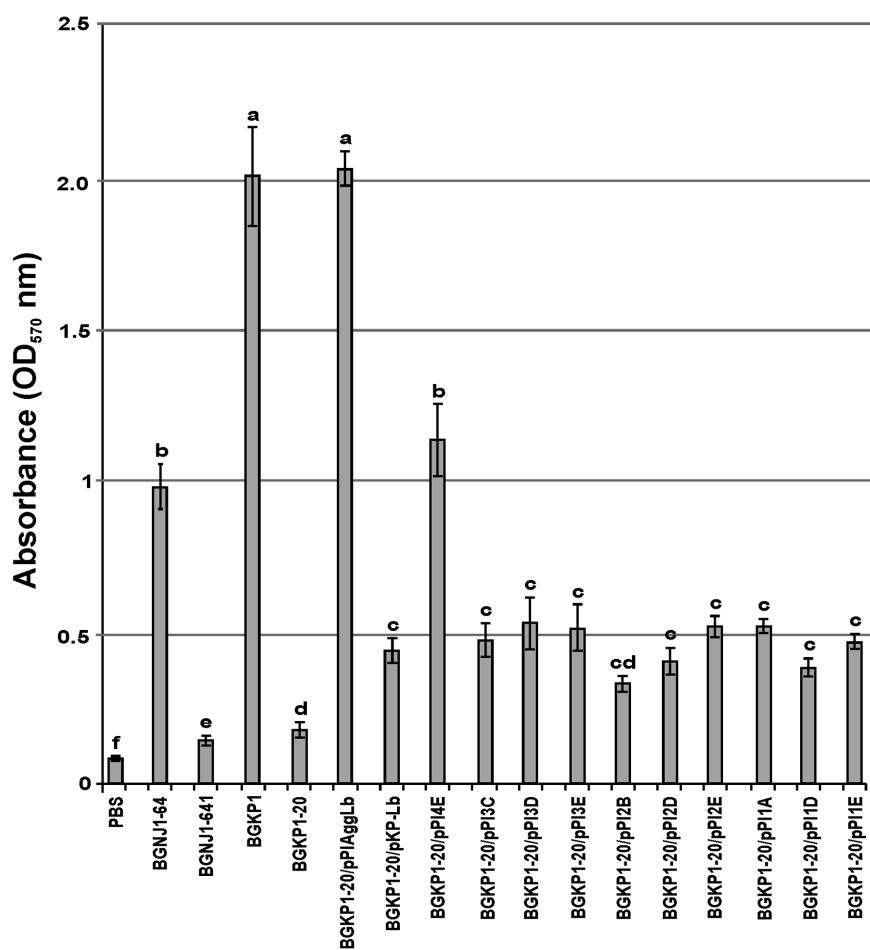


FIGURE 4 | Graphical presentation of results obtained in collagen-binding assay of selected strains and derivatives to immobilized collagen in microtiter plates. Results were expressed as average of normalized A₅₇₀ values. The error bars show the standard deviations. In each column, the values with different superscript letters differ significantly ($p < 0.001$).

of transformants carrying different variants of the *aggLb* gene. A comparative analysis of the variants led to the conclusion that the biofilm formation ability has a negative correlation with auto-aggregation – R² squared 0.312 (Supplementary Figure 3A), binding to collagen – R² squared 0.260 (Supplementary Figure 3B), binding to fibronectin – R² squared 0.242 (Supplementary Figure 3C). In addition using Python 2.7.8 and scipy library (version 0.14.0) we proved positive correlation between auto-aggregation and collagen binding – R² squared 0.652 (Supplementary Figure 3D) and aggregation and fibronectin binding – R² squared 0.636 (Supplementary Figure 3E).

DISCUSSION

The adhesion of lactic acid bacteria to epithelial and mucosal surfaces is thought to be a rather complex process involving many different factors (Buck et al., 2005). The ability of lactobacilli to aggregate has been linked to their role as probiotic factors

(García-Cayuela et al., 2014). The data of the literature suggest that the Apf-like proteins may contribute to the survival of *L. acidophilus* during its transit through the digestive tract and, potentially, may participate in the interactions with the host intestinal mucosa (Goh and Klaenhammer, 2010). Considering the importance of aggregation phenomena for human health, the experiments described in this study were mainly focused to determine the contribution of the different domains and repeats of the AggLb protein on the modulation of the aggregation phenotype. Additionally, our results have proven the existence of a direct relationship between strong auto-aggregation, collagen or fibronectin binding and biofilm formation.

Biofilms of lactobacilli can be found in many natural environments (Lebeer et al., 2007). Because the gastrointestinal tract is an important target for probiotics, some factors related to this niche have been investigated in the past decade. It was of interest to study the possible relationship between aggregation ability and biofilm formation. It has been reported that the agglutination protein AggA is required for the aggregation and increased biofilm formation of a hyper-aggregating mutant

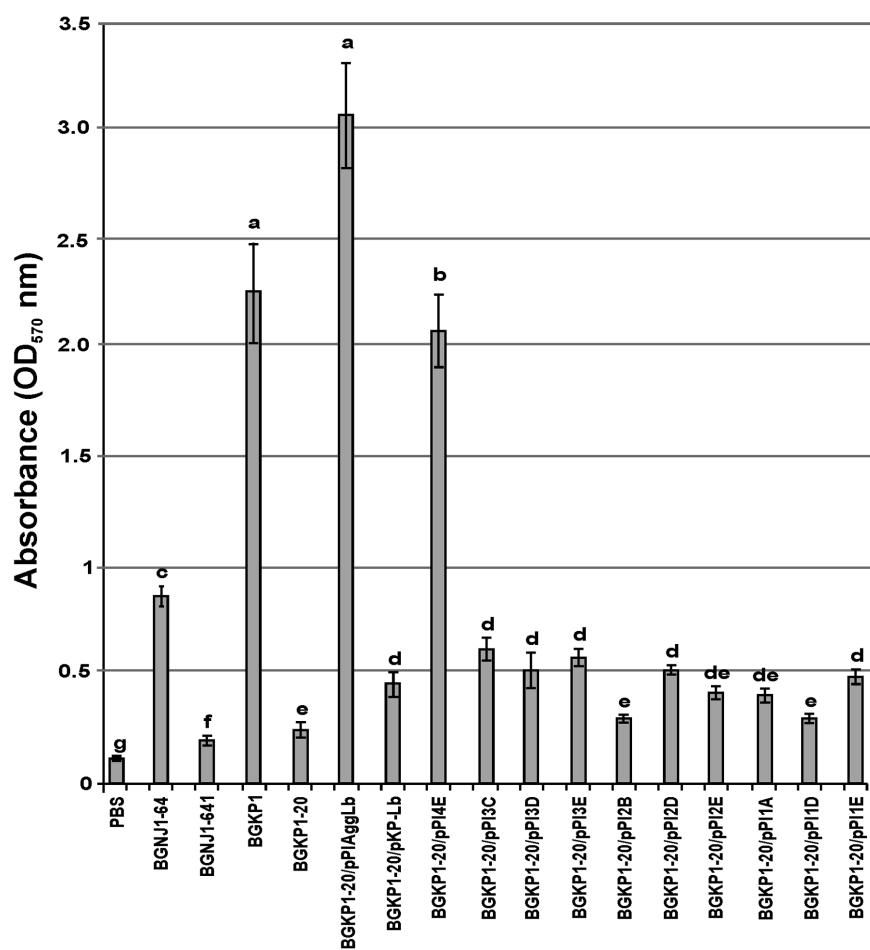


FIGURE 5 | Graphical presentation of results obtained in fibronectin-binding assay of selected strains and derivatives to immobilized fibronectin in microtiter plates. Results were expressed as average of normalized A₅₇₀ values. The error bars show the standard deviations. In each column, the values with different superscript letters differ significantly ($p < 0.001$).

of *Shewanella oneidensis* MR-1 (De Windt et al., 2006). An insertional mutant of *aggA* resulted in the loss of aggregation properties and ability to form a biofilm. Additionally, the SasC protein of a pathogenic *S. aureus* strain was involved in cell aggregation, biofilm formation and colonization during infection. The N-terminal domain of the SasC protein was involved in the production of large cell aggregates, in the attachment to polystyrene, and in increased biofilm formation (Schroeder et al., 2009). Aggregation and biofilm formation are multicellular processes that allow a community to be more resistant to stress conditions. Given that these are similar processes, it is not surprising that the same protein may be involved in both functions. Since biofilm formation is important in food spoilage and pathogenic bacteria because it results in high resistance to different treatments, it is important to identify and characterize the active components that could inhibit bacterial biofilm formation (Söderling et al., 2011; Furukawa, 2015).

The ability to strongly aggregate and adhere to collagen and fibronectin is inversely correlated with the biofilm formation,

(if the ability to strongly aggregate and bind collagen and fibronectin is stronger the ability of biofilm formation is less; Figures 3–5; Supplementary Figure 3). Therefore, it seems that the lack of collagen binding domains II, III, and IV in the AggLb protein results in the reduced auto-aggregation, collagen and fibronectin binding and increases the propensity of the cells to form a biofilm. A comparative regression analysis of AggLb variants containing a constant number of CnaB-like domains and a different number of collagen binding domains (pPI4E, pPI3E, pPI2E, and pPI1E; pPI3D, pPI2D, and pPI1D; Figures 4 and 5) showed a correlation of binding to collagen or fibronectin, and an increase in biofilm formation (Supplementary Figure 3).

Our results indicate that the region responsible for the strong auto-aggregation, collagen and fibronectin binding is located on the N-terminus of the AggLb aggregation protein; transformants that carried the construct pPI4E, which contained only the N-terminal part, exhibited a strong aggregation capability, as did as clones that harbored the complete gene. Deletion studies of the AggLb protein showed that all three

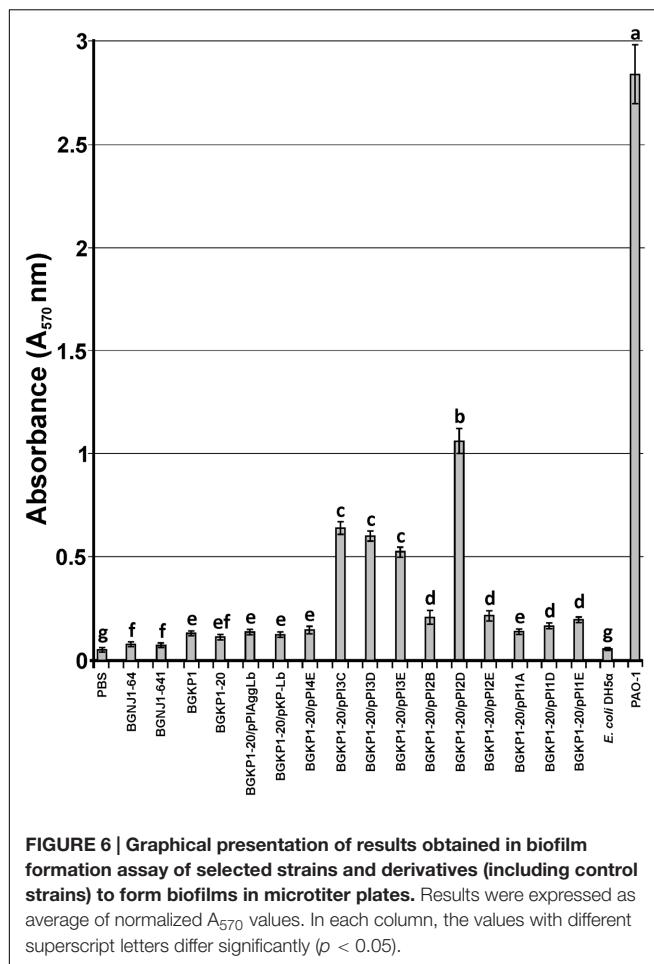


FIGURE 6 | Graphical presentation of results obtained in biofilm formation assay of selected strains and derivatives (including control strains) to form biofilms in microtiter plates. Results were expressed as average of normalized A_{570} values. In each column, the values with different superscript letters differ significantly ($p < 0.05$).

TABLE 2 | Representation of domain organization series of AggLb variants.

Name of construct	No. of collagen binding domains	No. of CnaB like domains	Molecular mass of expressed protein (kDa)
pPIAgLb	6	20	318.6
pPI4E	6	2	145.5
pPI3C	2 (hybrid of I-V, and VI)	7	132.0
pPI3D	2 (hybrid of I-V, and VI)	5	117.3
pPI3E	2 (hybrid of I-V, and VI)	2	87.6
pPI2B	1 + 1/2 (1/2 of V and VI)	10	139.3
pPI2D	1 + 1/2 (1/2 of V and VI)	5	94.8
pPI2E	1 + 1/2 (1/2 of V and VI)	2	65.0
pPI1A	0	20	207.3
pPI1D	0	5	63.9
pPI1E	0	2	34.2

functions dependent on the collagen binding domains II, III, and IV, and their deletion leads to a complete loss of strong aggregation ability. These three domains are critical for function of AggLb in strong auto-aggregation, binding to collagen and fibronectin, either through direct and specific interaction with proteins of the matrix or by changing the properties of the cell surface. Multiple CnaB-like domains

likely function as an antenna which exposes the collagen binding domains to the surface to improve target protein interactions. The CnaB-like domains in AggLb cannot be considered as the domains responsible for the direct interaction with collagen or fibronectin, but they can strengthen the interaction between the collagen binding domains and collagen or fibronectin. Also, we noted that because the first and last CnaB-like domains had sequence heterogeneity compared to the other 18 domains, it is possible they may have a different but not strong effect on AggLb function. We can conclude that the presence of the collagen binding domains predominantly determined the adhesive function of the AggLb protein. In addition, combination of domains from lactobacilli (AggLb) and lactococci (AggL; hybrid molecule – BGKPI-20/pKP-Lb) did not result in functional protein in strong auto-aggregation, collagen, or fibronectin binding. The results obtained in this study have demonstrated that a protein may exert different functions depending on physicochemical properties of the bacterial surfaces, and this probably depends on the structure and conformation variants of AggLb. The removal of certain domain(s) not only eliminated certain functions but also resulted in other domain(s) coming to the fore and allowing the protein to assume another function. In our previous publication we have noticed one strain BGGR2-68 that simultaneously exhibits both functions strong auto-aggregation and biofilm formation (Miljkovic et al., 2015). It would be interesting to determine whether these two functions in this strain are associated with one the same protein or independent. This will be the subject of further research.

These results bolster the hypothesis that in the *S. aureus* collagen-binding Cna protein, the collagen binding A region is responsible and sufficient for collagen binding, while the B region aids as a “stalk” that projects the A region from the bacterial surface to facilitate the bacterial adherence to collagen. Such a B region assembly could result in flexibility, stability, and positioning the ligand-binding A region away from the bacterial cell surface (Deivanayagam et al., 2000). The difference between AggLb and the Cna protein is that the aggregation promoting factor contains repetitive collagen binding domains (six very heterogeneous units with less than 26% identity) that have different contributions to strong auto-aggregation, collagen, and fibronectin binding (II, III, and IV showed the most significant effects), as well as to biofilm formation. It is important to note that even if AggLb is composed of two collagen binding domains, it is not able to provide strong auto-aggregation. In contrast in Cna, this is accomplished with a single domain, indicating that it is important which of the domains is/are present.

AUTHOR CONTRIBUTIONS

MK conceived, designed, and coordinated this study, interpreted all of results and contributed to the preparation of the figures and wrote this paper. MM designed, performed, analyzed the experiments and wrote this paper. BJ and KN provided

experimental assistance and contributed to the preparation of the figures. IB performed one part of experiments of production polyclonal antibody. DF and VV provided technical assistance and contributed to the preparation of this paper. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of Interest Statement: 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.

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Flow Cytometric and 16S Sequencing Methodologies for Monitoring the Physiological Status of the Microbiome in Powdered Infant Formula Production

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The aim of this study was to develop appropriate protocols for flow cytometric (FCM) and 16S rDNA sequencing investigation of the microbiome in a powdered infant formula (PIF) production facility. Twenty swabs were collected from each of the three care zones of a PIF production facility and used for preparing composite samples. For FCM studies, the swabs were washed in 200 mL phosphate buffer saline (PBS). The cells were harvested by three-step centrifugation followed by a single stage filtration. Cells were dispersed in fresh PBS and analyzed with a flow cytometer for membrane integrity, metabolic activity, respiratory activity and Gram characteristics of the microbiome using various fluorophores. The samples were also plated on agar plates to determine the number of culturable cells. For 16S rDNA sequencing studies, the cells were harvested by centrifugation only. Genomic DNA was extracted using a chloroform-based method and used for 16S rDNA sequencing studies. Compared to the dry low and high care zones, the wet medium care zone contained a greater number of viable, culturable, and metabolically active cells. Viable but non-culturable cells were also detected in dry-care zones. In total, 243 genera were detected in the facility of which 42 were found in all three care zones. The greatest diversity in the microbiome was observed in low care. The genera present in low, medium and high care were mostly associated with soil, water, and humans, respectively. The most prevalent genera in low, medium and high care were *Pseudomonas*, *Acinetobacter*, and *Streptococcus*, respectively. The integration of FCM and metagenomic data provided further information on the density of different species in the facility.

Keywords: powdered infant formula (PIF), systems microbiology, flow cytometry, 16S sequencing, viable but non-culturable (VBNC), microbial stress response, environmental sampling, microbial physiology

INTRODUCTION

Currently, culture-based methods such as agar plates are the most commonly used technique for assessing the microbiological status of food processing environments. In using agar plates, it is only possible to determine the presence of bacteria that the investigator is looking for (using selective agars) and the method gives little information on the physiological status of the cells, except that they are alive if they grow. Some additional information can also be obtained by using different nutrient-content agars and assessing the number of stressed cells. With advances in microbiological techniques, there are a number of different methodologies that can be applied to samples to gain more information about the bacterial population of a processing environment and the physiological state of the bacteria present. Two of these methodologies are 16S rDNA sequencing and flow cytometry (FCM), the former giving information about the type of bacteria present and the latter providing information on the physiological state of the bacteria. Combining the results of these two methods can give valuable information about the microbiological status of a food processing environment.

Though most micro-organisms are cultured using traditional culture-based methods, under stress conditions, many microorganisms, some yet unidentified, are unable to grow on conventional growth media due to lack of effective culture techniques and/or induction of the so-called viable but non-culturable (VBNC) state (see Quigley et al., 2011; Ramamurthy et al., 2014). This has made normal culture techniques ineffective in describing the entire microbiome of complex environments. This problem could be overcome by using 16S rDNA sequencing, which is a culture-independent next generation sequencing method and has been successfully used for describing the composition of the microbiome in depth. The 16S rDNA gene encodes for 16S ribosomal RNA which is universally present in prokaryotic microorganisms (Coenye and Vandamme, 2003). The variations within the 16S rDNA sequences facilitate identification of bacteria at the species level and, therefore, has made 16S rDNA gene sequencing an ideal tool for bacterial taxonomic studies (Neefs et al., 1993). Even though 16S rDNA gene sequencing is widely used to identify inter-species variations, the development of modern high throughput sequencing technologies combined with downstream bioinformatics analysis has made this tool ideal for species identification within complex communities (Logares et al., 2014). It has been shown that 16S rDNA amplicon fragments as short as ~82 base pairs are sufficient for classification at the phylum level (Lazarevic et al., 2009), and with good primer design and analysis methods, fragments of 100–200 base pairs could show the same clustering information as long fragments used in phylogenetic studies (Liu et al., 2007). Compared to other molecular microbiology techniques, 16S rDNA sequencing is not only cheaper in price, but also the interpretation of the resulting data is easier and faster. With some online analysis platforms, such as Illumina Basespace or software such as QIIME and MOTHUR (Schloss et al., 2009; Caporaso et al., 2010) one could easily generate very straightforward information and give an overview about detailed structures and

compositions of the target environment microbiome. However, such methodologies will detect gene sequences and will not differentiate live from dead cells.

Flow cytometry is a powerful and rapid technique for simultaneous quantification and multi-parameter analysis of the microbial populations at single cell level (Müller and Nebe-Von-Caron, 2010). Cells are focused and aligned one behind the other in a narrow stream with a diameter close to the diameter of the cells so that single cells can be introduced to the light beam (generally laser). When cells are subjected to the light, they scatter light in all directions, although it is generally detected in two directions: forward scatter (FSC), along the axis of the light source; and side scatter (SSC), perpendicular to the light beam. Data from FSC and SSC scatters are generally used to characterize the morphological state of the cells, as rough indicators of the cell size and granularity, respectively. In addition, the light absorbed by the cells can result in emission of fluorescence (either due to presence of naturally fluorescent compounds or staining with various fluorophores), the intensity of which could be detected by FCM (Shapiro, 2003). Consequently, staining the cells with various fluorophores or fluorescence-conjugated antibodies can be used for understanding a wide range of physiological parameters of the cell (e.g., viability, metabolic and respiratory activities, internal pH, etc.) as well as detection of specific microorganisms at an analysis rate of up to 10,000 cells per second. Furthermore, comparing the viability results obtained with FCM with those of the plate counting can be used to determine the number of VBNC cells in a sample.

The aim of this study was to develop appropriate protocols for flow cytometry and 16S rDNA sequencing investigation of the environmental microbiome in a powdered infant formula (PIF) production facility in the Republic of Ireland.

MATERIALS AND METHODS

Sampling

The PIF production unit consisted of three care zones of low, medium and high care. From each zone, twenty swab samples were collected, representing the critical production, storage and packaging sites within each PIF production zone (see Supplementary Table 1). Sponges pre-moistened with neutralizing buffer (Labplas Inc., Sainte-Julie, Canada) were used for environmental swabbing. Each hydrated sponge was used for swiping a single sampling zone of 50 × 50 cm. The zigzag wiping procedure for surface sampling was performed as described by Nicolau and Bolocan (2014). In total, the twenty samples taken represented 5 m² of that zone and were placed into five bags, each consisting of four sponges. To each bag, 40 mL of phosphate buffered saline (PBS; Sigma, St Louis, USA) was added not earlier than 30 min post-sampling. The purpose of the delay was to prevent the dilution of the neutralizing buffer, allowing the effective neutralization of the possible chlorine and quaternary ammonium compound residues in the sample. The bags were sealed with the tabs provided, kept on ice, transported to the laboratory and processed within 24 h. The sampling procedure and the overall protocol used in this study is schematically represented in **Figure 1**.

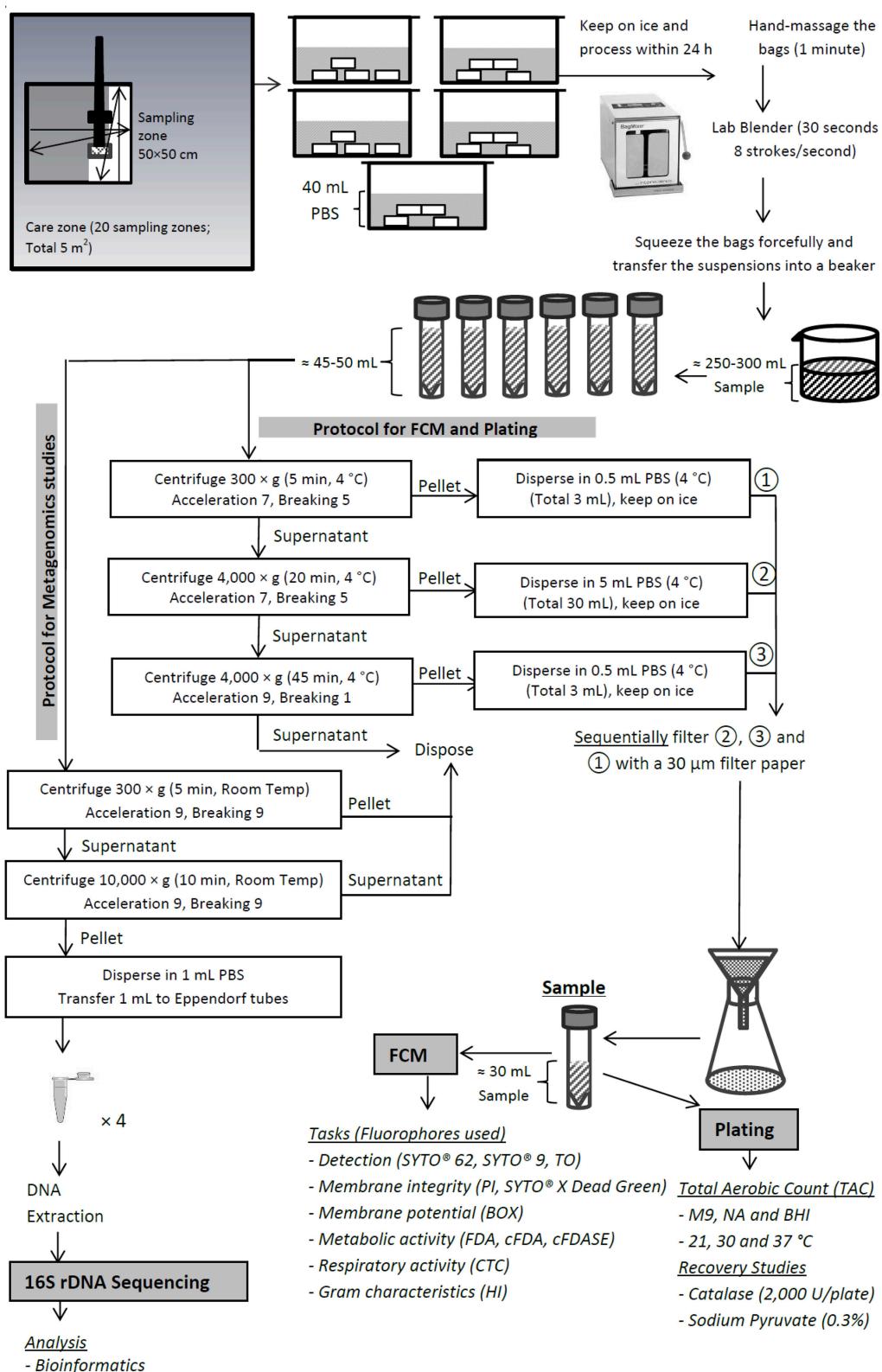


FIGURE 1 | Schematic representation of the steps involved in sampling and sample preparation for flow cytometric and 16S rDNA sequencing studies.

Sample Preparation

Sample Preparation for Flow Cytometric and Plating Studies

The bags were massaged vigorously by hand for 1 min and subsequently stroked in a lab blender (BagMixer® 400 P, Interscience, Saint Nom la Bretèche, France) at a fixed speed of 8 strokes/s for 30 s to release the cells into the PBS dispersion medium. The sponges were then forcefully squeezed by hand and the resultant suspension was transferred into a sterile beaker, representing three composite samples, one from each zone. Clarification of the suspension and separation of cells from debris were achieved by three-step centrifugation followed by a single-step filtration. The cell suspension from each composite sample was first sub-sampled into six polypropylene conical skirted centrifuge tubes (Sarstedt, Wexford, Ireland) and centrifuged at 300 ×g for 5 min at 4°C using a Sorvall Legend RT centrifuge (Thermo Electron Corporation, Waltham, USA) to remove the large dust particles and debris. The supernatant was subsequently transferred to a clean centrifuge tube and centrifuged at 4000 ×g for 20 min in order to harvest the cells. Finally, the supernatant was transferred again to a clean tube and centrifuged at 4000 ×g for 45 min to harvest the possible remaining cells. For the first two steps of centrifugation, the acceleration and breaking were set at 5 and 7, respectively, while for the last step they were set at 9 and 1, respectively. The pellets from steps one, two and three of centrifugation were dispersed in 0.5, 5, and 0.5 mL of 4°C PBS, respectively. The dispersed pellets of the second, third and first stages of centrifugation were then filtered sequentially through sterile Whatman™ Grade 113V prepleated qualitative filter papers (GE Healthcare, Little Chalfont, UK) with pore size of ~30 µm. This was done to ensure that the final sample was free from large particles which could block the fluidics system of the flow cytometer. The filtrate (final sample volume of ~32 mL) was then transferred into a clean centrifuge tube and kept at 4°C before analysis within 24 h.

Sample Preparation for 16S Sequencing Study

For 16S sequencing studies, the sample preparation was similar to that mentioned above with minor modifications. For each centrifugation, 50 ml of the suspension was used. Following the first centrifugation step (300 ×g for 5 min at 4°C) and the removal of large dust particles and debris, the supernatant was transferred to a new 50 mL centrifuge tube and centrifuged at 10,000 ×g for 10 min at 4°C to harvest the cells. For both steps of the centrifugation, the acceleration and breaking were set at 9. The supernatant was discarded and pellet was resuspended in 1 ml sterile PBS and transferred to a new 1.5 ml microcentrifuge tube. The two centrifuge steps were repeated until all the suspension in the sampling bag was centrifuged. The cell suspensions were stored at -80°C until analyzed.

Flow Cytometric Study

Instrument

A BD FACSCanto II flow cytometer (BD Bioscience) equipped with green (488 nm air-cooled solid state; 20 mW laser output) and red (633 nm HeNe; 17 mW power output) lasers was used

in this study. The fluorescence detector/filters relevant to this study were FITC (FL1; 530 ± 30 nm), PerCP-Cy5.5 (FL3; > 670 nm), APC (FL5; 660 ± 20 nm), and APC-Cy7 (FL6; 780 ± 60 nm). FSC and SSC detectors were also used for determining the light scatter parameters of forward scatter and side scatter, respectively. The instrument was cleaned before and after use and its performance was validated according to the manufacturer's instructions. In order to differentiate between the background noise and the signal (particles of interest), the threshold channel number was set at 200 on FSC. The number of background events (noise) detected by the instrument upon analysis of deionized water (deH₂O) at a fast flow rate (~80 µL/min) was less than 4 events/min (approximately less than 1 noise particle per 20 µL deH₂O) (data not shown). For each parameter, the height, width and area (integral) of the voltage pulses of each event was measured and recorded.

Determination of the Flow Rate

The exact flow rate of the instrument was determined on the day of the experiment. Ten flow tubes (Sarstedt) were filled with 1 mL of deH₂O. Each sample was acquired on the flow cytometer for 30 to 600 s at low, medium or high flow rate settings. The weights of the tubes were determined before and after analysis with the help of an analytical balance with accuracy of ± 0.001 g (Denver instruments, Göttingen, Germany). Assuming the density of deH₂O to be 1000 kg/m³, a calibration curve was generated by plotting time vs. volume, to calculate the flow rate (µL/s) of the instrument. Therefore, by knowing the time required for recording a certain number of cells within a sample, it was possible to determine the volume, hence the flow rate of the instrument using Equation 1 (see Supplementary Figure 1).

$$\text{Flow rate } \left(\frac{\mu\text{L}}{\text{min}} \right) = \frac{\frac{\text{Number of beads counted} \times \text{Sample volume (1,000 }\mu\text{L)}}{\text{Acquisition time (1 min)} \times \text{number of beads added (5,110 beads)}}}{(1)}$$

Identification of the Cells and Gating Strategy

Depending on the parameter and/or the type of fluorophore used in the study, the Photomultiplier tube (PMT) voltage for each parameter was adjusted so that the cells could be displayed in the center of the investigating plot. The samples were diluted to ensure that the flow rate was between 800 and 1200 events/s at medium speed setting (~45 µL/min). After displaying the data in a density plot of FSC vs. SSC and identifying the particles of interest, the latter was defined by creating a gating region (P1) around it, based on the light scatter properties of the particles (Plot a[1] and b[1]; Figure 2).

The defined gated population of P1 contained not only the presumed cells but also acellular particulates. Therefore, in order to differentiate between the two and detect the cells of interest, samples were stained with ~162 nM SYTO62 (Molecular Probes, Eugene, USA). This dye is a cell-permeant nucleic acid stain which is capable of staining most live and permeabilized (i.e., dead) bacteria. Its maximum excitation and emission wavelengths (λ_{max}) are 652 and 676 nm, respectively.

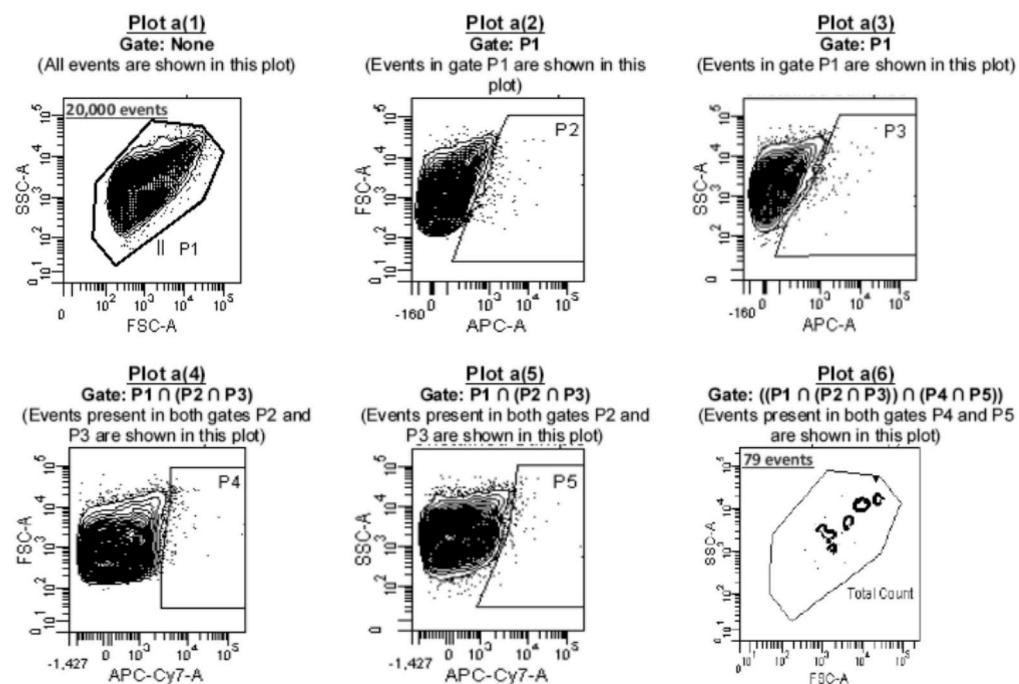
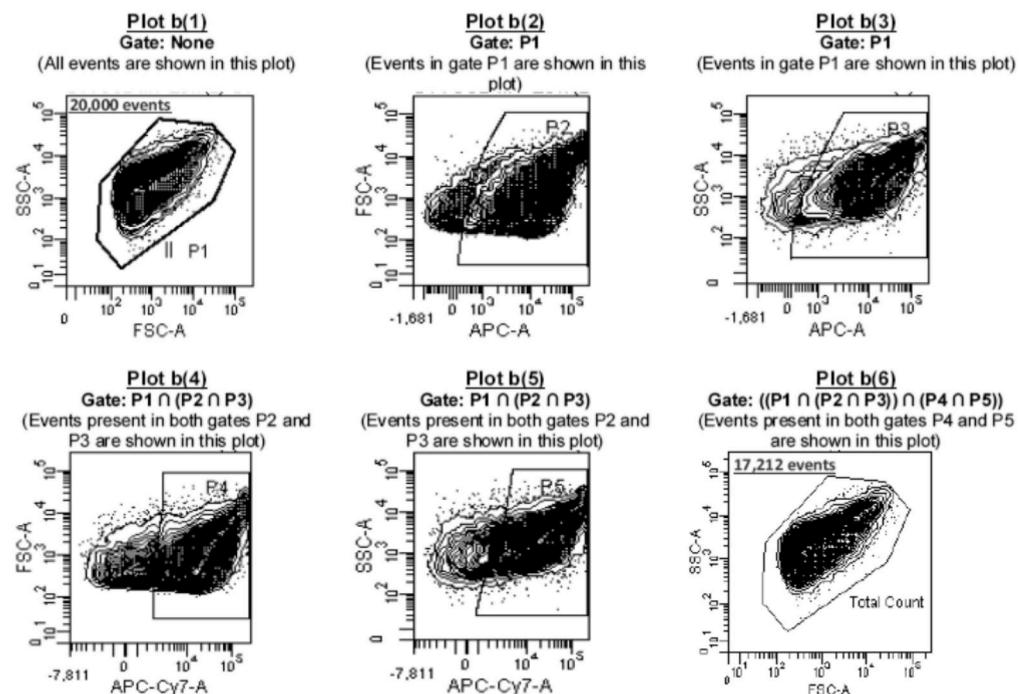
A Unstained Samples**B Stained (with SYTO® 62)**

FIGURE 2 | Gating strategy used in this study. Cells were acquired (**A**) before and (**B**) after staining with SYTO 62 dye. Based on Boolean logic, the events recorded within P1 (20,000 events) were passed through a series of gates (P2-P5) as shown in plots a(2)-a(5) (for unstained cells) and b(2)-b(5) (in the case of stained cells) to determine the number of noise particles (particles in gate P6 of plot a[6]; i.e., 79 noise particles) as well as cells of interest (particles in gate P6 of plot b[6] minus those shown in gate P6 of plot a[6], i.e., 17212–79 = 17133 cells).

Considering the fact that SYTO62 was the only fluorophore used in this study that could primarily be excited by the red-laser, this made it possible to make exclusive use of the red-laser and its two detectors, FL5 and FL6 for detection of SYTO62-positive particles, hence the cells of interest. The principle behind the multi-gating strategy used in this study and using both FL5 and FL6 for detecting SYTO62 positive particles was based on the one reported by Buzatu et al. (2014). As the λ_{max} emission of SYTO62 is 676 nm, it is primarily detected by FL5; however, due to its broad emission spectrum (620–800 nm), its fluorescence could also be detected by FL6 detector (with relative fluorescence intensity of less than 24% compared to the λ_{max} emission wavelength). The gating strategy used in this study is described in details in Supplementary Data 2.

Determination of Cell Density and Signal to Noise (S/N) Ratio

The cell density in the sample was calculated using the following equation:

$$\text{Cell density} \left(\frac{\text{Cells}}{\text{cm}^2} \right) = \frac{\left[(P6_{(\text{Stained})} - P6_{(\text{Unstained})}) \text{ (Cells)} \right] \times \text{Sample volume (mL)} \times 1000 \left(\frac{\mu\text{L}}{\text{mL}} \right) \times 60 \left(\frac{\text{s}}{\text{min}} \right)}{\text{Flow rate} \left(\frac{\mu\text{L}}{\text{min}} \right)^* \times \text{Acquisition time (s)} \times \text{Area sampled (cm}^2\text{)} \times \text{dilution factor}} \quad (2)$$

$P6_{(\text{stained})}$ and $P6_{(\text{unstained})}$ refer to the number of events within gate P6 for stained and unstained samples, respectively. By knowing the number of events for $P6_{(\text{unstained})}$ (i.e., noise particles), it was also possible to determine the S/N ratio by using

the following equation:

$$\begin{aligned} \text{Signal to noise ratio} & \left(\frac{S}{N} \right) = \\ & \frac{\text{Number of signal events (cells) within P6}}{\text{Number of noise events within P6}} \end{aligned} \quad (3)$$

Physiological Studies and Staining Strategy

Considering the heterogeneity of the microbiome in environmental samples and the variations between the stainability of different microorganisms with various dyes, it was decided to take a holistic multi-staining approach for studying the physiological status of the cells by staining the samples with a wide range of fluorescent dyes. Immediately prior to staining, 250 μL of diluted sample was transferred to 12 \times 25 mm flow tubes and supplemented with 20 μL of filter-sterilized 100 mM EDTA (Sigma, Wicklow, Ireland) and 20 μL of 0.1% (v/v in deH₂O) polyoxyethylene sorbitan monolaurate (Tween[®] 20) (Sigma) to improve the stainability of the cells.

The staining protocol for all the fluorochromes used in this study is shown in Table 1. In summary, samples were first stained with 10 μL of 5 μM working solution of SYTO62 to differentiate between the cells of interest and acellular

TABLE 1 | The staining protocol used in this study.

Fluorophore	Concentration of the working solution	Volume (μL) of working solutions added to the sample	Final concentration (M) of the dye in the sample*†	Incubation Time and temperature (post-staining, pre-analysis)**
SYTO [®] 62	5 μM	10	166.67 nM ⁽¹⁾ 163.93 nM ⁽²⁾ 161.29 nM ⁽³⁾ 158.70 nM ^(4, 5, 6, 7)	30 min (RT)
PI	299.23 μM	10	9.50 mM ^(4, 5, 6, 7)	5 min (RT)
BOX	19.36 μM	5	307.30 nM ⁽⁵⁾	5 min (RT)
SYTO [®] 9	250 μM	5	4.03 μM ⁽³⁾ 3.97 μM ⁽⁵⁾	15 min (RT)
TO	42 μM	5	666.67 nM ⁽⁶⁾	10 min (RT)
FDA	2.40 mM	5	39.37 μM ⁽²⁾	30 min (37°C)
cFDA	2.50 mM	5	40.98 μM ⁽²⁾	30 min (37°C)
cFDA-SE	25.11 nM	5	411.70 pM ⁽²⁾	30 min (37°C)
SYTOX Dead	3 μM	5	49.18 nM ⁽⁷⁾	20 min (RT)
HI	25.13 μM	5	405.32 nM ⁽³⁾	15 min (RT)
CTC	53.46 mM	30	4.86 μM ⁽⁸⁾	30 min (37°C)

RT: Room temperature (18 to 22°C).

*250 μL cell suspension, 20 μL of 100 mM EDTA, 20 μL of 0.1% Tween[®] 20.

**For instance, in the case of SYTO[®] 62/SYTO[®] 9/PI staining, cells were first stained with SYTO[®] 62 (at time 0 min), followed by staining with SYTO[®] 9 and PI at time 15 min and 25 min, respectively.

† The final concentration of the fluorochrome in the sample when stained with (1) SYTO[®] 62 only; (2) SYTO[®] 62/cFDA, SYTO[®] 62/cFDA or SYTO[®] 62/cFDA-SE; (3) SYTO[®] 62/SYTO[®] 9/HI; (4) SYTO[®] 62/PI/BOX; (5) SYTO[®] 62/PI/SYTO 9; (6) SYTO[®] 62/PI/TO; (7) SYTO[®] 62/PI/SYTO X[®]; and (8) SYTO[®] 62/CTC.

particulates, as previously described. Samples were then vortexed for 1–2 s and incubated at room temperature (18 to 22°C) in darkness for 30 min before analysis. Upon identification of the cells of interest, SYTO62-stained sub-samples were also stained with the following combination of dyes to determine the physiological status of the cells: (a) Propidium iodide (PI) and SYTOX Green Dead Stain to study the membrane integrity; (b) PI in combination with SYTO9 or thiazole orange (TO) to determine the membrane integrity and viability; (c) PI with DiBAC₄(3) (Bi-oxonol or BOX) to investigate the membrane potential and viability; (d) Fluorescein diacetate (FDA) and its derivatives [5-(and-6)-carboxyfluorescein diacetate (cFDA) and 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (cFDA-SE)] as indicators of esterase activity; (e) 5-Cyano-2,3-di-(p-tolyl)tetrazolium chloride (CTC) for semi-quantitative analysis of the respiratory activity; and (f) Hexidium iodide (HI) in combination with SYTO9 for Gram staining. All samples were protected from light during the staining process. The protocols used for preparing the fluorophore stock and working solutions as well as the rationale behind using each one is described in detail in Supplementary Data 1.

Color Compensation

Live and heat-killed samples of one Gram positive (*Lactobacillus rhamnosus* GG; LGG) and one Gram negative (*Escherichia coli*; EC) strain were used as compensation controls. The color compensation was performed for each fluorophore and its primary detector by first plotting a contour plot of the primary detector (the one used for measuring the fluorescence intensity of the fluorophore) vs. non-primary detector. The values for each fluorescence parameter on the plots were transformed bi-exponentially using the BD FACSDiva software version 6.1.3 (BD Biosciences). After gating the negative (non-fluorescent) and positive (fluorescent) cells, the median fluorescence intensity (FI) of the observed populations in primary and non-primary detectors was measured. When the FI of the positive cells in the non-primary detectors was larger than the one for negative cells, a percentage of the FI of the primary detector was subtracted from the affected non-primary FI in order to remove the spillover. The approximate values required for color compensation were calculated using the following equation:

$$\text{Spillover correction (\%)} = \frac{(\text{median FI of positive control} - \text{median FI of positive control})_{\text{non-primary detector}}}{(\text{median FI of positive control} - \text{median FI of positive control})_{\text{primary detector}}} \quad (4)$$

The color compensation was performed using the calculated values and verified by visualizing the effects of the applied values on the median FI of the non-primary detectors. If necessary, using the calculated value as a guide, an arbitrary percentage of the FI of the primary detector was subtracted from the affected non-primary FI until the median FI of the latter was relatively the same for both the positive and negative controls. The information on the control samples and the color compensation values used in this study could be shown in Supplementary Tables 2, 3, respectively.

Analysis and Display of FCM Data

The data were acquired and analyzed using BD FACSDiva software version 6.1.3. For FSC vs. SSC contour plots, the scale of the axis for each parameters was logarithmic. When samples were dual-stained (including SYTO62), histograms were used for displaying the FL1 (in the case of FDA, cFDA, cFDA-SE) or FL3 (for CTC). For histograms, the scales for the x-axis (fluorescence channel number) were transformed bi-exponentially (logical x-axis) while the y-axis (count) was displayed on a linear scale beginning at zero. In the case of triple-staining (including SYTO62), the channel numbers (x-axes) for all the fluorescence parameters were transformed bi-exponentially and plotted in two-dimensional quantile (probability) contour plots. All contour plots displayed the events with 99% probability as well as the outliers. The gates (rectangles, polygons, quadrants and vertical or horizontal markers) for each sub-population were set manually.

Plating Study

Growth Media

The growth media used in this study consisted of M9 minimal salt media agar (M9), nutrient agar (NA) (Oxoid, Altrincham, UK) and Brain Heart Infusion (BHI) agar (Sigma). M9, NA, and BHI were used as low, medium and rich nutrient media, respectively, and were used to investigate the effects of the nutrient content of the growth medium on recovery of cells from the processing environment samples. Unless stated otherwise, all media and diluents were prepared according to the manufacturer's instructions using distilled water and autoclaved at 121°C for 15 min. After autoclaving, the salt and agar solutions were allowed to cool to 50°C. The salt broth was then supplemented with 20 mL of 20% (w/v) D-(+)-glucose (Fisher Scientific, Loughborough, UK), 2 mL of 1 M MgSO₄ (Fisher Scientific), 0.2 mL of 0.5 M CaCl₂ (Reagecon, Shannon, Ireland), and 0.1 mL of 0.5% (w/v) thiamine hydrochloride (Sigma-Aldrich). All the supplements for M9 agar were filter sterilized using 0.22 μm syringe filters (Sarstedt). M9 minimal agar was finally prepared by mixing 500 mL of the supplemented M9 salt broth with 500 mL of 3% (w/v) sterile agar solution.

Recovery of Viable but Non-Culturable Cells (VBNC)

Prior to pouring as agar plates, M9, NA and BHI were also occasionally supplemented with the reactive oxygen species (ROS) scavengers, catalase (Sigma), and sodium pyruvate (SP) (Sigma-Aldrich). To investigate the possible resuscitation of injured and stressed VBNC cells, the supplementation of the agar plates (~20 mL) with 2000 units of catalase per plate (100 μL stock solution) or 0.3% of SP (200 μL stock solution) was performed around 10 min prior to plating the sample. Respectively, 20,000 units/mL and 300 mg/mL stock solutions

of catalase and SP were prepared by vigorous vortexing of the compounds in distilled water followed by filter sterilization with 0.22 μm filters. Stock solutions were prepared on the day of experiment and stored at 4°C until use.

Total Aerobic Count (TAC)

In order to determine the total aerobic count (TAC), samples were first decimaly serially diluted in maximum recovery diluent (MRD; Fluka), allowed to stand for 30 min after which 100 μL of each dilution was plated on the aforementioned growth media. The plates were incubated either at the room temperature ($\approx 21^\circ\text{C}$), 30°C or 37°C for 48 h. The colonies were examined and counted after 24 h and 48 h incubation.

16S rDNA Sequencing Study

Genomic DNA Extraction and Quality Check

In this study, the processing environment samples were collected from a PIF production site which had strict sanitary standards; therefore, they contained far less cells compared with environment samples collected from the natural environment such as water and soil. As a result, commercial kits with filter column genomic DNA extraction was not used, to avoid filter column clogging and genomic DNA loss during the binding-washing step. Genomic DNA was extracted using a chloroform-based method. Samples (see Section Sample Preparation for 16S Sequencing Study) were centrifuged at 8000 $\times g$, at room temperature for 2 min, the supernatant was discarded and the pellet was resuspended in 1 ml sterile PBS. This washing step was repeated three times after which the pellet was resuspend in 300 μl DNase/RNase free H₂O. The resuspended pellet was boiled at 100°C on a heating block for 5 min. After boiling, it was vortexed for ~ 5 s to ensure the disruption of cell walls and then centrifuged at 8000 $\times g$, for 5 min at room temperature. Subsequently, the supernatant was transferred to a new microcentrifuge tube, mixed with chloroform (Sigma) in a 1:1 ratio and vortexed for 5 s to ensure thorough mixing of the aqueous and chloroform phases. Finally, the mixture was centrifuged at 13000 $\times g$, 4°C for 10 min and $\sim 75\%$ of the aqueous (upper) phase that contained genomic DNA was transferred to a new tube. Nanodrop® Spectrophotomer ND-1000 (1 μl genomic DNA) and Qubit® 2.0 Fluorometer (1 μl genomic DNA; Thermo Fisher Scientific) were used to check genomic DNA concentration and quality, and the genomic DNA was stored at -20°C for further use.

Total RNA Extraction

As with DNA extraction, no commercial kit was used to avoid filter column clogging and total RNA loss during the binding-washing step. Samples were centrifuged at 8000 $\times g$, at room temperature for 2 min, the supernatant was discarded and the pellet was resuspended and washed with 1 ml sterile PBS. The centrifuge and wash steps were repeated twice and the pellet was resuspend in 1 ml sterile PBS, transferred to a 50 ml centrifuge tube and sterile PBS was used to adjust the final volume of the cell suspension to 4 ml. To this suspension, 1.6 mL of ice cold phenol-ethanol solution (95% ethanol and 5% acidic phenol; pH 4.3) was added and the tube was incubated on ice for at 30–120 min to stabilize the RNA and prevent degradation (Tedin

and Bläsi, 1996). The mixture was centrifuged at 3300 $\times g$ at 4°C for 10 min and most of the supernatant was discarded. The pellet was resuspended with the remaining supernatant in the tube and transferred to a 1.5 ml tube. The tube was centrifuged at 18,000 $\times g$, 4°C for 1 min, the supernatant was discarded and the pellet was resuspended in 1 ml of TRIzol (Ambion, Foster City, USA). The mixture was transferred to a 2 ml heavy phase lock tube (5 Prime) and supplemented with 400 μl chloroform (Sigma-Aldrich). The tube was immediately gently inverted for 10 s (no vortexing) and incubated at room temperature for 2–5 min. The mixture was centrifuged at 16,000 $\times g$, at room temperature for 15 min and the aqueous phase was transferred to a new microcentrifuge tube, supplemented with 450 μl isopropanol (Sigma-Aldrich) and mixed immediately. The mixture was incubated at room temperature for 20 min and stored at -20°C overnight to provide higher yields. After storage, the mixture was centrifuged at 16,000 $\times g$ at room temperature for 30 min, the supernatant was discarded, the pellet was washed with 350 μl of 70–75% ethanol and centrifuged at 16,000 $\times g$ at room temperature for 10 min. After air drying the pellet, 25 μl of pre-heated (65°C) DNA/RNase-free H₂O was added and the tube was incubated on a thermomixer at 900 rpm, 65°C for 5 min. During this time, the tubes were vortexed briefly 2–3 times to improve the dispersion of the pellet. The liquid in the tube contained total RNA. DNA removal was carried out using Ambion TURBO DNA-free™ kit (Thermo Fisher Scientific, USA). An Agilent 2100 Bioanalyzer (1 μl total RNA) and a Nanodrop® Spectrophotomer ND-1000 (1 μl total RNA) were used to check total RNA quantity and quality, and the sample was stored at -80°C for further use.

16S rDNA Sequencing and Bioinformatics Analysis

16S rDNA sequencing was carried out using the Illumina MiSeq platform. Such sequencing is amplicon-based, targeting the variable region V3-V4 of the 16S rDNA gene and generates PCR products with a length of ~ 460 bp. Paired-end, 300 $\times 2$ bp sequencing was carried out to cover the whole PCR product from the two opposite ends. For library construction, the Illumina official guide for 16S rDNA sequencing library preparation was used as reference. Bioinformatics analysis was carried out using the 16S Metagenomics app v1.0.1 provided in the Illumina BaseSpace online platform, including reads quality control, reads alignment, assembly and annotations. For taxonomic classification of the 16S rDNA reads, the Ribosomal Database Project (RDP) Classifier was used for classification (Wang et al., 2007) and an Illumina-curated version of the GreenGenes taxonomic database was used as reference database (DeSantis et al., 2006). Both applications were inbuilt in BaseSpace.

RESULTS AND DISCUSSION

Flow Cytometric and Plating Studies

Table 2 shows the FCM data obtained for three samples collected from low, medium and high care zones. The greatest number of cells (regardless of their physiological state) was detected in the low care zone, followed by the medium and high care zones ($p < 0.0001$). This was expected considering the implementation

TABLE 2 | Flow cytometric (FCM) total cell and viable cell density.

	Low care	Medium care	High care
KNOWN VALUES			
(A) Sample volume (mL)	32	33	33
(B) Dilution factor (ratio of sample to PBS)	1:12	1:12	1:6.6
(C) Area sampled (cm ² , approximately)	50000	50000	50000
ACQUIRED FCM DATA			
(D) Number of data recorded in gate P1	20000	20000	20000
(E) Number of SYTO® 62-stained particles in gate P6	16931 ± 398	13548 ± 33	6857 ± 194
(F) Number of unstained particles in gate P6	79 ± 0	71 ± 3	47 ± 3
(G) Number of presumed cells in P6 (E–F)	16852 ± 398	13477 ± 33	8610 ± 194
(H) Percentage noise particles (F/G)	0.47 ± 0.01%	0.53 ± 0.02%	0.55 ± 0.04%
(I) Percentage of presumed cells in P1 (G/A)	84.26 ± 1.99%	67.38 ± 0.17%	43.05 ± 0.97%
(J) Acquisition time (s)	186 ± 19	226 ± 11	193 ± 59
(K) Flow rate (μL/min)	45.82	45.82	45.82
TOTAL CELL DENSITY [L₁ = (G × A × 1000 × 60)/(K × J × C × B)]			
(L ₁) cells/cm ²	917 ± 73 ^X	621 ± 31 ^Y	267 ± 77 ^Z
(L ₂) log ₁₀ cells/cm ² (*)	(2.96 ± 0.03)	(2.79 ± 0.02)	(2.43 ± 0.12)
(M) Percentage viable (n = 8)(**; See Table 3 for calculations)	4.53 ± 2.71%	19.09 ± 3.12	16.12 ± 5.36
Viable cell density (L₁ × M)			
(N ₁) cells/cm ²	42 ± 25 ^X	118 ± 20 ^Y	43 ± 19 ^X
(N ₂) log ₁₀ cells/cm ² (*)	(1.62 ± 0.26)	(2.07 ± 0.07)	(1.63 ± 0.19)

Unless stated otherwise, the values shown here are the mean ± standard deviation (SD) of two technical replicates.

*: The SD values are the differential relative errors, i.e. absolute standard deviation divided by the mean value and multiplied by 0.434; e.g., 0.434 × (79/917) = 0.03;

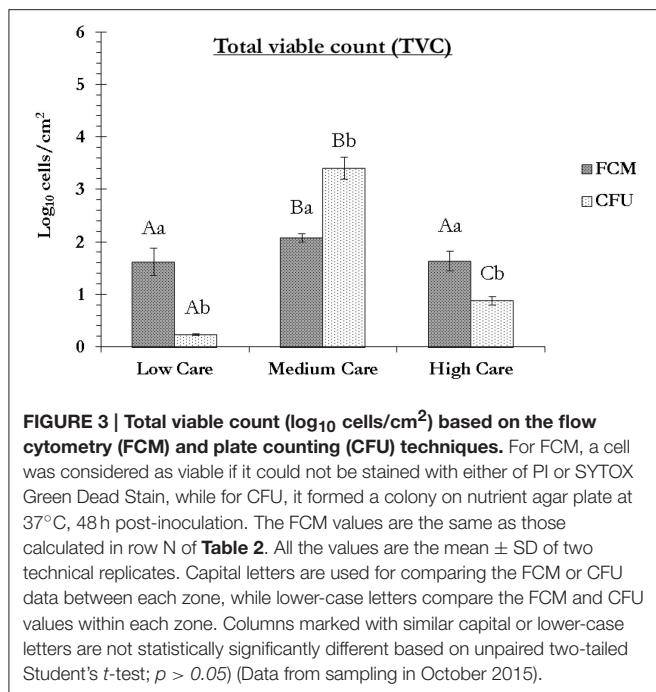
**: Viable cells were considered as those with intact membrane which either excluded PI (when used in combination with either of SYTO 9, TO or BOX) or were not stained with SYTOX Green Dead Stain dye (n = 8, two technical replicates per dye combination: SYTO 9/PI, TO/PI, BOX/PI and SYTOX Green only). In each row, the values with similar letters (X–Z) are not statistically significant (both p > 0.05; based on unpaired two-tailed Student's t-test) (Data from sampling in October 2015).

of stricter levels of hygiene and working practices by the PIF manufacturer in the latter two zones. However, the reduction in the total cell count, did not necessarily translate to a concurrent reduction in the number of viable cells as determined by FCM. By knowing the total cell count and the percentage of viable cells (based on the exclusion of PI viability dye), it was possible to calculate the density of viable cells per cm² of the sampling zone. The number of viable cells per cm² in medium care was nearly 3 times greater than that detected in both low and high care zones. This was probably due to greater level of humidity, hence greater access of microorganisms to available water in this zone. The lack of significance between the FCM viability results obtained for the two dry zones (low and high care) could be considered as further evidence of the primary role of humidity in improved viability of the cells in the wet medium care zone.

The results of the flow cytometry study were in agreement with those of the plate counting technique in the sense that the cells from medium care exhibited significantly greater culturability as well as viability compared to those from the other two care zones (Figure 3). In both dry zones, compared to the FCM, the plate counting significantly under-estimated the number of viable cells, while in the medium care, significantly lower number of cells were detected by FCM, compared to the plate counting. The under-estimation of viable cells by plate counting due to the presence of stressed and starved VBNC cells is well-documented (Oliver, 2005) and was, therefore, expected.

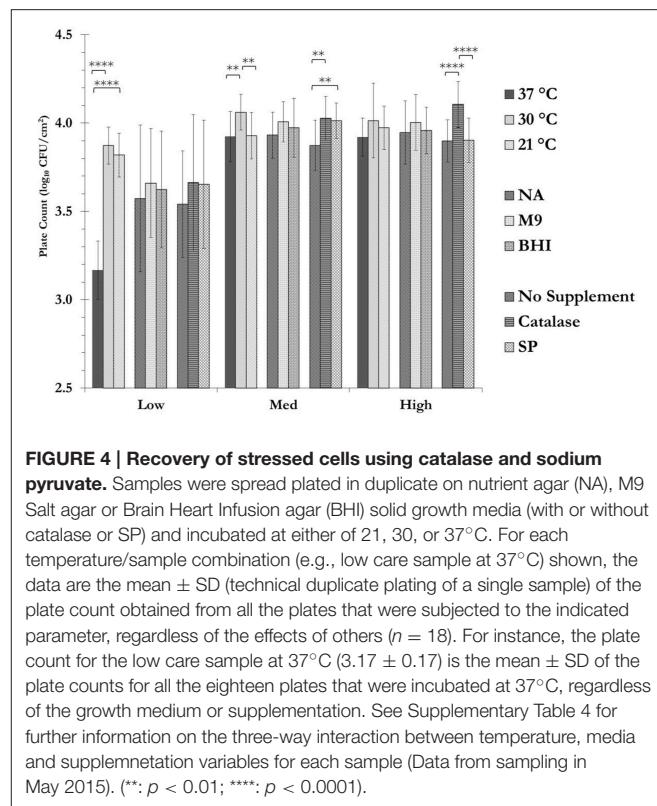
On the other hand, the under-estimation of viable cells by FCM in medium care may be due to the presence of ultramicrobacteria and ultramicrocells (e.g., bacteria of the genera *Flavobacterium*, *Bacteroides*, and *Chryseobacterium*) with sizes smaller than the detection limit of the flow cytometer used in this study (< 0.5 μm). This could have resulted in the cells being considered as background noise. This could also mean that the number of FCM viable cells, hence VBNC cells in dry care zones of low and high could have been significantly higher than determined in this study. Moreover, the possible presence of very high concentration of surfactants, detergents and washing solutions in that zone, could have rendered some of the cells un-stainable with SYTO62, hence not detectable based on the proposed protocol (Vives-Rego et al., 1999).

Table 3 shows the FCM data regarding the physiological status as well as Gram characteristics of the cells in each care zone. With regard to membrane integrity, membrane polarization, and metabolic activity, relatively similar results were obtained. The greatest mean percentage of cells with intact and polarized membranes and metabolic activity was observed in medium care, followed by high and low care zones. For instance, with the exception of SYTO9/PI staining, the percentage of cells with intact membranes was significantly lower in low care compared to the other two zones, regardless of the fluorophores used. The discrepancies observed between the results obtained for each fluorophore combination could be due to the difference between



their staining mechanism (Netuschil et al., 2014). Although, the greatest percentage of cells with respiratory activity was found in the medium care sample, the values obtained were significantly higher than the percentage of viable cells. The reason behind this observation is not clear, however, it could be due to the residual activity of the electron transfer chain in cells with depolarized and compromised cells. Moreover, the difference between the stainability of Gram negative and Gram positive cells could have played a role in the discrepancies observed (Holm and Jespersen, 2003).

Changing the incubation temperature and/or the supplementation of the growth media had a significant effect on the recovery of the cells on a care-zone basis (Figure 4 and Supplementary Table 4). For instance, decreasing the incubation temperature from 37 to 30°C or room temperature, had a highly significant effect on the total aerobic count for samples collected from the low care zone ($p < 0.0001$). This could indicate the presence of a significant number of psychrotrophic bacteria in low care samples (Hantsis-Zacharov and Halpern, 2007). The change in nutrient content of the media did not make a statistically significant difference in the recovery of the stressed cells. On the other hand, supplementation of the growth media with reactive oxygen species scavengers seemed to improve the recovery of the cells. Catalase and SP have previously been shown to be effective in recovery and resuscitation of injured and VBNC cells (Mizunoe et al., 2000; Bang et al., 2007). Catalase was effective in recovery of the cells collected from both medium and high care zones, while SP only improved the recovery in medium care samples. In the case of low care samples, the results were similar to those observed in the medium care zone, however, they did not reach the level of statistical significance.



16S rDNA Sequencing Study

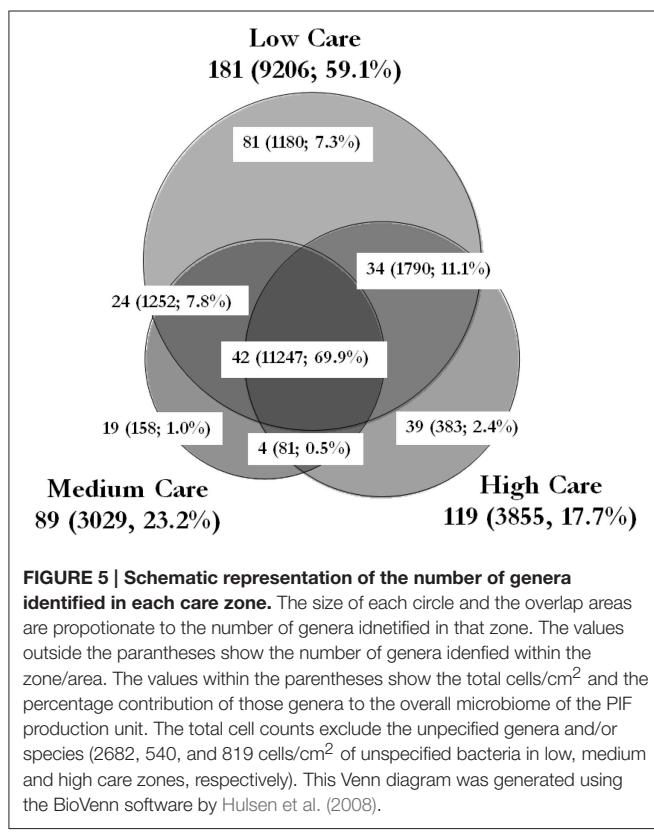
The mean DNA content in low, medium and high care zones was 278, 168, and 53 pg/cm², respectively. Considering the vast difference between the DNA content of different bacterial species, it was not possible to establish a direct correlation between the DNA content of the sample and the cell count. Nonetheless, the greatest DNA content was found in the low care sample, followed by medium and high care zone samples, which closely resembled the results for cell counts obtained using FCM total count, as previously described. In addition, the mean total RNA content for samples of low, medium and high care zones was 2, 29, and 1 pg/cm², respectively. By making the presumption that the presence RNA in the cell is an indicator of protein synthesis, hence a degree of cellular vitality, the RNA content of the cells was in agreement with both the FCM viable count and plate counts.

16S sequencing provided valuable information on the type of bacteria present in each care zone and the percentage contribution of each genera to the entire microbiome. This technique has been used successfully to characterize the microbiome in various environments such as soil, water, food and hospitals (Gomez-Alvarez et al., 2012; Oberauner et al., 2013; Rampelotto et al., 2013; de Boer et al., 2015). By integrating these data with that of the total FCM cell count, it was possible to calculate the number of cells belonging to each genera in each care zone. 16S sequencing of the samples revealed the presence of 243 bacterial genera (with more than 0.05% distribution) in the microbiome of the PIF production facility (Figure 5). The combination of 16S rRNA-targeted oligonucleotide probes

TABLE 3 | The physiological status and Gram characteristics of the microbiome.

Physiological status	Low care	Medium care	High care			
(a) Intact membrane ($n = 8$; I-IV)*	$4.53 \pm 2.71\%$	X	$19.09 \pm 3.12\%$	Y	$16.12 \pm 5.36\%$	Y
i.SYTO9 ⁺ /PI ⁻ ($n = 2$)	$1.72 \pm 0.04\%$	A;X	$18.83 \pm 1.58\%$	AB;Y	$9.33 \pm 0.59\%$	A;Z
ii.TO ⁺ /PI ⁻ ($n = 2$)	$2.70 \pm 0.03\%$	B;X	$16.72 \pm 0.19\%$	B;Y	$20.52 \pm 1.76\%$	B;Y
iii.BOX [±] /PI ⁻ ($n = 2$)	$8.17 \pm 0.48\%$	C;X	$23.76 \pm 1.59\%$	AC;Y	$20.75 \pm 3.57\%$	BCD;Y
iv.SYTOX ⁻ ($n = 2$)	$5.53 \pm 0.42\%$	D;X	$17.04 \pm 0.02\%$	B;Y	$13.87 \pm 1.05\%$	C;Y
(b) Polarized membrane ($n = 2$; BOX ⁻)	$0.27 \pm 0.06\%$	X	$10.29 \pm 0.04\%$	Y	$1.46 \pm 0.02\%$	Z
(c) Metabolically active ($n = 4$; VI-VII)**	$3.50 \pm 3.35\%$	X	$20.08 \pm 9.34\%$	Y	$10.59 \pm 2.29\%$	Y
v.FDA ⁺ ($n = 2$)	$0.25 \pm 0.02\%$	E;X	$2.80 \pm 0.38\%$	E;Y	$0.34 \pm 0.01\%$	E;Z
vi.cFDA ⁺ ($n = 2$)	$6.04 \pm 0.02\%$	F;X	$28.01 \pm 0.38\%$	F;Y	$12.56 \pm 0.15\%$	F;Z
vii.cFDA-SE ⁺ ($n = 2$)	$0.96 \pm 0.03\%$	G;X	$12.16 \pm 0.44\%$	G;Y	$8.62 \pm 0.43\%$	G;Z
(d) Respiratory activity ($n = 2$; CTC ⁺)	$10.18 \pm 0.46\%$	X	$44.21 \pm 3.56\%$	Y	$1.38 \pm 0.33\%$	Z
(e) Gram positive ($n = 2$; HI ⁺)	$12.40 \pm 0.01\%$	X	$19.70 \pm 1.58\%$	Y	$6.10 \pm 2.35\%$	X

In each column, the values with similar letters (A-D for membrane integrity and E-G for metabolic activity data) are not statistically significant. Moreover, in each row, the values with similar letters (X-Z) are not statistically significant (both $p > 0.05$; based on unpaired two-tailed Student's t-test). With the exception of rows marked with * and **, all the values are the mean \pm standard deviation (SD) of two technical replicates. *: The values in row (a) are the mean \pm SD of the percentage of cells that were not stained with either of PI or SYTOX Green (intact membrane; rows I to IV). **: The values in row (c) are the mean \pm SD of the percentage of cells that were stained with either of cFDA or cFDA-SE (esterase activity; rows VI and VII respectively). The data obtained for FDA⁺ were not regarded for the purpose of this calculation, due to apparent passive leakage of the fluorescein dye from the cells (Data from sampling in October 2015).



with flow cytometry for analyzing mixed microbial populations has been reported (Amann et al., 1990), although there are no reports of its use to characterize the microbial population of a food production environment. The results pointed to a striking similarity between the type of bacteria present in different care zones. For instance, 42 out of 243 genera were common to all

three care zone, contributing to nearly 70% of the microbiome. Similarly, 58 genera were common between low care and the other two care zone. On the other hand, although a third of the identified genera were unique to low care, they only made up 7.3% of the microbiome (total 1180 cells/cm²). Similarly, 19 and 39 genera were unique to medium and high care zones, making up 1.0 and 2.4% of the microbiome, respectively.

Looking at the top thirty genera, in terms of % occurrence, in the PIF production unit provided a better picture of the type of microorganisms associated with each care zone (Table 4). For the complete list, readers are referred to Supplementary Table 5. Twenty out of 30 genera which were predominantly present in low care zone, are mainly associated with soil and the general environment, which included species belonging to *Pseudomonas*, *Sprirosoma*, and *Sphingomonas* genera. On the other hand, those predominantly present in wet care zone such as *Acinetobacter*, *Chryseobacterium*, and *Paucibacter* are mainly associated with water and sewage, as well as soil and other general environment sources. In contrast, the greatest number of human and milk-associated genera such as *Streptococcus*, *Lactococcus*, *Corynebacterium*, *Lactobacillus*, and *Kocuria* were found in the high care zone. Washing the sponges of the low care zone in PBS resulted in the formation of very dark gray cell suspension with a significant soil and debris sedimentation. Unlike the other two care zones, the majority of drains and sampling points (mainly drains) within medium care were wet. Based on the current data, it was not possible to definitively determine the primary reason behind the greater prevalence of human-associated microorganisms in high care zone. However, it is plausible that, while strict segregation of the high care zone led to a substantial reduction in the entry of soil and drain-associated microorganisms from the other two care zones, human-associated microorganisms within that zone still contributed to the microbiome of high care zone.

TABLE 4 | The top 30 genera in the PIF production unit (all care zones).

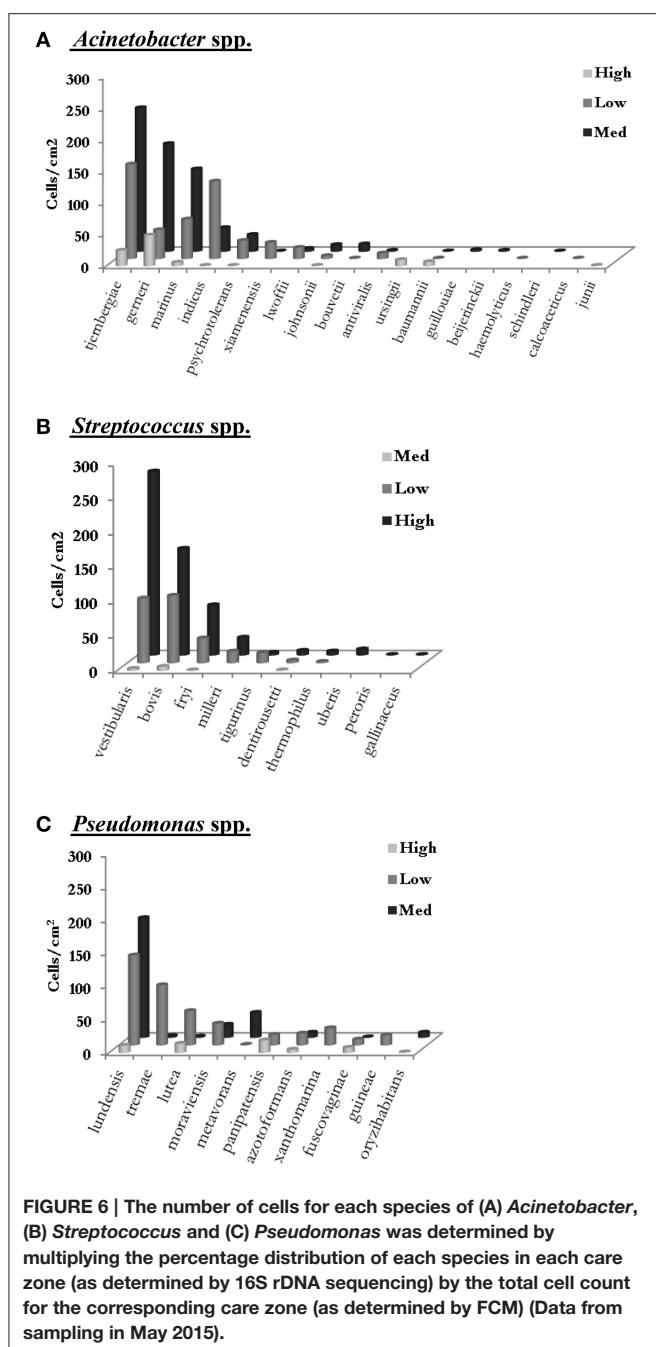
Overall ranking*	Genus	Low care		Medium care		High care		Mean Density (Cells/cm ²)
		Distribution (%)	Cell density (Cells/cm ²)	Distribution (%)	Cell density (Cells/cm ²)	Distribution (%)	Cell density (Cells/cm ²)	
1	Acinetobacter	5.64	670	27.76	991	2.90	136	599
2	Streptococcus	4.81	572	0.68	24	24.88	1163	586
3	Pseudomonas	6.60	785	13.02	464	4.82	225	492
4	Spirosoma	5.39	641	0.08	3	1.11	52	232
5	Sphingomonas	4.69	558	0.16	6	1.76	82	215
6	Lactococcus	1.47	175	2.03	72	6.96	326	191
7	Pedobacter	3.39	403	0.18	6	—	—	136
8	Chryseobacterium	1.29	154	6.68	238	0.20	9	134
9	Calothrix	2.38	283	—	—	2.01	94	126
10	Flavobacterium	2.56	304	0.49	17	0.71	33	118
11	Janthinobacterium	2.20	261	1.83	65	0.18	9	112
12	Enterobacter	0.17	21	7.02	251	0.98	46	106
13	Psychrobacter	0.90	107	4.29	153	0.81	38	100
14	Corynebacterium	0.75	89	0.16	6	4.26	199	98
15	Hymenobacter	1.83	218	—	—	0.24	11	76
16	Lactobacillus	0.70	83	0.16	6	2.82	132	74
17	Bacteroides	1.25	148	0.15	5	1.18	55	70
18	Paucibacter	0.36	43	3.19	114	0.20	9	55
19	Staphylococcus	0.77	91	—	—	1.49	70	54
20	Oxalobacter	1.17	139	0.19	7	0.25	12	53
21	Roseomonas	1.22	145	0.05	2	0.16	8	51
22	Arthrobacter	0.73	87	0.92	33	—	—	40
23	Sejongia	0.83	99	0.08	3	0.37	17	40
24	Kocuria	0.38	45	0.26	9	1.25	58	38
25	Stenotrophomonas	0.67	79	0.18	7	0.45	21	36
26	Bacillus	0.60	71	—	—	0.66	31	34
27	Dyadobacter	0.71	84	0.37	13	0.06	3	33
28	Novosphingobium	0.77	92	0.12	4	—	—	32
29	Tolumonas	0.32	39	1.02	36	0.29	13	29
30	Variovorax	0.71	84	0.08	3	—	—	29

*: The ranking (out of 243 genera identified in all three zones, with equal to or more than 0.05% distribution) is based on the calculated mean density of the specified genus in all three zones (i.e., the sum of the densities in each zone divided by three as shown in the last column). In each row (genus), the highlighted in bold cell indicate the zone at which the greatest density for that genus was observed. See Supplementary Table 5 for the list of all genera identified and their distribution in each care zone (Data from sampling in May 2015).

A closer look at the number of cells of the different species of the top three genera could be used as a good indicator of the possible transition of the cells between different zones (Figure 6). For instance, with regard to the *Acinetobacter*, the top five species of this genus in low care and the top three in low care were also among the top five species of this genus in medium care. Similar results were also obtained for *Streptococcus* spp. where *S. vestibularis*, *S. bovis*, and *S. fayi* were the top three species of this genus in all three zones. Furthermore, the rate of change in the number of cells for each species in one zone, closely

resembled the change observed in the other two zones. It is important to note that the aim of this study was to compare the microbiome of different care zones and therefore, sponges from different locations of a specific care zone were placed in a single bag. Consequently, this could have contributed to the variability observed between the results for each care zone. Further studies are needed to determine the microbiome of each sampling point.

16S sequencing also provided information on the pathogenic strains present in each care zone. Table 5 shows the percentage distribution of 18 pathogenic species and 1 pathogenic genus



in three different care zones. According to the official report of FAO/WHO (2004), these species are divided into three categories “based on the strength of evidence of a causal association between their presence in PIF and illness in infants.” Class A includes *Cronobacter* spp. and *Salmonella enterica* for which clear evidence of causality exist. No Class A microorganisms were detected in either of the three care zones. On the other hand, Class B (i.e., causality plausible, but not yet demonstrated) and Class C organisms (i.e., causality less plausible, or not yet demonstrated) were detected at both genus and species level in all three zones.

TABLE 5 | Pathogenic species identified in different care zones.

	Genus	Species	Low	Medium	High
CLASS A	<i>Cronobacter</i>	<i>sakazakii</i>	ND	ND	ND
	<i>Salmonella</i>	<i>enterica</i>	ND	ND	ND
CLASS B	<i>Citrobacter</i> spp.		0.001%	0.116%	0.048%
	<i>Citrobacter</i>	<i>freundii</i>	ND	0.057%	0.014%
	<i>Citrobacter</i>	<i>koseri</i>	ND	ND	ND
	<i>Enterobacter</i> spp.		0.173%	7.022%	0.978%
	<i>Enterobacter</i>	<i>cloacae</i>	ND	0.001%	ND
	<i>Hafnia</i>	<i>alvei</i>	ND	ND	ND
	<i>Klebsiella</i> spp.		0.002%	0.291%	0.001%
	<i>Klebsiella</i>	<i>pneumoniae</i>	ND	0.001%	ND
CLASS C	<i>Klebsiella</i>	<i>oxytoca</i>	ND	0.004%	ND
	<i>Pantoea</i>	<i>agglomerans</i>	ND	ND	ND
	<i>Escherichia</i>	<i>vulnaris</i>	0.012%	0.004%	ND
	<i>Escherichia</i>	<i>coli</i>	ND	ND	ND
	<i>Bacillus</i> spp.		0.601%	0.025%	0.656%
	<i>Bacillus</i>	<i>cereus</i>	0.103%	ND	ND
	<i>Clostridium</i> spp.		0.190%	0.089%	1.122%
	<i>Clostridium</i>	<i>botulinum</i>	ND	ND	ND
	<i>Clostridium</i>	<i>difficile</i>	ND	ND	ND
	<i>Clostridium</i>	<i>perfringens</i>	ND	ND	ND
	<i>Listeria</i>	<i>monocytogenes</i>	0.015%	0.001%	0.019%
	<i>Shigella</i>		ND	ND	ND
	<i>Staphylococcus</i>	<i>aureus</i>	0.769%	0.013%	1.493%
	<i>Yersinia</i>	<i>perstis</i>	0.118%	0.023%	ND

The values are the percentage distribution of the specified genus or species (rows) detected in the corresponding care zone (columns). The distribution values of greater than 0.05% (considered as significant presence of the specified genus/species) are shown in bold (Data from sampling in May 2015). ND: Not detected.

In summary, the results showed that the physical segregation of a production unit into different care zones has a positive impact on reducing the microbial load within a PIF production unit. However, the reduction in total cell count did not lead to a reduction in either the total viable count or the human associated pathogenic bacterial species. Therefore, better control measures such as stricter monitoring of staff and personal hygiene policies might be necessary to achieve a significant reduction in the human-associated microorganisms in high care. The results also demonstrated that combining the FCM and 16S rDNA sequencing data could be used successfully for hygiene monitoring in PIF production units.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the sampling protocol, AA and YC collected samples for FCM and 16S rDNA sequencing studies, respectively, prepared the samples, analyzed the data and drafted the manuscript. AA integrated the FCM and sequencing data, wrote the statistical analysis plan and analyzed the integrated data. SS contributed to the development of sample preparation protocol and analyzed the sequencing data.

SF and KJ initiated the study, obtained the funding, designed and supervised the collaborative project, monitored the data collection and contributed to the completion of the manuscript. AA and YC made an equal contribution (co first authors).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00968>

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Conflict of Interest Statement: 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.

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