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Real-Time PCR Method for the Rapid Detection and Quantification of Pathogenic *Staphylococcus* Species Based on Novel Molecular Target Genes

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Abstract: Coagulase-positive *Staphylococcus aureus* is a foodborne pathogen considered one of the causes of food-related disease outbreaks. Like *S. aureus*, *Staphylococcus capitis*, *Staphylococcus caprae*, and *S. epidermidis* are opportunistic pathogens causing clinical infections and food contamination. The objective of our study was to develop a rapid, accurate, and monitoring technique to detect four *Staphylococcus* species in food. Four novel molecular targets (GntR family transcriptional regulator for *S. aureus*, phosphomannomutase for *S. epidermidis*, FAD-dependent urate hydroxylase for *S. capitis*, and Gram-positive signal peptide protein for *S. caprae*) were mined based on pan-genome analysis. Primers targeting molecular target genes showed 100% specificity for 100 non-target reference strains. The detection limit in pure cultures and artificially contaminated food samples was 10^2 colony-forming unit/mL for *S. aureus*, *S. capitis*, *S. caprae*, and *S. epidermidis*. Moreover, real-time polymerase chain reaction successfully detected strains isolated from various food matrices. Thus, our method allows an accurate and rapid monitoring of *Staphylococcus* species and may help control staphylococcal contamination of food.

Keywords: *Staphylococcus aureus*; *Staphylococcus capitis*; *Staphylococcus caprae*; *Staphylococcus epidermidis*; real-time PCR; pan-genome analysis; detection

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1. Introduction

Staphylococci are common pathogens, widely distributed in nature and frequently isolated from food. *Staphylococcus aureus* is one of the most important foodborne pathogens, producing staphylococcal enterotoxins causing diarrhea and vomiting through direct invasion or systemic transmission, adversely affecting human health [1,2]. Unlike foodborne pathogenic *S. aureus*, which is a coagulase-positive *Staphylococcus*, *S. capitis*, *S. caprae*, and *S. epidermidis* are coagulase-negative staphylococci (CoNS) [3]. Traditionally considered commensals, CoNS species are now recognized as opportunistic pathogens [4]. Moreover, CoNS species contaminate foods and have become a prominent pathogenic strain in ready-to-eat foods [1]. In the CoNS group, *S. epidermidis* is the most common pathogen associated with human infections, such as bacteremia and endocarditis in immunocompromised patients [5]. *S. capitis* is occasionally associated with hospital-acquired meningitis and native and prosthetic valve endocarditis. At the same time, *S. caprae* infections include bacteremia and prosthetic infections [4–6]. *S. saccharolyticus* has been reported to be associated with human infection but has rarely been isolated from food. Phylogenetic analysis using the 16S rRNA gene classified *S. epidermidis* into the *S. epidermidis* group, including *S. capitis*, *S. caprae*, and *S. saccharolyticus* [5]. These species are closely phenotypically related, so it is difficult to distinguish them. Therefore, accurate methods to detect and discriminate *Staphylococcus* species contamination in food are needed to reduce disease outbreaks and ensure food safety.

Traditional methods for detecting pathogenic bacteria require multiple steps, including pre-enrichment, selective growth, and selective plating, which can then be characterized by analyzing additional biochemical tests [7]. The process is labor-intensive and time-consuming, is not cost-effective, and is also unable to detect viable but nonculturable cells [8,9]. Various staphylococci detection methods exist, both phenotypic and genotypic. Other detection methods, such as the Staph-Zym test, the API Staph test, and the BF Phenix Automated Microbiology system, have been used for the detection of staphylococci based on their phenotypic properties [10,11]. Recently, an attempt has been made to detect staphylococci using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry based on protein profiles [12]. However, the low accuracy (50–70%) of the biochemical reaction of the API kit and the high initial acquisition cost of automatic mass spectrometry systems restrict their application [13,14]. Compared to genotypic detection methods, such as amplified fragment length polymorphic fingerprinting and polymerase chain reaction (PCR)-based methods, phenotypic tests are less accurate [1,14,15].

The development of simple and rapid methods with high specificity and sensitivity is critical for detecting pathogenic bacteria. In recent decades, molecular detection methods, such as PCR, have been widely employed for pathogen detection because they are faster and simpler than conventional culture methods [16,17]. Among the PCR-based methods, real-time PCR has become a useful tool for detecting and quantifying bacterial species associated with foods due to its superiority, such as high sensitivity and efficiency [8,17–21]. At present, real-time PCR is used for monitoring staphylococci in food processing [1,22,23].

The selection of appropriate genes or sequences is critical for pathogenic bacteria detection using PCR. Various molecular target genes were used to detect *Staphylococcus* species, namely, 16S rRNA gene, *tuf* (elongation factor Tu), *sodA* (superoxide dismutase A), *nuc* (thermostable nuclease), and *dnaJ* (chaperone dnaJ) [11,24–26]. However, some of these genes cannot discriminate between phylogenetically closely related species, such as the *S. epidermidis* and *S. aureus* cluster groups. The lack of exclusivity and inclusivity of molecular target genes is one major drawback of implementing the PCR-based method in food inspection [27]. Advances in high-throughput sequencing technology have allowed increasing the number of whole-genome sequences available in public databases, making it easier to obtain target genes specific for *Staphylococcus* species using bioinformatics approaches [27]. This study aimed to develop a real-time PCR assay targeting molecular target genes to detect the staphylococci *S. aureus*, *S. capitis*, *S. caprae*, and *S. epidermidis*, thus allowing their accurate and rapid monitoring in food matrix.

2. Materials and Methods

2.1. Evaluation of Staphylococci Genomes

The genome sequences of 155 *Staphylococcus* strains were obtained from the National Center for Biotechnology Information (NCBI). *Staphylococcus* genomes consist of the *S. aureus* cluster group (8 *S. argenteus*, 35 *S. aureus*, 9 *S. schweitzeri*, 9 *S. simiae*), the *S. epidermidis* cluster group (16 *S. capitis*, 15 *S. caprae*, 28 *S. epidermidis*, 18 *S. saccharolyticus*), 9 *S. pasteuri*, 8 *S. warneri*. Detailed genome information is shown in Table S1. Phylogeny analysis was performed using anvi'o software version 7.0 [28] to confirm the taxonomic position of the genomes obtained from publicly available databases. Assembled genomes were first used to generate genome storage files using the "anvi-gene-genomes-storage" command. Genome storage was then used for phylogeny analysis based on pan-genome using the "anvi-pan-genome" command. The average nucleotide identity (ANI) value was calculated using the "anvi-compute-ani" command, which uses PyANI [29].

2.2. Mining of Molecular Target Genes

The molecular target genes were mined using bacterial pan-genome analysis (BPGA) v1.3 [30]. The criteria for selecting molecular target genes of four species were 100% presence in the respective target species and absence in non-target species. The genomes of staphylococci were constructed using two databases: a core gene database for target

species and a pan gene database for non-target species. Then, the two databases were compared to search for target genes with a cut-off value of 50%, default parameter. The candidate target genes were searched using BLAST to further identify the genes specific to each species. The genes that were absent, with 72,899,005 other bacterial sequences, were considered molecular target genes. The specificity of the discovered molecular target genes and of the reported target genes (*tuf*, *sodA*, *nuc*, and *dnaJ*) was confirmed by aligning them with 94 *S. aureus*, *S. capitis*, *S. caprae*, and *S. epidermidis* genomes.

2.3. Design of Specific Primers

Based on the sequences of the target genes, primer pairs were designed using Primer Designer (Scientific and Education Software, Durham, NC, USA) with the following criteria: G + C content, 40–60%, Tm value, 65 °C and 75 °C, and no ability to form dimers. The specificity of the primer pairs was checked using the primer-BLAST tool [31].

2.4. DNA Extraction

The reference strains, including 39 *Staphylococcus* strains and 73 non-*Staphylococcus* strains, are listed in Table 1. All reference strains were grown in tryptic soy broth (TSB, Difco, Becton & Dickinson, Sparks, MD, USA) at 37 °C for 24 h. Genomic DNA of all staphylococci and non-staphylococci strains was extracted using the G-spin genomic DNA extraction kit (Intron Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. The DNA purity and concentration were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Table 1. List of reference strains used in this study.

Classification	Species	Strain Number
Target *Staphylococcus*	*Staphylococcus aureus*	ATCC 29213
	Staphylococcus aureus	KCTC 1928
	Staphylococcus aureus	NCCP 14560
	Staphylococcus aureus	ATCC 25923
	Staphylococcus aureus	ATCC 29737
	Staphylococcus aureus subsp. *aureus*	ATCC 6538
	Staphylococcus capitis	NCCP 14663
	Staphylococcus capitis subsp. *capitis*	KACC 13242
	Staphylococcus caprae	KCTC 3583
	Staphylococcus caprae	NCCP 15629
	Staphylococcus epidermidis	NCCP 14723
	Staphylococcus epidermidis	KACC 13234
Non-target *Staphylococcus*	*Staphylococcus auricularis*	KACC 13252
	Staphylococcus carnosus subsp. *utilis*	KACC 13190
	Staphylococcus cohnii subsp. *cohnii*	KACC 13237
	Staphylococcus cohnii subsp. *urealyticus*	KCTC 3574
	Staphylococcus delphini	KACC 13258
	Staphylococcus equorum subsp. *equorum*	KACC 13255
	Staphylococcus fleurettii	KACC 13199
	Staphylococcus gallinarum	KACC 13253
	Staphylococcus haemolyticus	NCCP 14691
	Staphylococcus hominis	NCCP 10748
	Staphylococcus hominis	KACC 13409
	Staphylococcus kloosii	KACC 13256
	Staphylococcus lentus	KCCM 41469
	Staphylococcus lugdunensis	NCCP 15630
	Staphylococcus lugdunensis	KACC 11270
	Staphylococcus pasteuri	KCTC 13167
	Staphylococcus pettenkoferi	DSM 19554
	Staphylococcus saprophyticus	NCCP 14670
	Staphylococcus saprophyticus	KCTC 3345
	Staphylococcus saprophyticus	KACC 15799

Table 1. *Cont.*

Classification	Species	Strain Number
	Staphylococcus schleiferi subsp. *coagulans*	KCCM 41634
	Staphylococcus sciuri subsp. *rodentium*	KACC 13217
	Staphylococcus sciuri subsp. *sciuri*	KCCM 41468
	Staphylococcus warneri	KCTC 3340
	Staphylococcus warneri	KACC 10785
	Staphylococcus xylosus	NCCP 10937
	Staphylococcus xylosus	KACC 13239
Non-*Staphylococcus*	*Bacillus cereus*	KCTC 3624
	Bacillus cereus	KCTC 1661
	Bacillus cereus	KCCM 1173
	Bacillus cereus	KCCM 1174
	Bacillus cereus	KCCM 40133
	Bacillus cereus	ATCC 11778
	Bacillus cereus	ATCC 10876
	Bacillus cereus	ATCC 14579
	Bacillus circulans	KCTC 3347
	Bacillus licheniformis	KCTC 1026
	Bacillus megaterium	KCTC 3007
	Bacillus subtilis	KCTC 3725
	Clostridium perfringens	ATCC 14810
	Enterococcus avium	KACC 10788
	Enterococcus casseliflavus	KCTC 3552
	Enterococcus cecorum	KACC 13884
	Enterococcus durans	KCTC 13289
	Enterococcus faecalis	KCTC 5290
	Enterococcus faecalis	KACC 11859
	Enterococcus faecalis	KCTC 3206
	Enterococcus faecium	KACC 15681
	Enterococcus faecium	KACC 11954
	Enterococcus faecium	KCTC 13225
	Enterococcus faecium	KACC 14552
	Enterococcus gallinarum	NCCP 11518
	Enterococcus gilvus	KACC 13847
	Enterococcus hirae	KACC 16328
	Enterococcus hirae	KACC 10782
	Enterococcus hirae	KACC 10779
	Enterococcus malodoratus	KACC 13883
	Enterococcus mundtii	KCTC 3630
	Enterococcus raffinosus	KACC 13782
	Enterococcus saccharolyticus	KACC 10783
	Enterococcus thailandicus	KCTC 13134
	Escherichia coli	KCTC 1682
	Escherichia coli	ATCC 25922
	Escherichia coli	ATCC 23763
	Escherichia coli	ATCC 35150
	Escherichia coli	ATCC 43890
	Enteroaggregative *Escherichia coli*	NCCP 14039
	Enterohemorrhagic *Escherichia coli*	NCCP 11076
	Enteroinvasive *Escherichia coli*	NCCP 15663
	Enteropathogenic *Escherichia coli*	NCCP 13715
	Enterotoxigenic *Escherichia coli*	NCCP 15732
	Listeria ivanovii	ATCC 19119
	Listeria monocytogenes	ATCC 19115
	Listeria monocytogenes	KCTC 3569
	Proteus mirabilis	KCTC 2566
	Proteus vulgaris	KCTC 2579
	Pseudomonas aeruginosa	KCTC 1636
	Pseudomonas chlororaphis	KCCM 41854

Table 1. *Cont.*

Classification	Species	Strain Number
	Pseudomonas oryzihabitans	KCCM 42984
	Salmonella bongori	ATCC 43975
	Salmonella enterica subsp. *arizonae*	ATCC 13314
	Salmonella enterica subsp. *diarizonae*	ATCC 43973
	Salmonella enterica subsp. *enterica*	ATCC 19585
	Salmonella Choleraesuis	ATCC 13312
	Salmonella Gallinarum	ATCC 9120
	Salmonella Paratyphi B	ATCC 10719
	Salmonella Paratyphi C	ATCC 13428
	Salmonella Typhimurium	ATCC 14028
	Salmonella enterica subsp. *houtenae*	ATCC 43974
	Salmonella enterica subsp. *indica*	ATCC 43976
	Salmonella enterica subsp. *salamae*	ATCC 15793
	Shigella dysenteriae	ATCC 13313
	Shigella sonnei	KCTC 2518
	Vibrio cholerae	NCCP 13589
	Vibrio cholerae	ATCC 14033
	Vibrio cholerae	ATCC 14035
	Vibrio parahaemolyticus	ATCC 17802
	Vibrio parahaemolyticus	KCCM 41664
	Vibrio parahaemolyticus	ATCC 27969
	Vibrio vulnificus	ATCC 33814

2.5. Real-Time PCR Condition

Real-time PCR was performed in a 20 µL reaction mixture containing 10 µL of 2× Thunderbird SYBR® qPCR mix (Toyobo, Osaka, Japan), 1 µL of each primer (10 pmol/µL), 1 µL of template (20 ng/µL), and 7 µL of distilled water. A CFX96 Touch Deep (Bio-Rad, Hercules, CA, USA) was used for thermal cycling as follows: denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 5 s and 60 °C for 30 s. The melting curve was obtained by increasing the temperature between 60 °C and 95 °C in 0.5 °C increments while holding for 15 s at each step.

2.6. Specificity of the Primer Pairs

The specificity of the primer pairs was confirmed by 112 bacterial strains (Table 1). If a primer pair successfully produced an amplification plot from strains of the corresponding species, it was then tested against other *Staphylococcus* strains and non-*Staphylococcus* strains. For sensitivity testing, cultures of *S. aureus* ATCC 6538, *S. epidermidis* KACC 13234, *S. capitis* KACC 13242, and *S. caprae* KCTC 3583 were serially diluted (10^2–10^8 colony-forming units (CFU/mL), and genomic DNA was extracted as described in Section 2.4, followed by real-time PCR.

2.7. Application in Artificially Contaminated Food Samples

The reliability of the established real-time PCR assay for pathogenic *Staphylococcus* detection in the food matrix was determined according to previous studies [1,19]. All strains were grown in TSB for 24 h at 37 °C, and plate counting was performed in TSA medium. For the spiking experiment, milk samples were purchased, and the absence of four *Staphylococcus* species was confirmed. Spiked samples were prepared by inoculating the cocktail of *S. aureus* ATCC 6538, *S. epidermidis* KACC 13234, *S. capitis* KACC 13242, and *S. caprae* KCTC 3583 at a concentration of 10^2–10^8 CFU/mL each in 25 mL of milk samples. The prepared spiked samples were then homogenized for 1 min. The genomic DNA of each homogenized sample was extracted under the conditions described in Section 2.4. Real-time PCR analysis was conducted at the conditions described in Section 2.5.

2.8. Application in Isolated Staphylococcus Strains

Twenty-two samples (4 chicken, 3 beef, 5 pork, 3 fish, 5 salted fish, and 2 raw milk) were collected in Korea to confirm the feasibility of the detection of staphylococci by real-time PCR. Twenty-five grams of food sample was transferred to stomacher bags and then homogenized with 225 mL saline and mixed thoroughly to isolate *Staphylococcus* strains. Serial dilutions of the homogenized samples were prepared, and a small volume of each of them was spread on mannitol salt agar (Difco) media and incubated at 37 °C for 24 h. Then, the genomic DNAs of unknown isolates were used for identification by the real-time PCR method developed in this study.

2.9. Detection of Staphylococcus Species in Food Samples

Fifty samples, including 2 samples of ready-to-eat vegetables (1 cucumber and 1 lettuce), 10 samples of meat (2 beef and 8 pork), 34 samples of raw milk, and 4 samples of salted fish, were randomly collected from local markets in Korea. Ten grams of each sample was homogenized using a blender. The genomic DNA of the food samples was extracted under the conditions described in Section 2.4. Detection of four *Staphylococcus* species in unknown samples was performed under the conditions described in Section 2.5.

3. Results

3.1. Evaluation of Staphylococci Whole-Genome Sequences

Misclassified genomes for closely related bacteria are often reported in public database [20,32,33]. The phylogenetic tree based on pan-genome cluster frequencies was clustered according to the species name, and two clusters were generated (Figure 1). The first cluster included *S. aureus*, *S. argenteus*, and *S. schweitzeri*, and the second cluster included *S. epidermidis*, *S. simiae*, *S. warneri*, *S. pasteuri*, *S. saccharolyticus*, *S. caprae*, and *S. capitis*. However, some genomes, such as those of *S. pasteuri* SP1, *S. saccharolyticus* IIF7SG_B1, 151250007-1-258-46, and IIF6SC-B4A, clustered with those of other species. *S. pasteuri* SP1 clustered with *S. warneri*. *S. saccharolyticus* IIF7SG_B1, 151250007-1-258-46, and IIF6SC-B4A clustered with *S. pasteuri*. In OrthoANI analysis, *S. pasteuri* SP1 showed 95.95% identity with an *S. warneri*-type strain (NCTC 11044) and 83.37% identity with an *S. pasteuri*-type strain (DSM 10656). In addition, *S. saccharolyticus* IIF7SG_B1, 151250007-1-258-46, and IIF6SC-B4A showed 98.93–98.98% identity with an *S. pasteuri*-type strain (DSM 10656) and 76.72–76.95% identity with an *S. saccharolyticus*-type strain (NCTC 11807). These results suggest that these strains should be corrected in the public database to avoid further misidentification.

3.2. Pan-Genome Analysis

One hundred fifty-five genomes were retrieved for pan-genome analysis. To screen target genes for detecting the four *Staphylococcus* species, 388,852 coding genes from 155 staphylococci genomes yielded a pan-genome of 9461 genes consisting of 464 core-genes, 6772 accessory-genes, and 2225 unique-genes. Of the 6772 accessory genes, 4, 13, 19, and 18 genes were common to 35 *S. aureus*, 16 to *S. capitis* genomes, 15 to *S. caprae*, and 28 to *S. epidermidis*. Fifty-four candidate genes were aligned with other sequences through the blast program. The molecular target genes were mined based on their 100% presence in all genomes of the target species and their absence in the genomes of non-target staphylococci. The functions of the molecular target genes selected for the detection of *Staphylococcus* species are as follows: GntR family transcriptional regulator (accession no. ABD29409.1) for *S. aureus*, FAD-dependent urate hydroxylase (accession no. AKL93396.1) for *S. capitis*, Gram-positive signal peptide protein, YSIRK family (accession no. BBD88784.1) for *S. caprae*, and phosphomannomutase (accession no. AAO05683.1) for *S. epidermidis*.

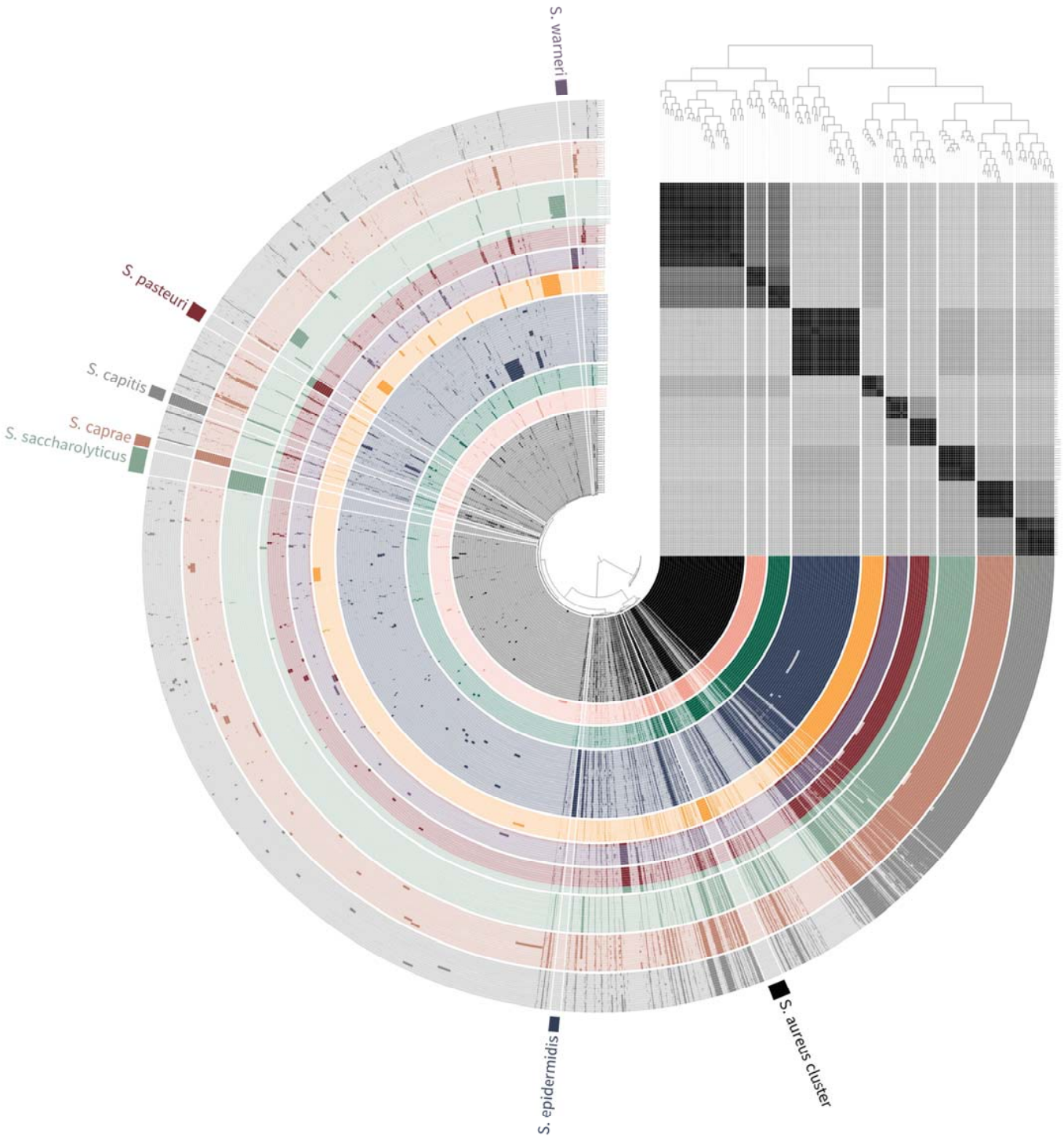

Figure 1. Pan-genome analysis generated with Anvi'o software (version 7.0) for 155 staphylococci genomes. The layers represent individual staphylococci genomes organized by their phylogenomic relationship. In the layers, the dark and bright areas within the bars indicate the presence and absence of genes, respectively. The ANI values are represented by a heatmap determined at high (black) and low (gray) similarities.

The specificity of the discovered molecular target genes in comparison with that of the previously reported target genes in detecting four *Staphylococcus* species was confirmed; we observed higher specificity for our molecular target genes (Table S2). All target genes except the *nuc* gene were present with high similarities (86–100%) not only in the target species but also in the non-target species. These results indicated that the four molecular target genes are suitable for the identification of *Staphylococcus* species.

Specific primers were designed for each molecular target gene, and the specificity was confirmed through in silico analysis. As a result, all primer pairs produced amplicons only in the target species (Table S3). For *S. aureus*-specific primers, we found that certain genome sequences, including those of *Staphylococcus* species MZ1, MZ3, MZ7, MZ8, MZ9, T93, SM3655, and SM9054, produced an amplicon of 145 bp. Eight genomes of *Staphylococcus* species showed 98.83 to 99.87% identity with an *S. aureus*-type strain (DSM 20231), suggesting that these genomes belong to *S. aureus*.

3.3. Specificity and Sensitivity of Real-Time PCR

A real-time PCR was designed to identify the four staphylococci species that cause food poisoning and are often isolated from food [34]. The specificity was tested using 12 target *Staphylococcus* strains, 27 non-target *Staphylococcus* strains, and 73 non-*Staphylococcus* strains (Table 1). The primer sequences and amplicon size are shown in Table 2. All *S. aureus*, *S. capitis*, *S. caprae*, and *S. epidermidis* strains yielded detectable amplicons for the corresponding primer pairs, whereas no amplifications were generated with the non-target *Staphylococcus* strains and non-*Staphylococcus* strains, indicating the high specificity of the four primer pairs (Figure 2).

Table 2. Specific primer information.

Species	Primer	Sequence	Size (bp)
S. aureus	Aureus_F	CAA GCA CAA GGC AGT GGT AT	145
	Aureus_R	GTG GCG TTG CAA TCT CCT TA	
S. capitis	Capitis_F	CGC AAG GTG GTC AAC TTG AT	150
	Capitis_R	GCG CAT CGT GAA GTA ATT CC	
S. caprae	Caprae_F	TCG TCG CAA CGA AGT TCA TC	146
	Caprae_R	CCT GGC GCA TAT GTA TGC TT	
S. epidermidis	Epidermidis_F	TGG CAC GGC TGG TAT TAG AG	121
	Epidermidis_R	GAC AGG ATG CGC GAT ACT TG	

The sensitivity analysis was performed using DNA from serial dilutions of the target bacterial species (10^2–10^8 CFU/mL) as a template. All tests were repeated thrice, and the standard curves are presented in Figure 3. For the detection of *S. aureus*, the sensitivity was 1.5×10^2 CFU/mL. Similarly, the limit of detection of *S. capitis*, *S. caprae*, and *S. epidermidis* was 2.6×10^2 CFU/mL, 1.4×10^2 CFU/mL, and 1.09×10^2 CFU/mL, respectively. The R^2 values were higher than 0.997, indicating that the amounts of DNA showed high linearity with the corresponding Ct values [35]. The equations for *S. aureus*, *S. capitis*, *S. caprae*, and *S. epidermidis* were $y = -3.582x + 41.759$, $y = -3.293x + 39.33$, $y = -3.522x + 41.263$, and $y = -3.134x + 40.753$ respectively. The amplification efficiencies for the four *Staphylococcus* species ranged from 90.20% to 108.50%, indicating high efficiency [35].

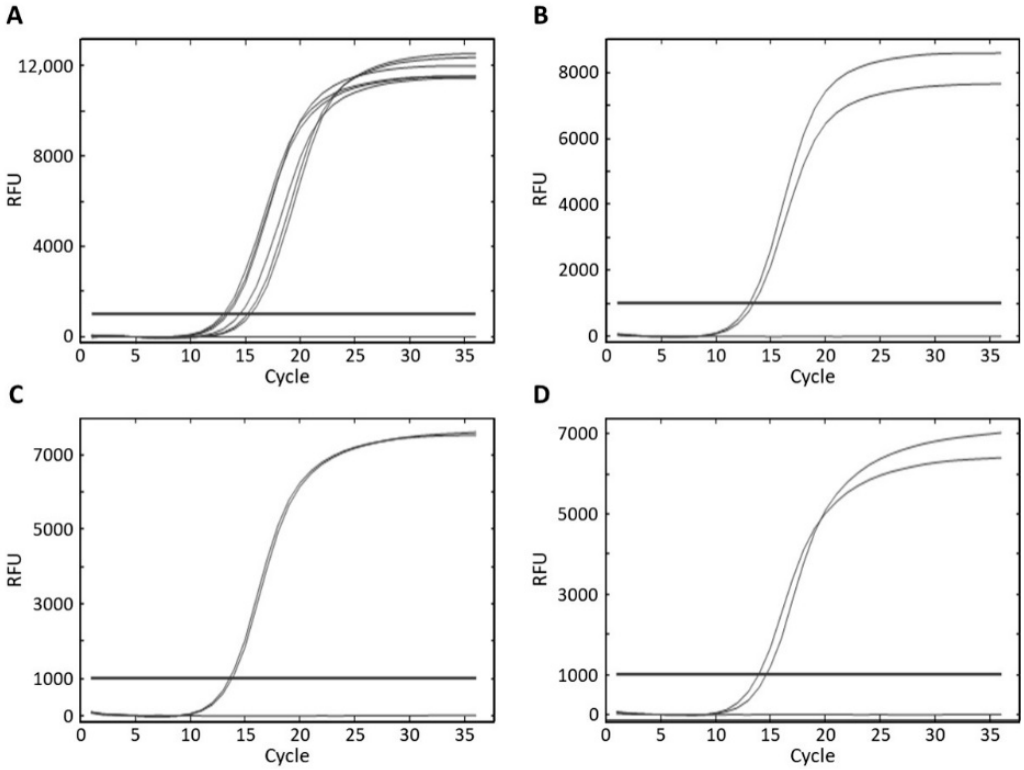

Figure 2. Specificity of the primer pairs for real-time PCR amplification. (**A**) *S. aureus* ATCC 29213, KCTC 1928, NCCP 14560, ATCC25923, ATCC 29737, and ATCC 6538 amplified using the *S. aureus* primer pair; (**B**) *S. capitis* NCCP 14663 and KACC 13242 amplified using the *S. capitis* primer pair; (**C**) *S. caprae* KCTC 3583 and NCCP 15629 amplified using the *S. caprae* primer pair; (**D**) *S. epidermidis* NCCP 14723 and KACC 13234 amplified using the *S. epidermidis* primer pair.

3.4. Detection of Staphylococcus Species in Artificially Contaminated Food Samples

DNA present in a food matrix can impair the efficiency of real-time PCR, as its concentration can be underestimated [36]. In this study, the quantification in food matrix was conducted by artificially adding four *Staphylococcus* species to milk, a food product they mainly inhabit. Simultaneously, the milk samples were inoculated with a cocktail of four pathogenic *Staphylococcus* species to induce competition between strains for the same nutrients. Food samples artificially inoculated with *Staphylococcus* species (average Ct values: 14.11–33.27) had Ct values similar to those of pure cultured bacteria (average Ct values: 13.7–33.82). All standard curves showed high efficiency, with R^2 of 0.998 for *S. aureus*, 0.997 for *S. capitis* and *S. epidermidis*, and 0.994 *S. caprae* (Figure 4). The limit of detection values were 1.5×10^2 CFU/mL for *S. aureus*, 2.6×10^2 CFU/mL for *S. capitis*, 1.4×10^2 CFU/mL for *S. caprae*, and 1.2×10^2 CFU/mL for *S. epidermidis*. The detection limits for the four *Staphylococcus* species in artificially contaminated milk samples were similar to those in pure cultures. These results suggested that the real-time PCR method could detect the four *Staphylococcus* species almost without any interference from the food matrix.

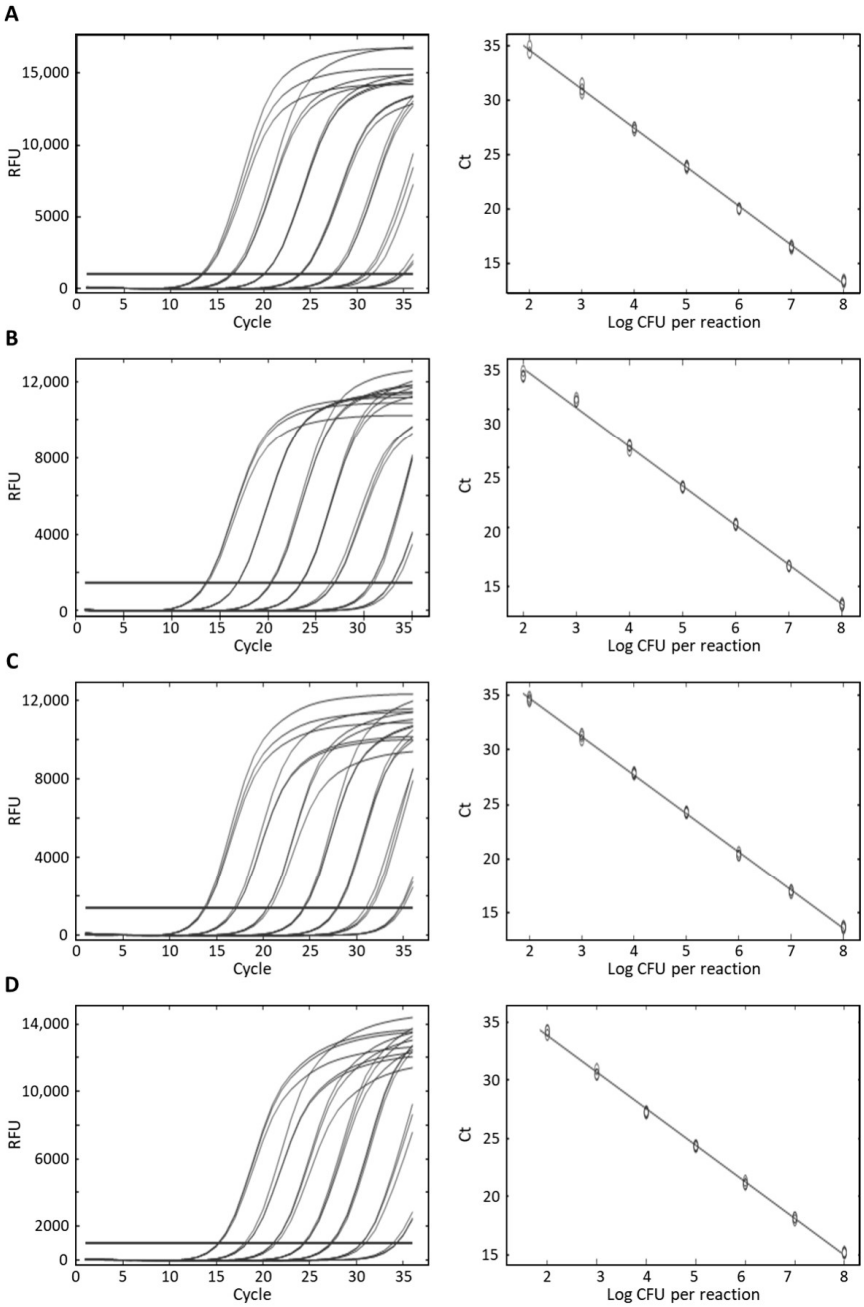

Figure 3. Standard curves by plotting cycle threshold (Ct) values against the logarithm of the number of cells of (**A**) *S. aureus*, (**B**) *S. capitis*, (**C**) *S. caprae*, and (**D**) *S. epidermidis* in pure culture.

3.5. Real-Time PCR Detection of Isolates

A total of 103 strains were isolated from chicken, beef, pork, fish, salted fish, and raw milk. All isolates produced one amplification curve: 36 isolates (34.95%), 63 isolates (61.17%), and 4 isolates (3.88%) were identified as *S. aureus*, *S. epidermidis*, and *S. capitis* (Table 3). *S. aureus* was isolated from meat such as chicken, beef, pork, and fish. *S. epidermidis* was isolated from pork, salted fish, and raw milk. *S. capitis* was isolated only from salted fishes, and *S. caprae* was not isolated from any sample.

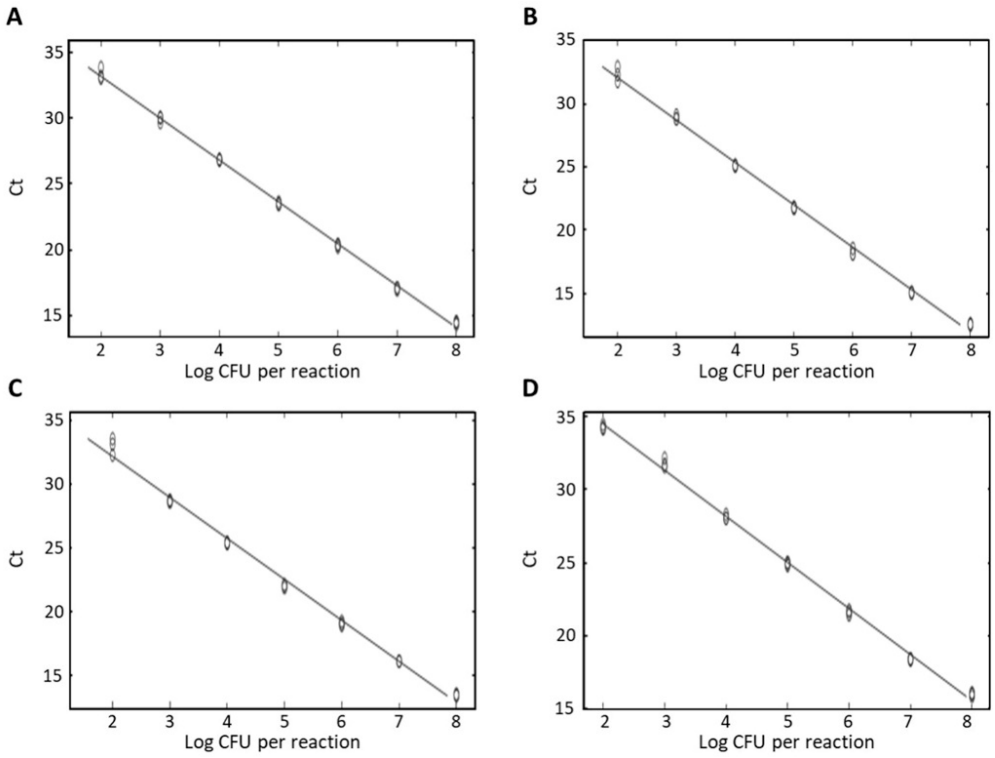

Figure 4. Limit of detection for (**A**) *S. aureus*, (**B**) *S. capitis*, (**C**) *S. caprae*, and (**D**) *S. epidermidis* in spiked milk samples.

Table 3. Identification of strains isolated from food samples.

Sample Type	No. of Isolates	No. of Positive Results by Real-Time PCR			
		S. Aureus	*S. Capitis*	*S. Caprae*	*S. Epidermidis*
chicken (n = 4)	12	12	0	0	0
beef (n = 3)	11	11	0	0	0
pork (n = 5)	38	7	0	0	31
fish (n = 3)	6	6	0	0	0
salted fish (n = 5)	22	0	4	0	18
raw milk (n = 2)	14	0	0	0	14
Total (n = 22)	103	36	4	0	63

3.6. Detection of Contamination by the Four Staphylococcus Species in Food Samples

To confirm the efficacy of real-time PCR for the detection of *Staphylococcus* species contamination in food samples, 50 samples were tested using the real-time PCR method developed in this study. *S. aureus* was detected in 11 samples of pork and raw milk, and *S. epidermidis* was detected in 9 samples of raw milk. *S. capitis* was detected in three samples of fermented fish and raw milk. *S. caprae* was not present in any of the food samples. The result showed that the detection rates of the *Staphylococcus* species were 22% for *S. aureus*, 6% for *S. capitis*, and 18% for *S. epidermidis* (Table 4). These results are consistent with a previous study suggesting that *S. epidermidis* and *S. aureus* are the main *Staphylococcus* species contaminating food [1].

Table 4. Identification of *Staphylococcus* contamination in food samples using the real-time PCR method developed in this study.

Sample Type	No. of Samples	No. of Positive Results by Real-Time PCR			
		S. Aureus	*S. Capitis*	*S. Caprae*	*S. Epidermidis*
beef	2	0	0	0	0
pork	8	1	0	0	0
lettuce	1	0	0	0	0
cucumber	1	0	0	0	0
raw milk	34	10	2	0	9
fermented fish	4	0	1	0	0
Total	50	11	3	0	9

4. Discussion

S. aureus and *S. epidermidis* are important pathogenic bacteria that cause clinical infections and food contamination [7,37]. These two pathogens are isolated in a wide range of foods, such as vegetables, meat, and fish [38]. *S. capitis* and *S. caprae* are species closely related to *S. epidermidis*, an opportunistic CoNS. They contaminate milk or meat and have been isolated from fermented foods such as cheese [34]. Therefore, developing reliable and rapid methods to detect these four pathogenic *Staphylococcus* species has become increasingly important to protect public health and ensure food safety [17]. Here, we developed a rapid and accurate detection method for four pathogenic *Staphylococcus* species based on novel molecular target genes.

Molecular detection methods play an important role in rapidly detecting pathogenic bacteria [27]. The usefulness of molecular detection methods is dependent on the target genes or sequences and the specificity of specific primer pairs [27]. The current PCR methods for pathogenic staphylococci target 16S rRNA genes, housekeeping genes, or virulence genes [26,38]. However, a previous study has reported that the 16S rRNA genes of the *S. epidermidis* group share high sequence similarities ($\geq$97%) and do not exhibit sufficient variability to allow differentiating the species [39]. In addition, the lack of virulence genes can result in misclassification, posing a potential threat of food poisoning [1,40]. As numerous whole-genome sequences become available with the development of genome sequencing technologies, many researchers are committed to the search for novel molecular target genes to replace the current markers that exhibit poor specificity [20,21,40,41]. In this study, pan-genome analysis was utilized for discovering molecular target genes of *Staphylococcus* species. We successfully identified molecular target genes specific for four *Staphylococcus* species via a pan-genome analysis. At the same time, we found misclassified staphylococci genomes. Through pan-genome analysis, we found that four molecular target genes were 100% specific for identifying *Staphylococcus* species and did not match other bacterial genes.

PCR methods are specific for detecting causative pathogens of infectious diseases and for discriminating closely related species [42]. Previous studies have reported that the PCR-based detection method of *Staphylococcus* species is more rapid, easier, and sensitive than traditional methods [1,7,11,43]. Real-time PCR methods provide a tool for the sensitive and accurate quantification of target bacteria, which could be applied to detect *Staphylococcus* species in foods [22,23,44]. Although several real-time PCR methods for detecting *Staphylococcus* species have been reported, their target genes or sequences have been shown poor specificity [1]. Recently, a real-time PCR method targeting novel specific genes obtained by a pan-genome analysis for the accurate detection of *Staphylococcus* species has been developed [1]. This method displayed a better specificity than the previous real-time PCR method. However, for monitoring *Staphylococcus* species using existing real-time PCR methods, previous studies focused on discovering novel genes for *S. aureus* and *S. epidermidis* [1], while no target genes for *S. capitis* and *S. caprae*, which are closely related species to *S. epidermidis*, have been reported. In this study, we successfully identified molecular target genes for *S. capitis* and *S. caprae* with high specificity and sensitivity.

More surprisingly, the detection limit of *S. aureus* (10^2 CFU/mL) was equivalent to that of previously reported target genes [1]. In contrast, the detection limit for *S. epidermidis* molecular target gene showed an obvious advantage in this study. The real-time PCR method developed in this study maintained a good consistency in detecting the four *Staphylococcus* species, without interference from the food matrix. Moreover, this method was successfully applied to 103 strains isolated from chicken, beef, pork, fish, salted fish, and raw milk. These results indicate that the molecular target genes discovered in this study have specificity in real-time PCR analysis, allowing the rapid, accurate, and sensitive detection of the four *Staphylococcus* species in food matrix.

In conclusion, we successfully mined four molecular target genes for the four *Staphylococcus* species *S. aureus*, *S. capitis*, *S. caprae*, and *S. epidermidis*. We developed a real-time PCR to detect the four *Staphylococcus* species with high specificity and high sensitivity. Our real-time PCR method was able to successfully detect the four pathogenic *Staphylococcus* species in food. Our data show that the method has a great potential as an accurate, rapid, and sensitive tool to monitor potential pathogenic *Staphylococcus* species in food samples.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10112839/s1, Table S1: Summary of the genome features of 155 staphylococci strains, Table S2: Presence of novel and reported molecular target genes for target and non-target genomes, Table S3: Specificity of the designed primer pairs using primer-BLAST tool.

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References

1. Zhou, B.; Ye, Q.; Chen, M.; Li, F.; Xiang, X.; Shang, Y.; Wang, C.; Zhang, J.; Xue, L.; Wang, J.; et al. Novel species-specific targets for real-time PCR detection of four common pathogenic *Staphylococcus* spp. *Food Control* **2022**, *131*, 108478. [CrossRef]
2. Argudín, M.Á.; Mendoza, M.C.; Rodicio, M.R. Food Poisoning and *Staphylococcus aureus* Enterotoxins. *Toxins* **2010**, *2*, 1751–1773. [CrossRef]
3. Becker, K.; Heilmann, C.; Peters, G. Coagulase-negative staphylococci. *Clin. Microbiol. Rev.* **2014**, *27*, 870–926. [CrossRef]
4. Cameron, D.R.; Jiang, J.H.; Hassan, K.A.; Elbourne, L.D.H.; Tuck, K.L.; Paulsen, I.T.; Peleg, A.Y. Insights on virulence from the complete genome of *Staphylococcus capitis*. *Front. Microbiol.* **2015**, *6*, 980. [CrossRef] [PubMed]
5. Watanabe, S.; Aiba, Y.; Tan, X.E.; Li, F.Y.; Boonsiri, T.; Thitiananpakorn, K.; Cui, B.; Sato'O, Y.; Kiga, K.; Sasahara, T.; et al. Complete genome sequencing of three human clinical isolates of *Staphylococcus caprae* reveals virulence factors similar to those of *S. epidermidis* and *S. capitis*. *BMC Genom.* **2018**, *19*, 810. [CrossRef]
6. Ahle, C.M.; Stødkilde, K.; Afshar, M.; Poehlein, A.; Ogilvie, L.A.; Söderquist, B.; Hüpeden, J.; Brüggemann, H. *Staphylococcus saccharolyticus*: An overlooked human skin colonizer. *Microorganisms* **2020**, *8*, 1105. [CrossRef] [PubMed]
7. Geng, Y.; Liu, S.; Wang, J.; Nan, H.; Liu, L.; Sun, X.; Li, D.; Liu, M.; Wang, J.; Tan, K. Rapid Detection of *Staphylococcus aureus* in Food Using a Recombinase Polymerase Amplification-Based Assay. *Food Anal. Methods* **2018**, *11*, 2847–2856. [CrossRef]
8. Xie, X.; Liu, Z. Simultaneous enumeration of Cronobacter sakazakii and *Staphylococcus aureus* in powdered infant foods through duplex TaqMan real-time PCR. *Int. Dairy J.* **2021**, *117*, 105019. [CrossRef]
9. Zhao, X.; Zhong, J.; Wei, C.; Lin, C.W.; Ding, T. Current perspectives on viable but non-culturable state in foodborne pathogens. *Front. Microbiol.* **2017**, *8*, 580. [CrossRef]
10. Ćirković, I.; Hauschild, T.; Ježek, P.; Dimitrijević, V.; Vuković, D.; Stepanović, S. Identification and antimicrobial susceptibility testing of *Staphylococcus* vitulinus by the BD Phoenix automated microbiology system. *Curr. Microbiol.* **2008**, *57*, 158–160. [CrossRef]
11. Kim, J.; Hong, J.; Lim, J.A.; Heu, S.; Roh, E. Improved multiplex PCR primers for rapid identification of coagulase-negative staphylococci. *Arch. Microbiol.* **2018**, *200*, 73–83. [CrossRef]

12. Kim, E.; Kim, H.J.; Yang, S.M.; Kim, C.G.; Choo, D.W.; Kim, H.Y. Rapid identification of *Staphylococcus* species isolated from food samples by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Microbiol. Biotechnol.* **2019**, *29*, 548–557. [CrossRef] [PubMed]
13. Koop, G.; De Visscher, A.; Collar, C.A.; Bacon, D.A.C.; Maga, E.A.; Murray, J.D.; Supré, K.; De Vliegher, S.; Haesebrouck, F.; Rowe, J.D.; et al. Short communication: Identification of coagulase-negative *Staphylococcus* species from goat milk with the API Staph identification test and with transfer RNA-intergenic spacer PCR combined with capillary electrophoresis. *J. Dairy Sci.* **2012**, *95*, 7200–7205. [CrossRef]
14. González-Domínguez, M.S.; Carvajal, H.D.; Calle-Echeverri, D.A.; Chinchilla-Cárdenas, D. Molecular Detection and Characterization of the *mecA* and *nuc* Genes From *Staphylococcus* Species (*S. aureus*, S. pseudintermedius, and S. schleiferi) Isolated From Dogs Suffering Superficial Pyoderma and Their Antimicrobial Resistance Profiles. *Front. Vet. Sci.* **2020**, *7*, 376. [CrossRef]
15. Szaluś-Jordanow, O.; Chrobak, D.; Pyrgiel, M.; Lutyńska, A.; Kaba, J.; Czopowicz, M.; Witkowski, L.; Kizerwetter-Świda, M.; Binek, M.; Frymus, T. PFGE and AFLP genotyping of *Staphylococcus aureus* subsp. anaerobius isolated from goats with Morel's disease. *Arch. Microbiol.* **2013**, *195*, 37–41. [CrossRef] [PubMed]
16. Mandal, P.K.; Biswas, A.K.; Choi, K.; Pal, U.K. Methods for rapid detection of foodborne pathogens: An overview. *Am. J. Food Technol.* **2011**, *6*, 87–102. [CrossRef]
17. Wei, S.; Daliri, E.B.M.; Chelliah, R.; Park, B.J.; Lim, J.S.; Baek, M.A.; Nam, Y.S.; Seo, K.H.; Jin, Y.G.; Oh, D.H. Development of a multiplex real-time PCR for simultaneous detection of Bacillus cereus, Listeria monocytogenes, and *Staphylococcus aureus* in food samples. *J. Food Saf.* **2019**, *39*, e12558. [CrossRef]
18. Yang, S.-M.; Kim, E.; Kim, D.; Kim, H.-B.; Baek, J.; Ko, S.; Kim, D.; Yoon, H.; Kim, H.-Y. Rapid Real-Time Polymerase Chain Reaction for Salmonella Serotyping Based on Novel Unique Gene Markers by Pangenome Analysis. *Front. Microbiol.* **2021**, *12*, 750379. [CrossRef] [PubMed]
19. Kim, E.; Kim, H.B.; Yang, S.M.; Kim, D.; Kim, H.Y. Real-time PCR assay for detecting Lactobacillus plantarum group using species/subspecies-specific genes identified by comparative genomics. *LWT* **2021**, *138*, 110789. [CrossRef]
20. Kim, E.; Yang, S.M.; Kim, H.Y. Differentiation of lacticaseibacillus zeae using pan-genome analysis and real-time pcr method targeting a unique gene. *Foods* **2021**, *10*, 2112. [CrossRef]
21. Kim, E.; Yang, S.M.; Kim, D.; Kim, H.Y. Real-time PCR method for qualitative and quantitative detection of Lactobacillus sakei group species targeting novel markers based on bioinformatics analysis. *Int. J. Food Microbiol.* **2021**, *355*, 109335. [CrossRef] [PubMed]
22. Rodríguez, A.; Gordillo, R.; Andrade, M.J.; Córdoba, J.J.; Rodríguez, M. Development of an efficient real-time PCR assay to quantify enterotoxin-producing staphylococci in meat products. *Food Control* **2016**, *60*, 302–308. [CrossRef]
23. Kilic, A.; Basustaoglu, A.C. Double triplex real-time PCR assay for simultaneous detection of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus* hominis, and *Staphylococcus* haemolyticus and determination of their methicillin resistance directly from positive blood cult. *Res. Microbiol.* **2011**, *162*, 1060–1066. [CrossRef]
24. Shah, M.M.; Iihara, H.; Noda, M.; Song, S.X.; Nhung, P.H.; Ohkusu, K.; Kawamura, Y.; Ezaki, T. *dnaJ* gene sequence-based assay for species identification and phylogenetic grouping in the genus *Staphylococcus*. *Int. J. Syst. Evol. Microbiol.* **2007**, *57*, 25–30. [CrossRef] [PubMed]
25. Zhang, Z.; Liu, W.; Xu, H.; Aguilar, Z.P.; Shah, N.P.; Wei, H. Propidium monoazide combined with real-time PCR for selective detection of viable *Staphylococcus aureus* in milk powder and meat products. *J. Dairy Sci.* **2015**, *98*, 1625–1633. [CrossRef] [PubMed]
26. Okolie, C.E.; Wooldridge, K.G.; Turner, D.P.J.; Cockayne, A.; James, R. Development of a heptaplex PCR assay for identification of *Staphylococcus aureus* and CoNS with simultaneous detection of virulence and antibiotic resistance genes. *BMC Microbiol.* **2015**, *15*, 157. [CrossRef] [PubMed]
27. Shang, Y.; Ye, Q.; Cai, S.; Wu, Q.; Pang, R.; Yang, S.; Xiang, X.; Wang, C.; Zha, F.; Ding, Y.; et al. Loop-mediated isothermal amplification (LAMP) for rapid detection of Salmonella in foods based on new molecular targets. *LWT* **2021**, *142*, 110999. [CrossRef]
28. Eren, A.M.; Esen, O.C.; Quince, C.; Vineis, J.H.; Morrison, H.G.; Sogin, M.L.; Delmont, T.O. Anvi'o: An advanced analysis and visualization platformfor 'omics data. *PeerJ* **2015**, *3*, e1319. [CrossRef]
29. Pritchard, L.; Glover, R.H.; Humphris, S.; Elphinstone, J.G.; Toth, I.K. Genomics and taxonomy in diagnostics for food security: Soft-rotting enterobacterial plant pathogens. *Anal. Methods* **2016**, *8*, 12–24. [CrossRef]
30. Chaudhari, N.M.; Gupta, V.K.; Dutta, C. BPGA-an ultra-fast pan-genome analysis pipeline. *Sci. Rep.* **2016**, *6*, 24373. [CrossRef]
31. Ye, J.; Coulouris, G.; Zaretskaya, I.; Cutcutache, I.; Rozen, S.; Madden, T.L. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinform.* **2012**, *13*, 134. [CrossRef]
32. Tran, P.N.; Savka, M.A.; Gan, H.M. In-silico taxonomic classification of 373 genomes reveals species misidentification and new genospecies within the genus Pseudomonas. *Front. Microbiol.* **2017**, *8*, 1296. [CrossRef]
33. Wuyts, S.; Wittouck, S.; De Boeck, I.; Allonsius, C.N.; Pasolli, E.; Segata, N.; Lebeer, S. Large-Scale Phylogenomics of the Lactobacillus casei Group Highlights Taxonomic Inconsistencies and Reveals Novel Clade-Associated Features. *mSystems* **2017**, *2*, e00061-17. [CrossRef]
34. Irlinger, F. Safety assessment of dairy microorganisms: Coagulase-negative staphylococci. *Int. J. Food Microbiol.* **2008**, *126*, 302–310. [CrossRef]

35. Broeders, S.; Huber, I.; Grohmann, L.; Berben, G.; Taverniers, I.; Mazzara, M.; Roosens, N.; Morisset, D. Guidelines for validation of qualitative real-time PCR methods. *Trends Food Sci. Technol.* **2014**, *37*, 115–126. [CrossRef]
36. Singh, P.; Mustapha, A. Multiplex real-time PCR assays for detection of eight Shiga toxin-producing Escherichia coli in food samples by melting curve analysis. *Int. J. Food Microbiol.* **2015**, *215*, 101–108. [CrossRef]
37. Sah, S.; Bordoloi, P.; Vijaya, D.; Amarnath, S.K.; Sheela Devi, C.; Indumathi, V.A.; Prashanth, K. Simple and economical method for identification and speciation of *Staphylococcus epidermidis* and other coagulase negative Staphylococci and its validation by molecular methods. *J. Microbiol. Methods* **2018**, *149*, 106–119. [CrossRef] [PubMed]
38. Marino, M.; Frigo, F.; Bartolomeoli, I.; Maifreni, M. Safety-related properties of staphylococci isolated from food and food environments. *J. Appl. Microbiol.* **2011**, *110*, 550–561. [CrossRef] [PubMed]
39. Ghebremedhin, B.; Layer, F.; König, W.; König, B. Genetic classification and distinguishing of *Staphylococcus* species based on different partial *gap*, 16S rRNA, *hsp60*, *rpoB*, *sodA*, and *tuf* gene sequences. *J. Clin. Microbiol.* **2008**, *46*, 1019–1025. [CrossRef] [PubMed]
40. Li, F.; Ye, Q.; Chen, M.; Zhang, J.; Xue, L.; Wang, J.; Wu, S.; Zeng, H.; Gu, Q.; Zhang, Y.; et al. Multiplex PCR for the Identification of Pathogenic Listeria in Flammulina velutipes Plant Based on Novel Specific Targets Revealed by Pan-Genome Analysis. *Front. Microbiol.* **2021**, *11*, 634225. [CrossRef]
41. Li, F.; Ye, Q.; Chen, M.; Zhou, B.; Xiang, X.; Wang, C.; Shang, Y.; Zhang, J.; Pang, R.; Wang, J.; et al. Mining of novel target genes through pan-genome analysis for multiplex PCR differentiation of the major Listeria monocytogenes serotypes. *Int. J. Food Microbiol.* **2021**, *339*, 109026. [CrossRef] [PubMed]
42. Xiong, D.; Song, L.; Pan, Z.; Jiao, X. Identification and Discrimination of Salmonella enterica Serovar Gallinarum Biovars Pullorum and Gallinarum Based on a One-Step Multiplex PCR Assay. *Front. Microbiol.* **2018**, *9*, 1718. [CrossRef]
43. Ma, B.; Li, J.; Chen, K.; Yu, X.; Sun, C.; Zhang, M. Multiplex recombinase polymerase amplification assay for the simultaneous detection of three foodborne pathogens in seafood. *Foods* **2020**, *9*, 278. [CrossRef] [PubMed]
44. Kilic, A.; Muldrew, K.L.; Tang, Y.W.; Basustaoglu, A.C. Triplex real-time polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and coagulase-negative staphylococci and determination of methicillin resistance directly from positive blood culture bottles. *Diagn. Microbiol. Infect. Dis.* **2010**, *66*, 349–355. [CrossRef] [PubMed]