Inhibition of *Listeria monocytogenes* in Cold-Smoked Salmon by *Carnobacterium piscicola* CS526 Isolated from Frozen Surimi

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**ABSTRACT**

Strain CS526 was isolated from frozen surimi and identified as a bacteriocin producer that had strong inhibitory activity against *Listeria monocytogenes*. Strain CS526 was identified as *Carnobacterium piscicola* by partial 16S rDNA sequence similarity. The ability of this bacteriocinogenic strain and nonbacteriocinogenic *C. piscicola* JCM5348 to inhibit the growth of *L. monocytogenes* was examined in culture broth incubated at 12°C and cold-smoked salmon stored at 4, 12, and 20°C. *L. monocytogenes* viable counts in the culture broth rapidly declined from 10⁶ colony-forming units per ml to less than 10 colony-forming units per ml within 1 day at 12°C in the presence of *C. piscicola* CS526. At 4 and 12°C, inhibition of *L. monocytogenes* on salmon depended on the initial inoculum level of *C. piscicola* CS526. However, *C. piscicola* CS526 was bactericidal to *L. monocytogenes* within 21 and 12 days at 4 and 12°C in cold-smoked salmon, respectively, even when the initial inoculum levels were low. *C. piscicola* CS526 suppressed the maximum cell number of *L. monocytogenes* by two and three log cycles, even at 20°C. However, *C. piscicola* JCM5348 did not prevent the growth of the pathogen, except at 4°C. Bacteriocin was detected in the samples coinoculated with *C. piscicola* CS526. The study shows that *C. piscicola* CS526 might have potential for biopreservation of refrigerated foods against *L. monocytogenes*.

**Listeria monocytogenes** is well-recognized as a foodborne psychrotrophic pathogen for humans, especially for pregnant women, infants, immunocompromised patients, and elderly people. This microorganism is commonly found in nature and can be detected from a wide variety of raw and ready-to-eat foods. Recently, raw fish and seafood products have been reported to be contaminated by *L. monocytogenes* (14). The isolation frequency of *L. monocytogenes* from smoked seafood is especially high (11, 27). Eklund et al. (12) showed that contamination could arise from water, raw materials, or the processing chain. However, it would be quite difficult to remove this microorganism completely from fish and processed seafood products because of its ubiquitous presence on fish surfaces and in the environment. The presence of psychrotrophic *L. monocytogenes* in smoked and lightly processed ready-to-eat fish products is a menace, because some of these products are often stored for a long period of time under refrigeration temperature and are commonly eaten without further heating.

Bacteriocin-producing lactic acid bacteria (LAB) and their bacteriocins have recently received greater interest because of their inhibitory activity against food spoilage and foodborne pathogenic bacteria, such as *L. monocytogenes* and *Clostridium botulinum* (9, 25). Natural antibacterial substances have been considered as food biopreservatives to reduce the wide use of chemical preservatives. Numerous studies about bacteriocins produced by LAB have been reported, but few have reported antagonism by bacteriocin producers in fisheries products (4, 8, 19, 21). The purpose of this study was to identify an antilisterial strain isolated from frozen surimi and to evaluate this strain for inhibition of *L. monocytogenes* in cold-smoked salmon stored at refrigeration temperatures.

**MATERIALS AND METHODS**

**Bacterial strains and media.** *L. monocytogenes* IID581 (serotype 4b) was used as the indicator strain for antilisterial antagonism experiments and activity measurements. *Carnobacterium piscicola* JCM5348, a bacteriocin-negative (Bac) strain isolated from a diseased adult cutthroat trout, was used as a control. All cultures were maintained as slants on tryptic soy agar (Difco, Sparks, Md.) supplemented with 0.6% yeast extract (TSYE agar, Difco) at 4°C and were transferred monthly. Before experimental use, all strains were propagated in tryptic soy broth (Difco) supplemented with 0.6% yeast extract (TSYE broth) at 30°C for 18 to 24 h.

**Isolation of bacteriocin-producing bacteria.** TSYE agar was used for the isolation of bacteria from retail raw fish and fisheries products. Five grams of the sample was aseptically transferred to a stomacher bag, and 45 ml of TSYE broth was added. The samples were homogenized for 1 min in a stomacher (Stomacher 400 Circulator, Seward, UK). Colonies of strains were grown on the agar plates at 30°C for 48 h. A lawn of 6 ml of TSYE soft agar (0.6% agar) containing *L. monocytogenes* IID581 (ca. 10⁶ CFU/ml) was then poured over the plates. After incubation for 24 h at 30°C, the colonies showed that growth inhibition zones around them were isolated.

**Identification of a bacteriocin producer.** From a total count of 3.0 × 10⁵ CFU/g surimi, three antilisterial strains were isolated.

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One isolate, designated CS526, showed strong antilisterial activity (Bac+) and was examined by phase-contrast microscopy for cell morphology, Gram stain reaction, catalase activity, and biochemical carbohydrate fermentation patterns with the API 50 CHL kit (bioMérieux, Marcy l’Etoile, France).

Total DNA was extracted and purified from the strain with InstaGene purification matrix (Bio-Rad Laboratories, Richmond, Calif.) according to the protocol for bacteria. The 16S rRNA gene was amplified by polymerase chain reaction (PCR). The sequences of primers used for amplification were 5'-AGAGTTTGATCCTGCGCTCAG-3' and 5'-AAGGAGGTTGATCAGCCCGCA-3', corresponding to positions 8 through 27 and 1,521 through 1,540, respectively, in the 16S rRNA sequence of Escherichia coli (3). The 1.5-kb PCR product was directly sequenced by the dideoxynucleotide chain termination method using a DNA sequencer (model 373A, Applied Biosystems, Foster City, Calif.). The similarity values of the sequence were calculated by the FASTA search program of DDBJ (DNA database of Japan).

**Coinoculation of C. piscicola CS526 and L. monocytogenes in culture broth.** Overnight TSYE broth cultures (1 ml) of C. piscicola CS526 or L. monocytogenes IID581 were centrifuged (5,000 × g, 2 min). The pellet was washed five times with sterile distilled water and resuspended in sterile distilled water to obtain the desired inoculum level. Aliquots of 0.1 ml of sterile distilled water containing the inoculum were added into TSYE broth (pH 7.0), giving a final inoculum level of 10^6 CFU/ml of C. piscicola CS526 and 10^6 CFU/ml of L. monocytogenes IID581. In each experiment, samples of C. piscicola CS526 inoculated alone at 10^6 CFU/ml and L. monocytogenes IID581 inoculated alone at 10^6 CFU/ml were used as controls. The inoculated samples were incubated at 12°C for 7 days.

L. monocytogenes was enumerated by surface plating appropriate 0.1-ml dilutions onto Oxford medium base (Difco) containing Oxford antimicrobial supplement (Difco) with incubation at 30°C for 48 h. C. piscicola populations were determined by surface plating on MRS (Difco) containing 1.5% agar (BBL, Cockeysville, Md.) at 30°C for 48 h.

**Coinoculation of C. piscicola and L. monocytogenes in cold-smoked salmon.** Smoked salmon (salt concentration, 4.0%; water activity, 0.98; wood burning) was purchased from a local supermarket. One hundred grams of smoked salmon were aseptically placed into individual stomacher bags. Overnight TSYE broth cultures of C. piscicola CS526, C. piscicola JCM5348, or L. monocytogenes IID581 were centrifuged (5,000 × g, 2 min). The pellets were washed five times with sterile distilled water and resuspended in sterile distilled water to obtain the desired inoculum level. One milliliter of each suspension was added to the smoked salmon, giving a final inoculum level of 10^6 CFU/g of C. piscicola CS526 or C. piscicola JCM5348 or L. monocytogenes or L. monocytogenes IID581. Five grams of the sample were aseptically transferred to a new stomacher bag, and 20 ml of sterile distilled water were added. The samples were homogenized for 1 min in a stomacher (Model 400, Dynatech Laboratories, Alexandria, Va.). L. monocytogenes and lactic acid bacteria, including C. piscicola CS526 or C. piscicola JCM5348, were enumerated using Oxford agar and MRS agar, respectively, as described above. Total aerobic plate counts were determined using TSA plates with incubation at 30°C for 48 h. Because TSA allows the growth of L. monocytogenes and the indigenous flora, the difference between counts on TSA and Oxford medium was used to determine the level of indigenous flora. The detection threshold was 50 CFU/g. The pH of each sample was determined by inserting a pH electrode into the homogenized sample.

**Agar diffusion assay for bacteriocin production.** Bacteriocin activity was quantified using the well diffusion method as follows. L. monocytogenes IID581 was grown in TSYE broth at 30°C overnight and diluted to 10^6 to 10^8 CFU/ml in tempered TSYE agar. The inoculated agar (18 ml) was pipetted into sterile petri dishes, and 6-mm wells were cut after the agar solidified. One milliliter of the homogenized sample or overnight culture was microcentrifuged at 10,000 × g for 5 min to obtain cell-free supernatant. Fifty microliters of serial 1.5-fold dilutions of the supernatant was pipetted into the wells. The bacteriocin titer was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator strain after incubating at 30°C for 18 h and expressed as arbitrary units (AU) per milliliter.

The proteinaceous nature of the bacteriocin was confirmed by adding 50 μl of 1.0 mg/ml proteinase K (Sigma), α-chymotrypsin (Sigma), or catalase (Sigma) in 5 mM sodium phosphate buffer (pH 7.2) into a 6.0-mm well cut adjacent to cell-free supernatant of the culture or the lowest dilution well of bacteriocin assay, as described by Winkowski and Montville (25). A negation of the clear zone around the protease indicated that the inhibitor was sensitive to the protease and confirmed the proteinaceous nature of the inhibitory substance.

**RESULTS AND DISCUSSION**

**Characterization of the bacteriocin-producing strain CS526.** Strain CS526, isolated from surimi, was a gram-positive, non-spore-forming, nonmotile, catalase-negative, rod-shaped bacterium. The strain fermented glucose but did not produce gas and was also able to ferment glycerol, ribose, D-galactose, D-fructose, D-mannose, mannitol, glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, saccharose, trehalose, and gentiobiose on the API 50 CHL test. The strain did not produce dextran from sucrose. It was able to grow at 4°C or pH 8.0, but no growth was observed at 40°C or pH 4.5 in TSYE broth.

Chromosomal DNA was extracted and used as the template for PCR performed with the two primers of 16S rDNA. The nucleotide sequence of the PCR product was determined directly using primer 8-27 (data not shown). The partial 16S rDNA sequence (318 bp) of strain CS526 exhibited 100 and 99.7% similarities to the 16S rDNA sequences of C. piscicola (accession no. M58812 and X54268, respectively). However, the 16S rDNA sequence of strain CS526 had 98.1 and 97.2% similarities to the 16S rDNA sequences of C. gallinarum (accession no. AJ387905) and C. maltaromicus (accession no. M58825), respectively. We concluded that strain CS526 was a strain of C. piscicola, and it was designated C. piscicola CS526.

The cell-free supernatant of C. piscicola CS526 inhibited L. monocytogenes IID581 in the well diffusion assay. Inhibition activity was removed by treatment with protein-
ase K or α-chymotrypsin, but not by treatment with catalase (Fig. 1). The cell-free supernatant of C. piscicola CS526 also inhibited other L. monocytogenes strains isolated from food and river water (data not shown). This indicated that the inhibitor was proteinaceous in nature. Thus, the antilisterial substance produced by C. piscicola CS526 was confirmed as a bacteriocin.

The genus Carnobacterium was proposed by Collins et al. (7) for certain members (nonaciduric lactobacilli) of the genus Lactobacillus. Four Carnobacterium species (C. divergens, C. gallinarum, C. mobile, and C. piscicola) were included in Bergey’s Manual of Determinative Bacteriology (13). They are commonly detected on vacuum-packaged meats, sh, and related products (7). In general, the addition of bacteriocin-producing LAB to food such as ready-to-eat products is a powerful tool in controlling the growth of foodborne pathogens, but LAB often affect flavor and texture by acidification of the products (19). Therefore, carnobacteria, which are nonaciduric organisms, might be better for biopreservation of food products because they will not acidify them.

Effect of coinoculation with C. piscicola CS526 on L. monocytogenes IID581 in culture broth and cold-smoked salmon. Growth of L. monocytogenes and C. piscicola CS526 coinoculated in TSYE broth at 12°C is shown in Figure 2. In the absence of C. piscicola, the L. monocytogenes population increased from $10^3$ to $10^6$ CFU/g after 42 days at 4°C (Fig. 3A and 3B), 17 days at 12°C (Fig. 3C and 3D), and 2 days at 20°C (Fig. 3E and 3F). Both C. piscicola CS526 (Bac− strain) and C. piscicola JCM5348 (Bac− strain) grew rapidly at each temperature in cold-smoked salmon from $10^6$ to $10^8$ CFU/g (initial inoculation level) to approximately $10^8$ to $10^9$ CFU/g throughout the storage period (Fig. 3A through 3F), because they are psychrotrophic and halophilic bacteria (7). The L. monocytogenes population decreased to below the detection limit (50 CFU/g) after 7 days at 4 and 12°C when the initial inoculum levels of L. monocytogenes IID581 were $10^6$ CFU/g (initial inoculation level) to approximately $10^4$ to $10^5$ CFU/g at 20°C. Some strains of this genus were reported as bacteriocin-producing strains that were active against L. monocytogenes (15, 24). In general, the addition of bacteriocin-producing LAB to food such as ready-to-eat products is a powerful tool in preventing the growth of foodborne pathogens, but LAB often affect flavor and texture by acidification of the products (19). Therefore, carnobacteria, which are nonaciduric organisms, might be better for biopreservation of food products because they will not acidify them.

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L. monocytogenes colonies were sensitive to the bacteriocin produced by C. piscicola CS526 (data not shown). The inhibitory effect of C. piscicola CS526 against L. monocytogenes was enhanced at refrigeration temperature. C. piscicola at 4, 12, and 20°C are shown in Figure 3. In the absence of C. piscicola, the L. monocytogenes population increased from $10^3$ to $10^6$ CFU/g after 42 days at 4°C (Fig. 3A and 3B), 17 days at 12°C (Fig. 3C and 3D), and 2 days at 20°C (Fig. 3E and 3F). Both C. piscicola CS526 (Bac− strain) and C. piscicola JCM5348 (Bac− strain) grew rapidly at each temperature in cold-smoked salmon from $10^6$ to $10^8$ CFU/g (initial inoculation level) to approximately $10^8$ to $10^9$ CFU/g throughout the storage period (Fig. 3A through 3F), because they are psychrotrophic and halophilic bacteria (7).
piscicola LK5, C. piscicola CP5, and C. piscicola DX also showed an enhanced antagonistic activity against L. monocytogenes at lower temperatures (5, 6, 17). The inhibition of L. monocytogenes by C. piscicola JCM5348 (a Bac^ strain) was not observed at 12 and 20°C throughout the storage period (Fig. 3C through 3F). However, a bacteriostatic effect was observed throughout the storage period at 4°C (Fig. 3A and 3B), although antilisterial activity was not detected (data not shown). Inhibition of L. monocytogenes by C. piscicola CS526 was also observed at 4°C in the early stages of mixed culture in spite of low levels of the bacteriocin (Figs. 3A, 3B, and 4). This inhibition of L. monocytogenes by Carnobacterium species at lower temperature might be caused by competitive antagonism or nutrient depletion, as reported by Buchanan and Bagi (4) and Degnan et al. (10).

Bacteriocin production profiles in cold-smoked salmon coinoculated with C. piscicola CS526 and L. monocytogenes are shown in Figure 4. Maximum yield of the bacteriocin were reached after 21 days at 4°C, 7 days at 12°C, and 2 days at 20°C. The titer was approximately 9,000 to 12,000 AU/g, except at an inoculum level (10^4 CFU/g) of C. piscicola CS526 at 20°C (1,000 AU/g). This suggests that bacteriocin synthesis reaches a maximum at the end of the exponential growth phase of the bacteriocin-producing strain in cold-smoked salmon (Fig. 3). However, bacteriocin activity decreased rapidly after bacterial populations in cold-smoked salmon reached a density of 10^8 to 10^9 CFU/g. In addition, after reaching maximum activity, the bacteriocin activity decreased more rapidly at the higher temperature. This could be because of (i) degradation of bacteriocins by proteolytic enzymes of C. piscicola, indigenous microorganisms, and salmon muscle (22), and (ii) activation of enzymes by higher temperature. Supernatants from
uninoculated samples or from samples coinoculated with the Bac \textsuperscript{1} strain. 

The pH of the initial cold-smoked salmon samples was 6.0 to 6.3. At the end of the storage period, the pH decreased to 5.5 to 6.2.

We isolated bacteriocin-producing \textit{C. piscicola} CS526 from frozen surimi and demonstrated the suppression of \textit{L. monocytogenes} growth even with a low initial inoculum level of \textit{C. piscicola} CS526 in cold-smoked salmon. This inhibitory ability was enhanced at lower temperature because of bacteriocin production and competitive antagonism by \textit{L. monocytogenes}. Therefore, \textit{C. piscicola} CS526 might be a promising strain for the biopreservation of refrigerated seafood products.

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