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Antimicrobial Activity of Foodborne *Paenibacillus* and *Bacillus* spp. against *Clostridium botulinum*

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ABSTRACT

The saprophytic *Paenibacillus* and *Bacillus* spp. found in cooked chilled foods may have an effect on the growth of *Clostridium botulinum,* a major microbiological hazard, especially for pasteurized vacuum-packaged products. Culture supernatants of 200 strains of *Paenibacillus* and *Bacillus* strains isolated from commercial cooked chilled foods containing vegetables were screened for activity against *C. botulinum* type A, proteolytic type B, and type E strains in a well diffusion assay. Nineteen strains were positive against *C. botulinum.* Among those, seven *Paenibacillus polymyxa* strains showed the highest antibotulinal activity and the largest antimicrobial spectrum against *C. botulinum* strains. The antibotulinal activity was evaluated throughout the growth of a representative strain of the positive *P. polymyxa* strains. The antimicrobial activity was detected in the culture supernatant from late-log/early stationary phase of the bacteria, which occurred after 7 to 10 days of incubation at 10°C and after 2 to 3 days at 20°C in nutrient broth and in vegetable purées under aerobic or anaerobic conditions. In co-cultures with the positive strain of *P. polymyxa* in nutrient broth and vegetable purées, a *C. botulinum* type E strain was inhibited whenever *P. polymyxa* reached stationary phase and produced its antimicrobial activity before *C. botulinum* began its exponential growth phase. The antimicrobial activity of *P. polymyxa* against *C. botulinum* was attributed to the production of antimicrobial peptides resistant to high temperature and acidity. Other gram-positive and -negative bacteria (*Escherichia coli, Streptococcus mutans, Leuconostoc mesenteroides,* and *Bacillus subtilis*) were also sensitive to these antimicrobial peptides.

Clostridium botulinum is a major hazard in cooked chilled foods, especially for pasteurized products packaged under vacuum conditions or low oxygen atmosphere *(12, 16, 17).* Psychrotrophic nonproteolytic strains are able to grow and produce toxins at temperatures as low as 3°C *(13)* and at 5°C in vegetable substrates *(3).* Among the factors in foods having an effect on *C. botulinum,* growth, pH, water activity, and storage temperature are well documented *(13).* By contrast, the influence of the saprophytic microflora found in foods on *C. botulinum* is poorly studied.

Bacillus spp., including some species recently renamed as *Paenibacillus* spp. *(8),* are the main bacterial components of a range of cooked chilled foods *(2, 14).* In cooked, pasteurized, and chilled vegetable purées, *Paenibacillus* spp. are among the most frequently isolated species in products stored at refrigeration temperatures (below 10°C), whereas *Bacillus subtilis, Bacillus cereus,* and *Bacillus licheniformis* are most frequently isolated from products stored at abuse temperatures (20°C) *(2).* Strains of these *Bacillus* spp. produce antimicrobial substances, some of which are active against *Clostridium* spp. *(26, 27).*

The objective of the present work was to determine whether foodborne *Paenibacillus* and *Bacillus* spp. might have antimicrobial activity against *C. botulinum* in foods. *Paenibacillus* and *Bacillus* spp. strains isolated from commercial cooked chilled foods containing vegetables were screened for their antimicrobial activity against *C. botulinum.* The antimicrobial activity of a selected *Paenibacillus polymyxa* strain was evaluated throughout its growth cycle in a range of nutrient broth and vegetable media. *C. botulinum* was then challenged with this selected *P. polymyxa* strain in co-cultures consisting of simultaneous inoculations of both strains in aerobic or anaerobic nutrient broth and vegetable substrates at 10 and 20°C. The molecular weight, the sensitivity to heat and acidity, and the bactericidal spectrum of antimicrobial substances produced by *P. polymyxa* strain were then evaluated.

MATERIALS AND METHODS

Bacterial strains. Strains of *Paenibacillus* and *Bacillus* spp. (INRA AV strains) were isolated in a previous work from commercial cooked, pasteurized, and chilled vegetable purées *(2).* Their identification was based on the API system (BioMerieux, Marcy-l'Etoile, France) and was confirmed by partial 16S ribosomal DNA sequencing *(8). Leuconostoc mesenteroides* INRA AV 90Lm1 was isolated from shredded carrot. Strains of *B. cereus* (CIP 51.27), *B. subtilis* (CIP 52.65T), *Lactobacillus plantarum* (CIP 103.140T), *Salmonella* Enteritidis (CIP 82.17), *Streptococcus mutans* (CIP 103.220T), and *Staphylococcus aureus* (CIP 57.10) were purchased from the Pasteur Institute collection (Paris, France). *Listeria monocytogenes* Scott A and *Escherichia coli* EC1 were kindly provided by Dr. B. M. Lund, Institute of Food Research, Norwich, UK, and Prof. J. Guiraud, University of Montpellier II, France, respectively. *Bacillus, Paenibacillus,* and reference strains were all stored at −20°C in a 30% (vol/vol)

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TABLE 1. Clostridium *spp. used in this study*

Clostridium spp.	Collection number
C. botulinum sp.	IP 8[a]
C. botulinum type A	CIP 104310T,[b] NCTC 7272[c]
Nonproteolytic *C. botulinum* type B	IFR 81/23,[d] IFR 83/1, IFR 86/17, IFR 87/4, IFR 87/7, IFR 90/4, IFR 93/6, IFR 93/10, IFR 93/11, ATCC 17844,[e] ATCC 25765
Proteolytic *C. botulinum* type B	NCTC 7273, ATCC 17844, ATCC 7949
C. botulinum type E	ATCC 9564, NCTC 8266 (Nanaimo), IFR 81/26, IFR 81/31, IFR 86/21, IFR 87/1, IFR 93/7, IFR 93/8
Nontoxigenic *C. botulinum* type E-like	DSM 1985[f]
C. botulinum type F	IFR 86/32, IFR 86/33, IFR 86/34
C. pasteurianum	ATCC 6013
C. sporogenes	CIP 793
C. butyricum	CIP 6051

[a] Gift from Prof. M. Sebald, Institut Pasteur, Paris, France; negative in the mouse bioassay for toxin production.
[b] CIP, Pasteur Institute collection, Paris, France.
[c] NCTC, National Collection of Type Cultures, London, UK.
[d] IFR, Institute of Food Research, Norwich, UK.
[e] ATCC, American Type Culture Collection, Rockville, Md.
[f] DSM, German collection of microorganisms and cell cultures, Braunschweig, Germany.

glycerol solution. Each strain was checked for purity on agar plates before inoculation. *Clostridium* strains used in this study are listed in Table 1. They were stored at 4°C in cooked meat medium (Bacteriological Meat Medium, Biokar Diagnostic, Beauvais, France).

Production of *C. botulinum* spores. Spores were produced in two-phase cooked meat medium (Oxoid, Basingstoke, UK) according to the method of Peck et al. *(18).* After incubation for 7 to 10 days at 30°C, spores were harvested by centrifugation (5,000 × *g,* 4°C, 10 min), washed 4 to 10 times in sterile distilled water, resuspended in 4 ml of sterile distilled water, and stored at 4°C. Before use, spore suspensions were resuspended in a 0.1% (vol/vol) Tween 80 solution and bath sonicated for 10 min up to four times to break up spore clumps.

Bacterial counts. *Paenibacillus* cells were enumerated by serially diluting in physiological water containing tryptone 1 g/liter (Biokar) and plating on Nutrient agar (tryptone 5 g/liter, beef extract [Biokar] 3 g/liter, and agar 15 g/liter). Serial dilutions of *C. botulinum* were realized in anaerobic half-strength tryptone glucose yeast (TGY) broth and plated on Differential Reduced Clostridial Medium (Difco Laboratories, Detroit, Mich.) supplemented with 4 mg/liter of trimethroprim antibiotic (Sigma Chemical Co., St. Louis, Mo.) and 100 ml/liter of a 50% saline suspension of egg yolk. Previous experiments showed that this medium supported the growth of *C. botulinum* but inhibited the growth of the *Paenibacillus* strains used in co-cultures.

Culture media. J-medium *(5)* containing tryptone 5 g/liter (Biokar), yeast extract 15 g/liter (Biokar), K_2HPO_4 3 g/liter, and glucose 2 g/liter at pH 7.4 was used for *Bacillus* and *Paenibacillus* spp. cultures. The TGY medium contained tryptone 30 g/liter (Biokar), yeast extract 20 g/liter (Biokar), glucose 5 g/liter, HCl-cystein 0.5 g/liter, and resazurin 4 mg/liter, adjusted to pH 7.3. No cystein was added in aerobic TGY medium. Tryptone-Soyase Broth contained 15 g/liter tryptone (Biokar), 3 g/liter soyase (Biosoyase, BioMerieux), 5 g/liter NaCl, 2.5 g/liter K_2HPO_4, and 2.5 g/liter glucose.

Vegetable purées were prepared from fresh vegetables purchased in a local supermarket. Vegetables were trimmed and cooked at 100°C in demineralized water for 15 min for potato and 30 min for broccoli and mushroom. Before blending in a domestic blender, demineralized water was added to the vegetables to achieve a final proportion of 0.4 kg of broccoli, 0.5 kg of mushroom, and 0.33 kg of potato per kg of purée. Vegetable purées were stored in plastic pouches at −20°C until used. Anaerobic purées contained HCl-cystein (0.5 g/liter) and resazurin (4 mg/liter).

Ten milliliters of culture media (vegetable purée or TGY broth) was aliquoted to vials (15-ml Penicillin bottles). For anaerobic cultures, culture media were boiled and aliquoted under a headspace of N_2/H_2 95:5 (vol/vol) to remove oxygen before sterilization. Vegetable purées were sterilized by tyndallization by three successive heatings at 90°C at 1-day intervals. Tryptone-Soyase Broth, TGY, and J-broth were autoclaved at 121°C for 20 min.

Co-culture experiments. Co-cultures consisted of simultaneous inoculations of spores of *C. botulinum* and vegetative cells of *Paenibacillus* in TGY broth or vegetable purée. Vegetative cells of *Paenibacillus* were grown in TGY broth at the same temperature as the co-culture. Freshly prepared spore suspensions (≤1 month old) of *C. botulinum* were used for co-cultures in vegetable purées. Suspensions of *C. botulinum* spores and *Paenibacillus* cells were diluted before inoculation in reduced half-strength TGY broth whenever necessary.

For each co-culture experiment, 100 μl of *C. botulinum* spore suspension and 100 μl of *Paenibacillus* cell suspension at the appropriate concentrations were inoculated in 10 ml TGY broth or vegetable purée in a series of vials and sealed with a butyl septum. Inoculation of anaerobic media was performed in an anaerobic cabinet. In parallel, a series of vials of control pure cultures were inoculated with 100 μl of either *C. botulinum* or *Paenibacillus* inoculum.

One vial of co-culture, one vial of *P. polymyxa* pure culture, and one vial of *C. botulinum* pure culture were sampled at regular intervals for bacterial counts and measurement of redox potential.

Whenever inhibition against *C. botulinum* in a co-culture was observed, experiments were replicated using other spore suspensions of *C. botulinum* and other preparations of vegetable purée.

Supernatants of bacterial cultures. Supernatants of co-cultures and pure cultures of *C. botulinum* and *Paenibacillus* spp. were collected after centrifugation at 7,800 × *g* for 10 min. One-

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milliliter aliquots were stored at −20°C until used for antimicrobial activity assays, *C. botulinum* neurotoxin enzyme-linked immunosorbent assay (ELISA), and pH measurements.

Antimicrobial activity of *Bacillus* and *Paenibacillus* spp. culture supernatants. The antimicrobial activity of culture supernatants of *Paenibacillus* and *Bacillus* spp. was tested against indicator strains in diffusion assays. For screening of foodborne *Paenibacillus* and *Bacillus* strains, supernatants of 48-h-old cultures at 30°C in 10 ml J-broth were tested against spore suspensions of *C. botulinum* strains IP 104310T, NCTC 7273, and Nanaimo. A spore suspension of *C. botulinum* was mixed with 15 ml of molten TGY agar at 50°C to obtain a final concentration of 10^4 to 10^6 spores per ml and poured into a petri dish. Aliquots of 20 μl of *Paenibacillus* and *Bacillus* spp. culture supernatants were deposited in 4-mm-diameter wells cut in the agar. The plates were then incubated for 24 h at 30°C under anaerobic conditions. The inhibitory activity of each supernatant was recorded by measuring the diameter of the zone of clearing surrounding the wells. A culture supernatant giving a zone of clearing of a diameter larger than 5 mm was considered positive against *C. botulinum.*

Tests with vegetative cells of *C. botulinum* gave the same results as those with spores in the diffusion assays. Consequently, a slightly simplified protocol using vegetative cells of *C. botulinum* was used to assay the antimicrobial activity of *P. polymyxa* strains in the subsequent part of the work. Ten-microliter aliquots of *P. polymyxa* culture supernatant were spotted on a bacterial lawn of *C. botulinum* obtained by spreading 100 μl of an overnight TGY culture at 30°C on TGY plates. Plates were checked for inhibition zones after a 24-h incubation at 30°C under anaerobic conditions. A culture supernatant giving a zone of clearing of a diameter larger than 3 mm was considered positive against *C. botulinum.* Antimicrobial activity of culture and co-culture supernatants of *P. polymyxa* strain Z1189 was assayed against *C. botulinum* strain Nanaimo. The antimicrobial activity of *P. polymyxa* Z1189 supernatants against other bacterial species was tested using the same protocol. The bacterial lawn of each indicator strain was obtained by spreading 100 μl of a 24- to 48-h Tryptone-Soyase Broth culture at 30°C.

Serial dilutions of culture supernatants of *P. polymyxa* Z1189 were made in distilled water.

Sterile medium and distilled water were used as negative controls, and the supernatant of a 48-h culture of *P. polymyxa* Z1189 in J-broth was used as a positive control. Aliquots of this positive control were stored at −20°C until use.

ELISA detection of *C. botulinum* neurotoxin. The presence of *C. botulinum* neurotoxin was tested by ELISA in the co-culture and pure culture of *C. botulinum* supernatants according to the method of Potter et al. *(21),* with minor modifications. Briefly, trivalent antitoxin (against types A, B, and E) antibodies, supplied by Dr. J. Austin (Health Canada, Ottawa, Canada), were coated on microtiter plates and incubated overnight at 4°C. After washing in a Tris-buffered saline solution (0.79% [wt/vol] Tris-HCl, 1.17% [wt/vol] NaCl, 0.0224% [wt/vol] KCl, and 0.05% [vol/vol] Tween 20, pH 7.5), nonspecific binding sites were blocked with a solution of 1% (vol/vol) calf serum (heat inactivated) (Gibco BRL, Life Technologies, Cergy Pontoise, France) and 0.5% casein (Sigma) in the Tris-buffered saline solution above for at least 90 min at 30°C. Culture supernatants were added to the well and incubated for 90 min at 30°C. After washing in Tris-buffered saline solution, an aliquot of a 1,000-fold diluted biotinylated trivalent antitoxin antibodies (Biotin protein labeling kit, Roche Diagnostic, Meylan, France) was added to the well and incubated for 90 min at 30°C. Positive ELISA reactions were revealed with Extravidin alkaline phosphatase diluted 1:20,000 in Tris-buffered saline solution (90 min, 30°C) and amplified using the ELISA Amplification System (Gibco, Life Technologies, Rockville, Md.) (incubations 15 min at 25°C).

Distilled sterile water and TGY broth were used as negative controls, and supernatants of *C. botulinum* cultures in TGY broth and pure toxin types A, B, and E (Sigma) were used as positive controls.

The threshold for a positive ELISA reading was determined by calculating the mean of the absorbance at 492 nm (A_{492}) of each negative control and adding twice the standard deviation. A_{492} values for the negative control were always $<0.4 \pm 0.04$.

Redox potential and pH measurements. Redox potential (E_h) of aerobic pure cultures of *Paenibacillus* spp. was measured at each sampling point using a platinum electrode combined with an Ag/AgCl reference system (Mettler Toledo, Urdorf, Switzerland) connected to a pH meter Schott CG825 (Schott, Hofheim, Germany). E_h values (mV) were recorded at approximately 25°C after 30 min of stabilization in an anaerobic cabinet. They were expressed at pH 7.0 using the following equation *(12)*: $E_{h7} = E_h + 235 + 59.1(\text{pH} - 7)$, where pH was the pH of the culture supernatant, measured using a microelectrode (Fisher Bioblock Scientific, Illkirch, France) connected to a pH meter Schott CG825.

Crude preparations of the antimicrobial substances produced by *P. polymyxa*. Culture of *P. polymyxa* strain Z1189 was obtained in 500 ml of J-broth at 30°C with agitation at 200 rpm for 48 h. The culture supernatant was obtained by centrifugation at 16,000 × *g* for 5 min and then filter sterilized on a 0.2-μm (pore diameter) polyvinylidene fluorure filter membrane (Durapore, Millipore, Saint Quentin en Yveline, France). Antimicrobial substances were recovered from the culture supernatant by a two-step ammonium sulfate precipitation. The supernatant from a first precipitation with 30% (wt/vol) ammonium sulfate (1 h at 7°C) was collected by centrifugation at 27,000 × *g* for 40 min and reprecipitated with 80% ammonium sulfate overnight at 7°C. After centrifugation, pellets were resuspended in 10 ml of 20 mM phosphate buffer, pH 6.8.

Estimation of the molecular weight of *P. polymyxa* antimicrobial substances. The molecular weight of antimicrobial substances produced by *P. polymyxa* strain Z1189 was estimated by gel filtration on a polyacrylamide (5%)-agarose (4%) gel (Ultrogel AcA 54, IBF Biotechnics, Clichy, France) in a column (1.5 by 34 cm) equilibrated with distilled water. Molecular weight markers (Gel filtration calibration kit, Pharmacia, Uppsala, Sweden) were ribonuclease A (13,700 Da), chymotrypsin (25,000 Da), ovalbumin (43,000 Da), and bovine serum albumin (67,000 Da) detected by absorbance at 280 nm. Crude preparations of antimicrobial substances (3 ml) were eluted through the column with distilled water under a flow rate of 0.5 ml/min. Forty-microliter aliquots of each 3-ml fraction were assayed with 400 μl of a 1% ninhydrin solution in methanol. After heating at 60°C for 30 min, the coloration was revealed by its absorbance at 560 nm. In addition, each fraction was assayed for antimicrobial activity. Positive fractions were pooled and concentrated under vacuum (Speed Vac) or by ultrafiltration on a 3-kDa membrane (Macrosep Filtron, Merck, Strasbourg, France).

Sensitivity of *P. polymyxa* antimicrobial substances to proteolytic enzymes, pH, and heat. Aliquots (20 μl) of pooled positive fractions from gel filtration were submitted to enzymatic treatment for 5 h at 37°C with 10 μl of the following proteolytic enzyme solutions: trypsin (Sigma) (100 mg/ml), proteinase K (Sigma) (100 mg/ml), and protease type IX (Sigma) (200 mg/ml).

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TABLE 2. *Antimicrobial activity of foodborne* Paenibacillus *and* Bacillus *spp. supernatants against* Clostridium botulinum *spp. in well diffusion assays*

Species	*Paenibacillus* and *Bacillus* spp.		Antagonistic to *C. botulinum* strain[a]:		
	No. positive strains against *C. botulinum*/no. of strains tested for each species	Strain number	Type A IP 104310	Type B NCTC 7273	Type E Nanaimo
Paenibacillus polymyxa[b]	7/12	INRA AV B4204	++	++	++
		INRA AV B2106	++	++	++
		INRA AV B1156	++	++	++
		INRA AV Z1186	++	++	++
		INRA AV Z1189	++	++	++
		INRA AV Z1191	++	++	++
		INRA AV Z2167	++	++	++
Bacillus subtilis	8/33	INRA AV B2113	+	−	+
		INRA AV P2106	+	+	−
		INRA AV B0051	−	+	+
		INRA AV K2101	−	−	+
		INRA AV C2101	−	−	+
		INRA AV K21100	−	−	+
		INRA AV Z2103	−	−	+
		INRA AV B0041	−	+	−
B. cereus	2/28	INRA AV P21 S	+	+	−
		INRA AV Z421	+	+	−
B. subtilis/ *amyloliquefaciens*[b]	2/7	INRA AV P2105	+	−	−
		INRA AV L2102	−	−	+
Other species[c]	0/120				

[a] ++, inhibition zone ≥ 1-cm diameter; +, 0.5 cm < inhibition zone < 1-cm diameter; −, no inhibition zone.
[b] Bacterial name given by API LAB System software.
[c] Including *B. circulans 2, B. macerans, B. circulans/macerans/polymyxa, B. megaterium, B. pumilus, B. amyloliquefaciens,* and *B. liqueniformis.*

Enzymes were inhibited by heating at 65°C for 15 min before samples were assayed for antimicrobial activity.

For pH sensitivity, crude preparations of antimicrobial substances (500 μl) were exposed to 20 μl of a 12 M HCl solution (pH 1.5) for 5 h at room temperature. One hundred forty microliters of 2 M NaOH (pH 7.5 to 8) was added to alkalinize the samples before antimicrobial activity was determined.

For heat sensitivity, 500-μl aliquots of *P. polymyxa* culture supernatants in J-broth were heated in a water bath at 65°C for 30 min or at 100°C for 10 min, or they were heated in an autoclave at 120°C for 10 min. Heat-treated samples were cooled before antimicrobial activity was assayed.

RESULTS

Antimicrobial activity of foodborne *Paenibacillus* and *Bacillus* spp. against *C. botulinum* spp. Culture supernatants of 200 *Bacillus* and *Paenibacillus* strains isolated from vegetable purées were tested against *C. botulinum* type A strain IP 104310T, proteolytic *C. botulinum* type B strain NCTC 7273, and *C. botulinum* type E strain Nanaimo. Among the 200 *Paenibacillus* and *Bacillus* strains tested, 19 inhibited at least one strain of *C. botulinum* (Table 2). Positive strains against *C. botulinum* belonged to the species *P. polymyxa, B. subtilis, B. cereus,* and *Bacillus amyloliquefaciens/subtilis.* For *B. subtilis, B. cereus,* and *B. amyloliquefaciens/subtilis* strains, small inhibition zones (≤1 cm) were observed, and none of these strains inhibited all three *C. botulinum* strains tested. In contrast, 7 of the 12 strains of *P. polymyxa* tested showed a marked antimicrobial activity against the three *C. botulinum* strains tested, with inhibition zones larger than 1-cm diameter. In addition, positive *P. polymyxa* strains strongly inhibited (inhibition zone ≥ 1-cm diameter) the 32 *Clostridium* spp. strains listed in Table 1.

Changes in the antibotulinal activity of *P. polymyxa* strain INRA AV Z1189 during growth in nutrient broth and vegetable purées. The antimicrobial activity of *P. polymyxa* strain Z1189 against *C. botulinum* was followed during its growth in TGY broth and vegetable purées (broccoli, potato, and mushroom) under anaerobic and aerobic conditions. In all of the media and conditions tested, the antimicrobial activity was detected in the culture supernatant from late-log/early stationary phase, i.e., after 2 to 3 days at 20°C and after 7 to 11 days at 10°C. In prolonged cultures at 20°C, antibotulinal activity was detected in two-fold-diluted, but not fourfold-diluted, supernatant and was detected only in undiluted supernatant in cultures at 10°C.

Co-cultures of *C. botulinum* Nanaimo with *P. polymyxa* Z1189 positive against *C. botulinum* in nutrient broth and vegetable purées. In anaerobic co-cultures of *C. botulinum* and *P. polymyxa* both inoculated at 10^3 to 10^4 CFU/ml in nutrient broth, growth of *C. botulinum* was detected at 10 and 20°C (Fig. 1A). In both pure cultures and co-cultures, *C. botulinum* reached a maximal population of 10^7 to 10^8 CFU/ml and produced toxin (ELISA $A_{492} > 1.0$) within 2 days of incubation at 20°C and within 7 days at

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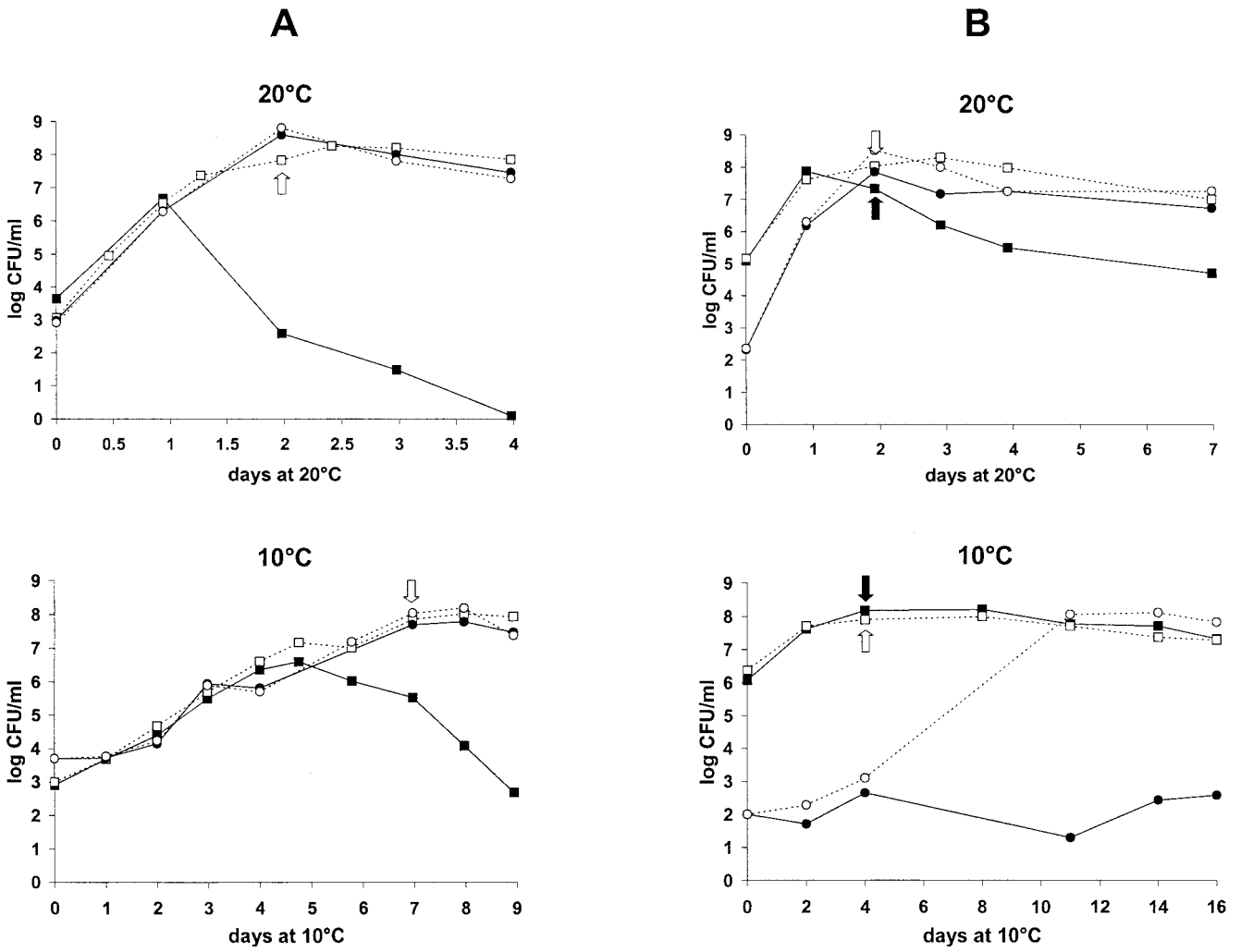

FIGURE 1. *Co-cultures of* C. botulinum *type E strain Nanaimo (●) with the strain P. polymyxa* Z1189 positive against C. botulinum *(■) inoculated at the same concentration (A) and at a 1,000- to 10,000-fold higher concentration of* P. polymyxa *cells than that of* C. botulinum *spores (B) in anaerobic TGY broth at 10 and 20°C. Control cultures were pure cultures of* P. polymyxa *(□) and* C. botulinum *(○). Arrows indicate the time to the first detection of antibotulinal activity in supernatants of co-cultures (black arrows) and of* P. polymyxa *pure cultures (white arrows). These results were confirmed in replicate experiments.*

10°C. No antimicrobial activity against *C. botulinum* was detected in co-cultures, and the size of the *P. polymyxa* population dropped before reaching a stationary phase at both temperatures. In pure cultures of *P. polymyxa,* antibotulinal activity was detected in the supernatant in early stationary phase. When co-cultures were inoculated with higher concentrations of *P. polymyxa* cells than *C. botulinum* spores (i.e., 1,000- to 10,000-fold more *P. polymyxa* vegetative cells than *C. botulinum* spores), the growth of *C. botulinum* was inhibited at 10°C but not at 20°C (Fig. 1B). In co-cultures at 20°C, *C. botulinum* started to grow before *P. polymyxa* reached a maximal population of 10^8 CFU/ml and produced its antibotulinal activity (Fig. 1B). By contrast, at 10°C, *P. polymyxa* reached stationary phase, and the first detection of antibotulinal activity in the co-culture supernatant was observed before the initiation of growth of *C. botulinum.* Under the same conditions of culture (similar inoculation level, anaerobic TGY broth, 10°C), growth of *C. botulinum* was observed in co-culture with a negative strain of *P. polymyxa* (strain INRA AV Z2160), but maximum population sizes were markedly lower than in pure cultures (data not shown).

Conversely, in aerobic TGY broth, *C. botulinum* strain Nanaimo grew in co-culture with a negative strain of *Paenibacillus* (*Paenibacillus odorifer* INRA AV B2185) (Fig. 2A), while no growth was detected when *C. botulinum* was cultured with the positive strain of *P. polymyxa* Z1189 (Fig. 2B) at 20°C. The redox potential of the pure culture of *P. odorifer* B2185 and *P. polymyxa* Z1189 dropped after the first 2 days of incubation from positive values between +200 and +250 mV to negative ones within −50 and −250 mV.

There were no marked differences between growth kinetics of *C. botulinum* strain Nanaimo in broccoli, potato, and mushroom purées at 20°C; growth was detected from 1 to 3 days after inoculation (data not shown). Broccoli purée was selected as the test vegetable substrate in further experiments. In co-cultures of *C. botulinum* strain Nanaimo with the positive strain of *P. polymyxa* Z1189 in anaerobic broccoli purée (Fig. 3), counts of *C. botulinum* increased

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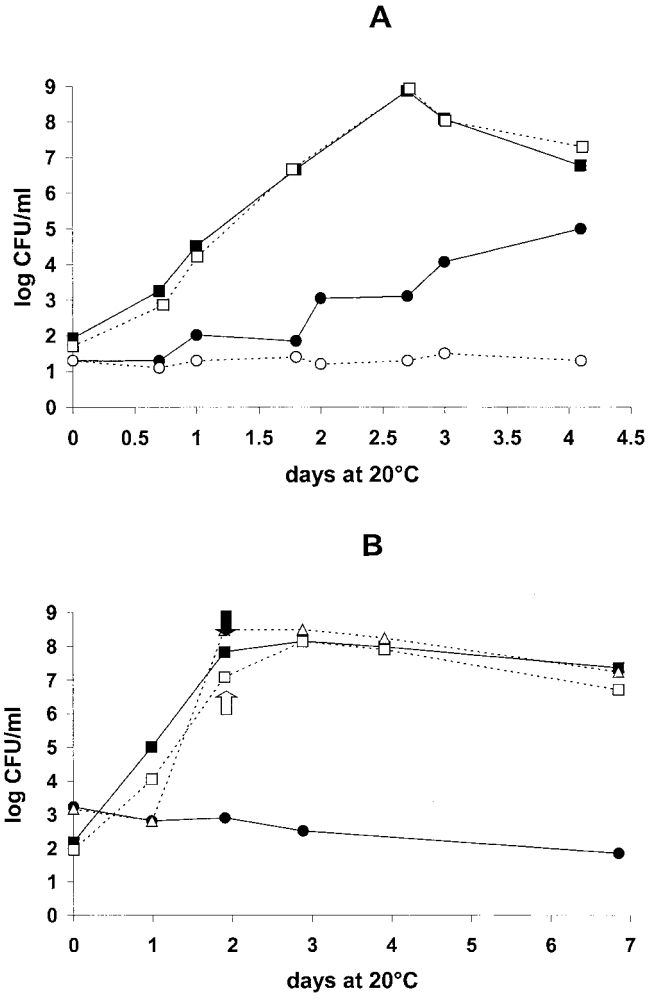

FIGURE 2. *Co-cultures of* C. botulinum *type E strain Nanaimo (●) with the negative* Paenibacillus *strain B2185 (■) (A) and the positive* P. polymyxa *strain Z1189 (■) (B) in TGY broth under aerobic conditions at 20°C. Pure cultures of* Paenibacillus *spp. (□) and* C. botulinum *under aerobic (○) and anaerobic (△) conditions were used as control cultures. Arrows indicate the time to the first detection of antibotulinal activity in supernatants of co-cultures (black arrow) and of* P. polymyxa *pure cultures (white arrow). These results were confirmed in replicate experiments.*

slightly after 2 days of incubation at 20°C. The actual growth of *C. botulinum* in co-culture was confirmed by the detection of a *C. botulinum* toxin in the culture medium from the third day of incubation (ELISA $A_{492} > 1.0$). After that time, counts of *C. botulinum* dropped dramatically as *P. polymyxa* reached stationary phase and produced its antibotulinal activity. In a duplicate experiment using other preparations of *C. botulinum* spore suspensions and broccoli purées, no growth of *C. botulinum* strain Nanaimo was observed in co-culture with the positive strain of *P. polymyxa* Z1189. *P. polymyxa* reached a maximal population of 5×10^7 CFU/ml within the first 2 days of incubation, while growth of *C. botulinum* in pure culture was observed only after 2 days. Co-cultures with a negative strain of *P. polymyxa* (strain INRA AV Z2160) showed that *C. botulinum* could grow in the presence of *P. polymyxa* in anaerobic broccoli purée at 20°C.

In vegetable purées (broccoli, potato, and mushroom)

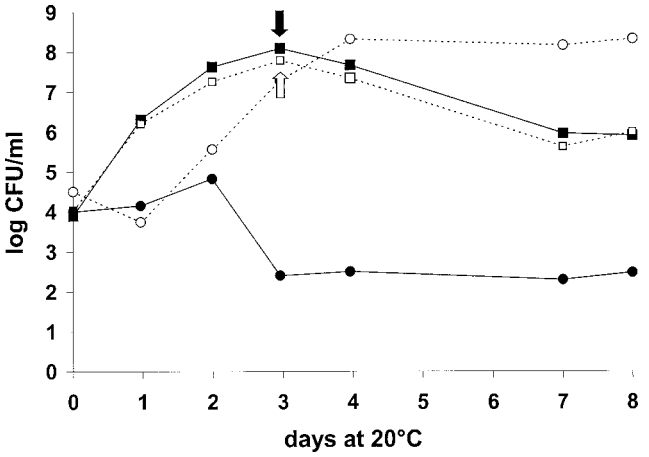

FIGURE 3. *Co-cultures of* C. botulinum *type E strain Nanaimo (●) with the strain* P. polymyxa *Z1189 positive against* C. botulinum *(■) in broccoli purée under strict anaerobic conditions at 20°C. Control cultures were pure cultures of* P. polymyxa *(□) and* C. botulinum *(○). Arrows indicate the time to the first detection of antibotulinal activity in supernatants of co-cultures (black arrow) and of* P. polymyxa *pure cultures (white arrow).*

at 10°C, growth of *C. botulinum* strain Nanaimo in pure culture was detected after more than 12 days of incubation (data not shown), long after the detection of antibotulinal activity in pure cultures of *P. polymyxa* Z1189 (i.e., after 7 to 11 days).

Partial characterization of the antimicrobial activity of *P. polymyxa* Z1189. In nutrient and vegetable media, the pH of co-cultures and control cultures of the positive strain *P. polymyxa* Z1189 remained above 5.5, ruling out the possibility that the antimicrobial activity of culture supernatants was solely due to acidification. The antimicrobial activity of the *P. polymyxa* supernatant was retained on a 3-kDa ultrafiltration membrane, showing that low-molecular-weight peptide antibiotics were not responsible for this activity. This subsequently excluded polymyxins with molecular weights of approximately 1.2 kDa *(24).* The molecular weight of the antimicrobial substances of *P. polymyxa* Z1189 was estimated at 9 to 10 kDa by gel filtration. The gel filtration fractions active against *C. botulinum* were positive for the ninhydrin test and lost their antimicrobial activity after treatment with proteases (trypsin, proteinase K, and protease type IX), thus suggesting their peptidic nature. Supernatants of *P. polymyxa* Z1189 cultures remained active after heating at 65°C for 30 min and at 100°C for 10 min but lost their antimicrobial activity after heating at 120°C for 10 min. Similarly, acidic treatment (pH 1.5) did not affect the antimicrobial activity of *P. polymyxa* supernatants.

Culture supernatants of *P. polymyxa* Z1189 were tested against several bacterial species, including foodborne pathogens (Table 3), and were active against gram-positive as well as gram-negative species (i.e., *E. coli, S. mutans, L. mesenteroides,* and *B. subtilis*). Conversely, the tested strains of *L. monocytogenes, B. cereus, S. aureus,* and *Salmonella* Enteritidis were not sensitive to *P. polymyxa* antimicrobial activity.

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TABLE 3. *Antimicrobial activity of* Paenibacillus polymyza *Z1189 supernatant against gram-positive (Gram +) and -negative (Gram −) bacterial species in diffusion assays*

	Bacterial species[a]	Inhibition by *P. polymyxa* supernatant[b]
Gram +	*Bacillus cereus* (CIP 51.27, INRA AV K1231, INRA AV L2104, INRA AV Z415, INRA AV P21S, INRA AV P2174)	−
	B. subtilis (CIP 52.65T)	+
	Lactobacillus plantarum (CIP 103.140T)	−
	Leuconostoc mesenteroides (INRA AV 90Lm1)	+
	Listeria monocytogenes (Scott A)	−
	Staphylococcus aureus (CIP 57.10)	−
	Streptococcus mutans (CIP 103.220T)	+
Gram −	*Escherichia coli* (EC1)	+
	Salmonella Enteritidis (CIP 82.17)	−

[a] See "Materials and Methods" for the origins of the bacterial strains.
[b] +, inhibition zone ≥ 3-mm diameter; −, no inhibition zone.

DISCUSSION

Our study constitutes the first report in vegetable foods of naturally occurring *Paenibacillus* and *Bacillus* spp. strains active against *C. botulinum,* particularly in cooked, chilled vegetable products. The presence of *Bacillus* strains inhibiting *C. botulinum* has already been reported in soils *(22, 25)* and in cooked, chilled foods containing fish *(14); Bacillus* spp. with antimicrobial activity against *C. botulinum* include *B. cereus, Bacillus mycoides/cereus, B. licheniformis, Bacillus macerans/polymyxa, Bacillus pumilus,* and *B. subtilis.* In vegetable products, isolates positive against *C. botulinum* were identified as *B. cereus, B. subtilis, B. subtilis/amyloliquefaciens,* and *P. polymyxa* (formerly classified in the *Bacillus* genus *(1)*). *P. polymyxa* strains showed the highest inhibitory activity and the largest antimicrobial spectrum against *C. botulinum* strains.

The antimicrobial activity of *P. polymyxa* Z1189, representative of the positive strains of *P. polymyxa* isolated from vegetable products, was attributed to the production of peptidic substances in the culture medium. Strains of *P. polymyxa* produce a variety of small antimicrobial peptides with molecular weights <3.5 kDa *(10, 11, 15, 19, 23, 24).* Only polyxin, produced by a *P. polymyxa* strain isolated from sausage, has a molecular weight of 10 kDa *(20),* in the range of the molecular weight of the antimicrobial substances produced by *P. polymyxa* Z1189. The production of polyxin was detected in the culture supernatant from early stationary phase and increased during late stationary phase. *P. polymyxa* Z1189 antimicrobial activity was always detected at the same stage of growth. *P. polymyxa* Z1189 antimicrobial substances were highly resistant to heat and acid conditions, and they inhibited *E. coli* and *B. subtilis* strains but failed to inhibit strains of *Salmonella* spp. and *S. aureus.* Polyxin has the same physicochemical and antimicrobial properties. For other bacterial species, such as *B. cereus* and *L. plantarum,* the antimicrobial activity of the *P. polymyxa* Z1189 substances and polyxin differed; culture supernatants of *P. polymyxa* Z1189 were negative against strains of *B. cereus* and *L. plantarum,* whereas crude preparation of polyxin inhibited both species. However, the two antimicrobial substances were not tested on the same indicator strains, and variations in susceptibility of these strains cannot be ruled out.

Besides the production of antimicrobial substances by *P. polymyxa,* nutrient depletion could also have contributed to the inhibition of *C. botulinum* growth in co-cultures. Nevertheless, rapid growth of *C. botulinum* was observed in the presence of negative strains of *P. polymyxa,* particularly in vegetable substrates, suggesting that nutrient insufficiency is probably not the main factor involved in the inhibition of *C. botulinum* in co-culture with positive strains of *P. polymyxa.*

In food products packaged under not strictly anaerobic conditions, saprophytic *Bacillus* and *Paenibacillus* spp. could promote *C. botulinum* development. Growth of these aerobic bacteria can produce conditions favorable for *C. botulinum* growth under aerobic conditions, presumably due to the decrease of the redox potential of the culture medium *(4, 9).* Under initially aerobic conditions, we observed that the growth of positive and negative strains of *Paenibacillus* decreased the redox potential of the medium, allowing *C. botulinum* to grow in the presence of negative strains. Nevertheless, under these conditions, growth of *C. botulinum* was delayed compared to that of the aerobic bacteria and therefore would be more likely stopped if inhibiting strains, such as positive *P. polymyxa* strains, were present in the microflora of the products.

Conversely, when *P. polymyxa* growth was delayed in co-cultures, *C. botulinum* growth was high, while *P. polymyxa* populations dropped rapidly. The decrease in *P. polymyxa* populations may be partly attributed to the production of antimicrobial peptides by *C. botulinum,* as shown recently by Dineen et al. *(6).*

The prevalence of *C. botulinum* in vegetables is low. The highest concentrations reported are about 2,100 spores/kg, which seem to be quite exceptional; the average is approximately 2.5 spores/kg *(7).* The initial total counts of *Bacillus* and *Paenibacillus* spp. in cooked, pasteurized, and chilled vegetable purées are about 10 to 200 CFU/g and can reach 10^6 to 10^8 CFU/g within 10 to 15 days of storage

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at 10°C *(2). P. polymyxa* strains are frequently isolated after storage at 10°C, and their population sizes are therefore likely to be higher than those of *C. botulinum.* The presence of antagonistic naturally occurring microorganisms, such as *P. polymyxa* strains, may be considered a factor to be accounted for in food risk assessment. However, levels of, and changes in, population sizes of these antagonistic microorganisms in foods must be better understood to further evaluate their impact on food safety.

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