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Bifidobacterium Species Isolated from Animal Feces and from Beef and Pork Meat

F. GAVINI,[1]* V. DELCENSERIE,[2] K. KOPEINIG,[3] S. POLLINGER,[3] H. BEERENS,[4] C. BONAPARTE,[3] AND M. UPMANN[3]

[1]*Institut National de la Recherche Agronomique, Laboratoire de Génie des Procédés et Technologie Alimentaires, 369 Rue Jules Guesde, F-59651 Villeneuve d'Ascq, France;* [2]*Food Sciences Department, Faculty of Veterinary Medicine, University of Liege, Sart Tilman B43b, 4000 Liege, Belgium;* [3]*Institute of Meat Hygiene, Meat Technology and Food Science, University of Veterinary Medicine Vienna, Veterinärplatz 1, A-1210 Wien, Austria; and* [4]*Centre d'Enseignement et de Recherche en Microbiologie Pharmaceutique, F-59260 Hellemmes Lille, France*

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ABSTRACT

Bifidobacteria were isolated from 122 of 145 samples of animal feces (from cattle, swine, sheep, goats, horses, rabbits, chickens, geese, and pigeons) from farms in France and Austria and from 92 of 955 production and processing chain samples of beef and pork (obtained at slaughter, cutting, and retail). Bacterial strains were identified to species by phenotypic numerical classification based on API 50CH and ID 32A tests and DNA-DNA hybridization. *Bifidobacterium pseudolongum* was present in 81% (99 of 122 samples) of all *Bifidobacterium*-positive fecal samples and predominated in samples from all animal species except those from swine from Austria. In these Austrian swine samples, the majority of strains were identified as *Bifidobacterium thermophilum* (78%), followed by *B. pseudolongum* (48%). The distribution of *B. thermophilum* and *B. pseudolongum* differed significantly between Austrian swine and cattle samples such as those collected along beef and pork production and processing chains. *Bifidobacterium animalis* was isolated from swine feces, and *Bifidobacterium ruminantium* was isolated from cow dung. Six fecal isolates (from cattle, swine, rabbits, goats, and horses) were identified as belonging to *Bifidobacterium* species of predominantly human origin: *B. adolescentis, B. bifidum,* and *B. catenulatum.* Only one other species, *Bifidobacterium choerinum,* was detected with low frequency in a pork processing chain. *B. pseudolongum* subsp. *pseudolongum* was predominant in pig feces, whereas *B. pseudolongum* subsp. *globosum* was predominant in feces from other animal species. Four strains closely related to both subspecies (58 to 61% DNA reassociation) formed a distinct genomic group. PCR techniques, which are more rapid and sensitive than culture-based methods, could be used to detect directly *B. pseudolongum* and *B. thermophilum* as indicators of fecal contamination along the meat processing chain.

Bifidobacteria are gram-positive, nonmotile, and non–spore-forming bacteria. They are part of normal intestinal flora of humans and animals and are generally regarded as nonpathogenic. Until recently, they were considered anaerobic, but in 2004 Simpson et al. *(30)* defined the new species *Bifidobacterium psychraerophilum,* which was able to grow on agar medium under aerobic conditions.

Currently, the genus *Bifidobacterium* is composed of 9 species that are found predominantly in human feces and 19 species that have been isolated from animal feces or environmental samples. Taxonomic studies resulted in recent changes in nomenclature. *Bifidobacterium longum* was divided into three biotypes: the infantis type, the longum type, and the suis type *(25). Bifidobacterium lactis* was recognized as a subspecies of *Bifidobacterium animalis (16),* and *Bifidobacterium denticolens* and *Bifidobacterium inopinatum* were transferred to the new genera *Scardovia* and *Parascardovia,* respectively *(13).* A new group of strains isolated from pig feces and having fructose-6-phosphate phosphoketolase activity was named *Aeriscardovia aeriphila (30).*

Because bifidobacteria represent one of the most important bacterial groups in the human and animal intestine *(18, 19),* their use as fecal indicators was studied in water *(12, 14, 21),* meat, raw milk, and raw milk cheese *(2, 3, 6, 9).* Because the dominant *Bifidobacterium* species in human and animal gut flora are different *(11),* it should be possible to distinguish between human and nonhuman sources of contamination. In water, *Bifidobacterium adolescentis (14)* and *Bifidobacterium dentium (21)* have been designated as indicators a human fecal pollution. In contrast, strains isolated from meat *(2)* and raw milk *(3)* have been used as indicators of fecal pollution of animal origin. However, it has been difficult to correlate a distinct *Bifidobacterium* species with a specific animal species. In contrast to the distribution of *Bifidobacterium* species in human feces, which has been well studied *(10, 17),* little is known about the most frequently occurring species in animal feces. Such information is necessary to decide whether certain *Bifidobacterium* species may serve as fecal indicators. The aim of this work was to isolate and identify *Bifidobacterium* species from the production and processing chains of fresh beef and pork. Identification of the isolates was compared with identification of *Bifidobacterium* isolates from feces of several other animal species.

MATERIALS AND METHODS

Samples and strains: farm animals. One hundred forty-five samples of animal feces were collected on farms in France and Austria. Most samples were from cattle and swine, but some were

* Author for correspondence. Tel: 33 3 20 43 54 03; Fax: 33 3 20 43 54 26; E-mail: gavini@lille.inra.fr.

collected from sheep, goats, horses, rabbits, chickens, geese, and pigeons.

Samples and strains: beef and pork production and processing chains. A total of 427 beef and 428 pork samples were collected from the respective fresh meat production and processing chains during slaughter, cutting, and deboning and from retail establishments in Austria. At the slaughterhouse, samples were collected from animal feces, freshly slaughtered carcasses, and slaughter equipment. In the cutting and deboning areas, raw materials (i.e., chilled meat carcasses), meat cuts, and cutting equipment were examined for the presence of bifidobacteria. Meat samples also were collected in several retail supermarkets. The number of samples for each type are presented later in Table 6.

Detection of bifidobacteria. The bifidobacterial detection method included two steps: an enrichment procedure and an isolation step. For bifidobacteria enrichment, brain heart infusion (Bio-Rad, Marnes la Coquette, France; French samples) or RCM medium (Oxoid, Basingstoke, UK; Austrian samples) was used; both media were supplemented with propionic acid *(1)* (final concentration of 5 ml liter^{-1}). Isolation was performed on Columbia agar medium (Difco, Elancourt, France) with selective supplements paromomycin *(2)* (final concentration of 7 mg liter^{-1}, French samples) or mupirocin (final concentration of 50 mg liter^{-1}, Austrian samples; GlaxoSmithKline, Research Triangle Park, N.C.) *(6).* The method was performed as described by Delcenserie et al. *(6).*

Identification of bifidobacteria: phenotypic characterization and numerical analysis. The type and reference strains used as representatives of human, animal, and environmental contaminants and included in the phenotypic numerical analysis are presented in Table 1. The type strains of *B. adolescentis, Bifidobacterium bifidum, Bifidobacterium catenulatum, B. animalis, Bifidobacterium choerinum,* and *Bifidobacterium ruminantium* were used for DNA-DNA hybridization experiments.

Strains were considered members of the genus *Bifidobacterium* or of the closely related genera *Scardovia, Parascardovia,* and *Aeriscardovia* on the basis of their fructose-6-phosphate phosphoketolase activity *(26).* Enzymatic and carbohydrate fermentation tests utilized the ID32A and 50CH kits (bioMérieux Department API, La Balme les Grottes, France). Tests for growth at 46°C within 48 h were carried out in Trypticase phytone yeast broth *(26).*

The similarity between strains was calculated with the Jaccard index. The aggregation method was the unweighted pair group method with averages *(32).*

Identification of bifidobacteria: DNA-DNA hybridization. Experiments were carried out on 11 strains from the numerical analysis that were phenotypically close to *B. adolescentis* (4 strains), *B. bifidum* (1 strain), *B. catenulatum* (1 strain), *B. animalis* (2 strains), *B. ruminantium* (1 strain), or *B. choerinum* (2 strains) and on 23 *B. pseudolongum* strains to determine their subspecies (*B. pseudolongum* subsp. *pseudolongum* or *B. pseudolongum* subsp. *globosum*).

DNA was prepared with the simultaneous use of achromopeptidase (5,000 U/500 mg of bacteria) and lysosyme (400,000 U/500 mg of bacteria) as lytic enzymes. DNA extraction was carried out according to Marmur's method *(15).*

The degree of DNA-DNA binding was determined quantitatively by spectrophotometry from renaturation rates in accordance with a modification of the method of De Ley et al. *(7).* The temperature of renaturation was 25°C below the midpoint. DNA-DNA relatedness was calculated after incubation for 21 and 24 min after data for the first 3 min of renaturation were removed. Identification of a strain to species was confirmed when the DNA-DNA reassociation value between the study strain and the species type strain was more than or equal to 70%.

TABLE 1. *Type and reference bifidobacteria strains used for phenotypic numerical analysis and DNA-DNA hybridization*

Species (name as received)	Collection and reference no.[a]
Bifidobacterium adolescentis	CCUG 18363^T
B. angulatum	DSMZ 20098^T
B. animalis	NCFB 2242^T
B. animalis	ATCC 27674
B. asteroides	DSMZ 20089^T
B. bifidum	DSMZ 20082
B. boum	DSMZ 20432^T
B. breve	NCFB 2257^T
B. catenulatum	CCUG 18366^T
B. choerinum	DSMZ 20434^T
B. coryneforme	DSMZ 20216^T
B. cuniculi	DSMZ 20435^T
B. dentium	CCUG 18367^T
B. gallicum	DSMZ 20093^T
B. gallinarum	ATCC 33777^T
B. gallinarum	ATCC 33778
B. globosum	ATCC 25864
B. indicum	DSMZ 20214^T
B. longum	NCTC 11818^T
B. magnum	DSMZ 20222^T
B. merycicum	RU 915 B^T
B. minimum	DSMZ 20102^T
B. pseudocatenulatum	DSMZ 20438^T
B. pseudolongum	DSMZ 20094
B. pseudolongum subsp. *globosum*	RU 224^T
B. pseudolongum subsp. *pseudolongum*	MB7^T
B. psychraerophilum	LMG 21775^T
B. pullorum	DSMZ 20433^T
B. ruminantium	RU 687^T
B. saeculare	DSMZ 6531
B. scardovii	DSMZ 13734^T
B. subtile	DSMZ 20096^T
B. suis	SU 859^T
B. suis	ATCC 27532
B. thermacidophilum subsp. *thermacidophilum*	LMG 21395^T
B. thermacidophilum subsp. *porcinum*	LMG 21689^T
B. thermophilum	MB1^T
B. thermophilum	DSMZ 20212
Aeriscardovia aeriphila	LMG 21773^T

[a] T, type strain. CCUG, Culture Collection of University of Göteborg, Sweden; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Göttingen, Germany; NCFB, National Collection of Food Bacteria, Shinfield, Reading, Berkshire, UK; ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK: RU, SU, and MB, B. Biavati, Bologna, Italy; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Belgium.

RESULTS AND DISCUSSION

Distribution of the *Bifidobacterium* species in fecal samples on the farm. Bifidobacteria were isolated from 84% of the total fecal samples examined (122 of 145 samples; Table 2). Ninety-six percent (108 of 113) of the cattle,

TABLE 2. *Concentration and identification of* Bifidobacterium *species in different types of animal feces on farms in France and Austria*

Animal species	No. of samples	No. (%) of samples positive for bifidobacteria	Mean (SD) concentration of bifidobacteria in positive samples (log CFU g^{-1})	*Bifidobacterium* species isolated (no. of samples)
France[a]				
Cow	40	39 (97)	5.87 (1.98)	*B. pseudolongum* (39) *B. thermophilum* (7) *B. ruminantium* (1) *B. adolescentis* (2)
Sheep	9	7 (78)	4.29 (2.87)	*B. pseudolongum* (7) *Bifidobacterium* sp. (1)
Goat	2	2 (100)	3.0 (0)	*B. pseudolongum* (2) *B. bifidum* (1)
Chick	2	0 (0)		
Chicken	5	2 (40)	2.50 (0.71)	*B. pseudolongum* (2) *Bifidobacterium* sp. (1)
Hen	4	2 (50)	0.50 (0.71)	*B. pseudolongum* (2) *B. thermophilum* (1)
Horse	6	4 (67)	1.75 (0.96)	*B. pseudolongum* (1) *B. catenulatum* (1) *Bifidobacterium* sp. (3)
Swine	6	4 (67)	5.75 (3.95)	*B. pseudolongum* (4) *B. thermophilum* (1) *B. adolescentis* (1) *Bifidobacterium* sp. (1)
Goose	1	0		
Rabbit	8	2 (25)	2.50 (2.12)	*B. pseudolongum* (1) *B. adolescentis* (1)
Pigeon	4	2 (50)	1.0 (0)	*B. thermophilum* (2) *Bifidobacterium* sp. (1)
Austria[b]				
Cattle	35	35 (100)	4.4 (1.2)	*B. pseudolongum* (30)[c] *B. thermophilum* (2)[d] *Bifidobacterium* sp. (3)
Swine	23	23 (100)	7.0 (0.4)	*B. pseudolongum* (9)[c] *B. thermophilum* (18)[d] *B. animalis* (9) *Bifidobacterium* sp. (2)
Total	145	122 (84)	ND[e]	*B. pseudolongum* (99) *B. thermophilum* (31) *B. adolescentis* (4) *B. animalis* (9) *B. bifidum* (1) *B. catenulatum* (1) *B. ruminantium* (1) *Bifidobacterium* sp. (12)

[a] Bifidobacteria detected using BHI as the basal medium for enrichment and paromomycin as the selective agent in isolation medium.
[b] Bifidobacteria detected using RCM as the enrichment medium and mupirocin as the selective agent in isolation medium.
[c] χ^2, $P < 0.0005$.
[d] χ^2, $P < 0.0001$.
[e] ND, not determined.

swine, and sheep fecal samples but only 44% (14 of 32) of the fecal samples collected from other animals contained bifidobacteria. The most frequently occurring species was *B. pseudolongum*, which was present in 81% (99 of 122) of all *Bifidobacterium*-positive fecal samples and predominated in samples from all animal species except swine fecal samples collected in Austria.

Although it had previously been split into two different species, the species *B. pseudolongum* consists now of two subspecies: *B. pseudolongum* subsp. *pseudolongum* and *B. pseudolongum* subsp. *globosum (33).* In pig feces, Yaeshima et al. *(33)* found only *B. pseudolongum* subsp. *pseudolongum*; *B. pseudolongum* subsp. *globosum* did not occur.

TABLE 3. *Number of strains assigned to* B. pseudolongum *subsp.* pseudolongum *and* B. pseudolongum *subsp.* globosum *and DNA-DNA reassociation percentage of these strains with the type strains of each subspecies (MB7 and RU224)*

Fecal sample	*n*	No. of strains identified to: *B. pseudolongum* subsp. *pseudolongum* (% reassociation with MB7)	No. of strains identified to: *B. pseudolongum* subsp. *globosum* (% reassociation with RU224)
Swine (farm)	8	7 (78–91)	7 (50–75)
		1 (62)	1 (99)
Swine (slaughter)	2	2 (89–100)	2 (64–75)
Cattle (farm)	5	4 (59–74)	4 (84–96)
		1 (63)	1 (61)
Rabbit (farm)	1	1 (62)	1 (58)
Chicken (farm)	3	2 (62–75)	2 (77–92)
		1 (63)	1 (60)
Horse (farm)	1	1 (51)	1 (59)
Sheep (farm)	3	3 (68–74)	3 (78–92)

In accordance with this observation, DNA-DNA hybridization analyses between DNA of type strains MB7 and RU224 of these two subspecies and DNA of 23 isolates from animal fecal samples revealed that *B. pseudolongum* subsp. *pseudolongum* was present in 9 of the 10 swine fecal samples (Table 3). *B. pseudolongum* subsp. *globosum* was recognized in one swine fecal sample, four cattle samples, three sheep samples, and two chicken samples. Four strains (one each from cattle, rabbit, chicken, and horse) belonged to neither *B. pseudolongum* subsp. *pseudolongum* nor *B. pseudolongum* subsp. *globosum*, although they were closely related to both subspecies (58 to 61% DNA reassociation) and to each other (77 to 92% DNA reassociation) (Table 4). These four strains formed a distinct genomic group close to *B. pseudolongum* as described by Yaeshima et al. *(33)*. Phenotypically, a few characteristics that could be used in numerical identification systems were selected to differentiate *B. pseudolongum* subsp. *pseudolongum*, *B. pseudolongum* subsp. *globosum*, and the close genomic group X (Table 5).

The species distribution in cattle was similar in France (40 fecal samples; Table 2) and Austria (35 fecal samples).

TABLE 4. *DNA-DNA reassociation percentage between strains Lap4/2, Poul1/2, Chev2/4, and Catt2843 from rabbit, chicken, horse, and cattle, respectively*

Strain	Lap4/2	Poul1/2	Chev2/4
Lap4/2			
Poul1/2	77		
Chev2/4	77	92	
Catt2843	89	79	88

B. pseudolongum was the most frequently occurring species followed by *B. thermophilum* regardless of the detection method used (paromomycin in France and mupirocin in Austria). Mupirocin as a selective agent in bifidobacteria research was introduced by Rada and Petr *(23, 24)* and Petr and Rada *(22)*, who used it efficiently to detect bifidobacteria from ceca and crops of hens.

B. thermophilum was isolated from 25% (31) of the total 122 samples and therefore was the second most frequently occurring species. In swine fecal samples collected in Austria, *B. thermophilum* was present in 78% of the samples, thus representing the main species in swine samples from this country, ahead of *B. pseudolongum* (48%; Table 2). The distribution of *B. thermophilum* and *B. pseudolongum* was significantly different in Austrian swine and cattle fecal samples (χ^2, $P < 0.0005$).

The first description of *B. thermophilum* was published by Mitsuoka *(20)* based on strains isolated from swine feces. At the same time, Scardovi et al. *(28)* defined the species *Bifidobacterium ruminale*, which was determined to be synonymous with *B. thermophilum (5, 27, 29)* isolated from bovine rumen. In the present study, *B. thermophilum* was found in both swine and cattle feces. One strain of this species was isolated from chicken samples and one was isolated from pigeon samples, but none were found in samples from the other animal species (Table 2).

Less frequently identified *Bifidobacterium* species were *B. animalis* (nine swine fecal samples), now known as *B. animalis* subsp. *animalis (16)*, and *B. ruminantium* (one cattle fecal sample) *(4)*, both species being of animal origin. Six strains were identified as belonging to species of predominantly human origin: *B. adolescentis* (two from cattle fecal samples, one from swine, and one from rabbits), *B.*

TABLE 5. *Phenotypic characteristics for the differentiation of* B. pseudolongum *subsp.* globosum, B. pseudolongum *subsp.* pseudolongum *and the genomic group X*[a]

Biochemical test	*B. pseudolongum* subsp. *globosum* ($n = 12$)	*B. pseudolongum* subsp. *pseudolongum* ($n = 11$)	Genomic group X ($n = 4$)
Acidification of[b]:			
L-Arabinose	− or + (42)	+ (91)	+ (100)
D-Xylose	− or + (25)	+ (91)	+ (100)
Enzymatic tests			
α-Arabinosidase	− or + (25)	+ (100)	+ (100)
Alanine arylamidase	− or + (17)	− or + (82)	− or + (25)

[a] Results are given as negative or positive for each test. Values in parentheses are the percentage of positive reactions.
[b] Acidification within 48 h.

TABLE 6. *Distribution of* Bifidobacterium *species along beef and pork production chains in Austria*

Production stage	*n*	No. (%) of samples positive for bifidobacteria	*Bifidobacterium* species[a]
Cattle			
Feces at slaughterhouse	30	17 (57)	*B. pseudolongum* (9/14) *B. thermophilum* (7/14)
Slaughter environment	42	6 (14)	*B. pseudolongum* (3/6) *B. thermophilum* (4/6)
Freshly slaughtered carcasses	122	11 (9)	*B. pseudolongum* (7/7)
Carcasses before cutting	120	4 (3)	*B. pseudolongum* (2/2)
Cutting environment	83	0	
Retail meat	30	0	
Total cattle	427	38 (9)	*B. pseudolongum* (21/28)[b] *B. thermophilum* (11/28)[c]
Swine			
Feces slaughterhouse	30	24 (80)	*B. pseudolongum* (6/24) *B. thermophilum* (20/24) *B. choerinum* (1/24)
Slaughter environment	48	9 (19)	*B. pseudolongum* (4/6) *B. thermophilum* (3/6)
Freshly slaughtered carcasses	121	19 (16)	*B. pseudolongum* (9/19) *B. thermophilum* (10/19) *B. choerinum* (1/19) *Bifidobacterium* sp. (1/19)
Carcasses before cutting	120	11 (9)	*B. pseudolongum* (4/11) *B. thermophilum* 8/11) *B. choerinum* (2/11)
Cutting environment	79	2 (3)	*B. thermophilum* (2/2)
Retail meat	30	2 (7)	*B. pseudolongum* (1/2) *Bifidobacterium* sp. (1/2)
Total swine	428	67 (16)	*B. pseudolongum* (24/64)[b] *B. thermophilum* (43/64)[c] *B. choerinum* (4/64) *Bifidobacterium* sp. (2/64)

[a] Values in parentheses are no. of samples in which the species was identified/no. of samples in which bifidobacteria were identified at the species level.
[b] χ^2, $P < 0.005$.
[c] χ^2, $P < 0.0005$.

bifidum (one from goats), and *B. catenulatum* (one from horses). These primarily human-associated species were uncommon but present in the animal feces. Another 12 strains isolated from sheep (one strain), chicken (one), horse (three), swine (one from France and two from Austria), pigeon (one), and cattle (three from Austria) fecal samples could not be assigned to any known *Bifidobacterium* species. According to our analyses, these isolates did not belong to the most recently described species *Bifidobacterium thermacidophilum,* which was isolated from an anaerobic digester by Dong et al. *(8),* nor to *B. psychraerophilum* or *Aeriscardovia aeriphila,* both species isolated from swine feces and able to multiply at low temperatures and under aerobic conditions *(30, 31).* The nature of these isolates should be examined further.

Distribution of the *Bifidobacterium* species along cattle and pork processing chains. In Austria, bifidobacteria were isolated from cattle fecal samples obtained at the slaughterhouse much less often (57%) than in samples obtained at the farm (100%).

For freshly slaughtered carcasses, 9% of cattle and 16% of swine carcasses contained bifidobacteria (Table 6), whereas for chilled carcasses at cutting and deboning, 3% of cattle and 9% of swine carcasses were contaminated. No retail beef cuts but 7% of retail pork cuts contained bifidobacteria. The two most frequently found species were the same as in those found in feces (*B. pseudolongum* and *B. thermophilum*). These results indicate that fecal contamination occurred mainly during the slaughter stage. A significant difference was still observed between the beef and the pork processing chains with respect to the occurrence of *B. thermophilum* (χ^2, $P < 0.0005$) and *B. pseudolongum* (χ^2, $P < 0.005$). Only one other species, *B. choerinum,* was detected with low frequency (one swine fecal sample and

one chilled swine carcass). This species was described by Scardovi et al. *(27)* based on strains isolated from swine feces. The occurrence of this bacterial species confirmed contamination of meat by feces at the slaughter stage.

The two species *B. pseudolongum* and *B. thermophilum* can be used as indicators of fecal contamination along beef and pork meat processing chains. However, their occurrence was low at different stages of the chain. Further investigations using methods such as PCR assays are needed to confirm these results. PCR techniques were suggested by Delcenserie et al. *(6)* as methods for detection of bifidobacteria as fecal indicators in raw milk and raw milk cheese samples. The culture-based methods for detecting bifidobacteria are time-consuming and are less sensitive than PCR techniques *(6)*. When PCR assays are performed directly on samples without an enrichment step, information on the number of killed bifidobacteria present along the meat chains can be obtained.

Identification of the *Bifidobacterium* species in fecal samples does not differ much among animal species. Two of the 16 known *Bifidobacterium* species of animal origin, *B. pseudolongum* and *B. thermophilum*, dominated in the fecal samples and throughout the beef and pork production and processing chains. Beerens et al. *(3)* found that these species also were predominant in raw milk samples contaminated by cow feces on farms in France and Belgium.

Considering the low number of meat samples that were positive for bifidobacteria, further studies using culture-based or PCR methods should be performed to determined the occurrence of *B. pseudolongum* and *B. thermophilum* in animal feces and other meat processing chains.

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