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Identification and characterisation of halotolerant bacteria in spoiled dry-cured hams

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Abstract

Twenty bacterial strains isolated from Italian dry-cured hams affected by the so-called 'vein defect', were Gram positive, catalase and oxidase negative non-spore-forming rods. Twelve strains were identified by molecular characterisation as *Marinilactibacillus psychrotolerans*. These strains were demonstrated to survive at high salt concentrations (up to 25% w/w, with growth up to 12% w/w), low temperatures (0–3 °C) and a pH range (6–7), which is encountered within the leg arterial vein. If strains of *Marinilactibacillus* are confirmed as causative agents of the 'vein defect', new manufacturing guidelines can be addressed to ham producers.

1. Introduction

Dry-cured ham is a product prepared according to a recipe whose roots lie deep in antiquity. The recent introduction of automation and mostly the application of HACCP systems improved quality and general safety of productions. However, a variety of technological problems remain unsolved preventing the production of cured ham completely free from defects. This results in rejects and financial losses.

Microbial defects affecting dry-cured ham are usually detected by the "probe and sniff technique" carried out around the 10th–12th month of ageing (Parolari, 1996). The defects of microbial origin typically encountered in hams can be grouped into two categories: (1) deep spoilage (typically at shank level) and (2) surface defects (vein, knob and aitch bone). Many studies carried out on "deep defect" proved that their onset can be ascribed to the growth of *Enterobacteriaceae* belonging to the genus *Enterobacter*, *Proteus* and *Serratia* (Campanini & Casolari, 1983). The "phenol-like odour" defect was attributed to the growth of *Penicillium puberulum* (syn. *Penicillium commune*) in the aitch bone (Spotti, Mutti, & Campanini, 1988). Strains of *Pseudomonas cepacia* were isolated from hams affected with the "potato defect" below the aitch bone (Blanco, Barbieri, Mambriani, Spotti, & Barbuti, 1994). In vitro studies (Campanini, Barbuti, Ghisi, & Baldini, 1985) on the isolated strains allowed definition of the limiting values of a_w, salt concentration and temperature for growth and inactivation.

In comparison to data for the defects previously mentioned, identification and characterisation of specific spoilage organisms in the "vein defect" have not been sufficiently studied. Therefore, the "vein defect" still remains a difficult problem to understand. The surface area of the femoral vein in defective dried hams was recently analysed for the presence of possible microorganisms involved in spoilage (Barbuti et al., 2003; Rastelli, Grisenti, Quintavalla, & Barbuti, 2002). Results from those studies revealed that halotolerant bacteria

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could be present at the exit of the femoral vein of spoiled hams.

In order to obtain more information on the role of such bacteria in the development of the "vein defect", strains of halotolerant bacteria in spoiled hams have been identified and characterised in this study.

2. Materials and methods

2.1. Strains, media, and cultivation conditions

Twenty bacterial strains isolated from Italian hams affected by the so-called 'vein defect' were stored at −20 °C in Brain-heart infusion (BHI) (Oxoid) broth containing 10% glycerol. Colonies were purified by streaking on Tryptone Soya Agar (TSA) (Oxoid) at 30 °C for 48 h and routinely cultivated in Tryptone Soya Broth (TSB) (Oxoid).

2.2. Preliminary phenotypic characterisation

All the isolates were evaluated for morphology, spore-forming ability and ability to grow on Mannitol Salt Agar (MSA) (Oxoid) and MRS agar (Oxoid), and subjected to Gram staining, catalase and oxidase tests. The growth ability on TSA at different temperatures (3, 9, 25, 37, and 42 °C) was also tested.

To evaluate the interaction of NaCl, pH and temperature upon bacterial growth, all the strains were inoculated on TSA with added 0.3% "Lab Lemco" powder (Oxoid) and modified to obtain a combination of factors including temperature (3, 9, 25 °C) pH (6, 7) and NaCl (8, 10, 12, 14, 16, 18 as percent w/w values); the inoculation of media was by multipoint technique (Faiers, George, Jolly, & Wheat, 1991). The growth was scored as positive if colony formation was observed within seven days. Isolate IV1 was then chosen as a representative strain among those isolated from spoiled hams for further tests.

2.3. Biochemical identification

Biochemical identification was based on the ability of isolates to oxidase different carbon sources, as determined by Biolog® GP2 Microplate method (AES Laboratoire).

2.4. Molecular identification

2.4.1. Genomic DNA extraction

About 100–200 µl of each strain (approximately 10^7 cfu), which had grown overnight at 25 °C in TSB, were pelleted by centrifugation at 12,500*g* for 5 min. The pellets were washed twice with sterile water in a clean 1.5-ml microcentrifuge tube and re-pelleted by centrifugation. Genomic DNA was extracted from washed cell pellets by a standard alkaline lysis method as described previously (Giraffa, Rossetti, & Neviani, 2000).

2.4.2. 16S rRNA gene sequence determination and phylogenetic analysis

Direct sequencing of the PCR amplified 16S rRNA gene of strain IV1 was performed using the MicroSeq Full Gene 16S rDNA Bacterial Sequencing and Identification kit System (Applera Italia, Monza, Italy) according to the protocol suggested by the manufacturer. Briefly, 50 µl PCR samples containing 25 µl of genomic DNA (diluted to obtain a final concentration of 1 ng μl^{-1} PCR sample) and 25 µl of PCR master mix included in the kit were amplified. PCRs consisted of an initial denaturation step of 95 °C for 10 min followed by 30 cycles of: 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s; a final elongation step of 72 °C for 10 min was performed. Prior to sequencing, amplified products were purified with the Microcon YM100 filter units (Millipore S.p.A, Milan, Italy) according to the manufacturer's recommendations. Cycle sequencing consisted of 11 steps of: six cycles of 10 s at 96 °C; 1 min at 65–55 °C (one degree less in each step).

In other experiments, sequencing of the hypervariable region (first 500 bp) in the 5′ end of the 16S rRNA gene was performed for all the other isolates using the MicroSeq 500 16S rDNA Bacterial Sequencing and Identification kit System (Applera Italia). A 500-bp 16S ribosomal DNA fragment was amplified from the 5′ end of the gene using primers forward GCYTAACACATGCAAGTCGA (46 *Escherichia coli* numbering) and reverse GTATTACCGCGGCTGCTGG (536 *E. coli* numbering). PCRs were carried out in a volume of 50 µl containing 200 $\mu mol\,l^{-1}$ each of dNTP, 5.0 µl of 10× *Taq* reaction buffer, 0.5 $\mu mol\,l^{-1}$ each of the two primers (Biotez, Berlin, Germany), 1.5 $mmol\,l^{-1}$ of $MgCl_2$, 1.25 U of AmpliTaq Gold DNA polymerase (Applera Italia), and 50 ng of genomic DNA, extracted as described above. PCRs were similar to those described above for full 16S rRNA gene amplification except the annealing temperature, which was set at 59 °C instead of 60 °C. After Microcon purification, forward and reverse sequencing reactions were performed for each 500-bp amplified product. The sequencing reactions consisted of 13 µl of MicroSeq sequencing mix, 4 µl of sterile distilled water, and 3 µl of purified amplified product. Cycle sequencing consisted of 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Rapid thermal ramps (1 °C/s) between steps were performed. Both PCR amplification and PCR sequencing reactions were performed in a Perkin Elmer (model 9700) thermal cycler (Applera Italia).

Excess dye terminators were removed by purification of PCR products using Centri-Sep™ spin columns (Applera Italia) as indicated by the manufacturer. Sequence analysis was performed on an ABI PRISM

310 Genetic Analyser (Applied Biosystems, Foster City, CA). The sequence data were analysed and multiple alignment was performed by using the MicroSeq software version 1.36. Consensus sequences obtained were compared to the main lactic acid bacteria species entries in the MicroSeq database using the Full Alignment Tool of the software. When the 500-bp sequences were analysed, a cut-off of <0.80% difference between the isolate and the database entries was chosen for species identity (Patel et al., 2000). A phylogenetic dendrogram with the 500-bp sequence data was constructed using the neighbour joining method. The identification was refined after BLAST (www.ncbi.nlm.nih.gov/BLAST) alignment of the obtained 16S rDNA sequences with the 16S rDNA sequences of bacteria and lactic acid bacteria (LAB) available from the EMBL database.

2.5. Growth studies

Microbial survival/inactivation properties as affected by salt, pH and temperature in such a range as is common in ham curing technology were studied for strain IV1. In order to focus on the influence of salt concentration, which can range over a wide concentration during the key stages of salting and resting, two trials were run covering a lower (8–16%, w/w) and an upper (18–25%, w/w) salt range. Those ranges are typically encountered on the ham surface during the resting and salting phases, respectively. Strain IV1 was inoculated into two series of tubes of TSB set at pH 6 and 7 each and containing 0,3% Lab Lemco and increasing NaCl concentrations, ranging from 8 to 25% w/w. In order to estimate inactivation and survival/growth ability of the IV1 strain, two different inoculum levels were used, they were 6.18 log cfu/ml for the inactivation study in the higher salt range and 5.18 log cfu/ml for survival/growth study in the lower salt range. After incubation of inoculated tubes at 0, 3, and 9 °C, growth was evaluated on TSA plates. Finally, to evaluate the effect of temperature increase occurring in ham manufacturing after the resting phase, the tubes were removed from the previous storage temperatures and the growth regaining ability at 20 °C within 7 days was determined.

3. Results and discussion

3.1. Preliminary characterisation of strains

The 20 isolates were found to be Gram positive, catalase and oxidase negative, non-spore-forming rods and were able to grow on MSA and not on MRS media. All the isolates were able to grow on TSA at 3, 9, 25, and 37 °C but not at 42 °C (data not shown). The growth ability of the isolates was also evaluated as a function of salt concentration (five levels ranging from 8 to 18 %, w/w), temperature (3, 9 and 25 °C), and pH (6 and 7). At pH 7, the 20 isolates were found to grow at the three temperatures tested only when salt was $\leqslant 8\%$, while at 10 and 12% salt concentrations growth was observed only at 9 and 25 °C; no growth occurred when salt was $\geqslant 14\%$ (Table 1). Growth ability was reduced at pH 6, with no growth at 3 °C while at 9 and 25 °C it was shown only if salt concentration was $\leqslant 8\%$ and $\leqslant 12\%$, respectively (Table 2).

3.2. Strain identification

After Biolog® GP2 Microplate identification, 12 out of 20 isolates were identified as *Carnobacterium* spp. with a similarity percent ranging from 40 to 72; eight of them were identified at species level (Table 2). The remaining eight isolates, though not identified by the Biolog database system, were found to be similar to *Carnobacterium* (six isolates) and *Globicatella* (two isolates), respectively. The "metabolic fingerprints" of the isolates are shown in Table 3.

Table 1
Effect of salt concentration and temperature on bacterial growth at pH 7 and 6

pH	Temperature (°C)	NaCl % (w/w)					
		8	10	12	14	16	18
7	3	+	–	–	–	–	–
	9	+	+	+	–	–	–
	25	+	+	+	–	–	–
6	3	–	–	–	–	–	–
	9	+	–	–	–	–	–
	25	+	+	+	–	–	–

Growth is scored as +, positive; –, negative.

Table 2
Halotolerant strains as identified by Biolog®

Strains	Species	ID	% similarity
GR2	*Carnobacterium mobile*	Species	72
GA6	*Carnobacterium divergens*	Species	67
IV1	*Carnobacterium gallinarum*	Species	62
BV	*Carnobacterium divergens*	Species	61
GA2	*Carnobacterium gallinarum*	Species	59
VE	Carnobacterium divergens	Species	52
FR5	*Carnobacterium divergens*	Species	52
GA1	*Carnobacterium divergens*	Species	50
FR1	*Carnobacterium divergens*	Genus	48
FR4	*Globigatella sanguinis*	No	48
SCB	Carnobacterium gallinarum	No	46
AN2	*Carnobacterium divergens*	No	45
B8D	*Carnobacterium mobile*	Genus	43
BM	*Globigatella sanguinis*	No	42
AN1	*Carnobacterium divergens*	Genus	40
GA4	*Carnobacterium divergens*	Genus	33
2B	*Carnobacterium alterfunditum*	No	28
SCD	Carnobacterium mobile	No	24
AN3	*Carnobacterium gallinarum*	No	22
GA5	*Carnobacterium gallinarum*	No	21

Table 3
Carbon compounds used by strains isolated from spoiled hams

Carbon compound	Strains																			
	SCD	SCB	B8D	2B	GR2	BV2	BM	IV1	AN1	AN2	AN3	VE	FR1	FR4	FR5	GA5	GA1	GA2	GA4	GA6
N-Acetyl-D-mannoside	+	+	+	+					+		+									
Amigdaline	+	+						+			+								+	
D-Galactose							+				+			+						
Gentiobiose	+	+		+		+	+	+	+	+	+	+	+			+	+	+	+	+
D-Gluconic-acid	+		+	+						+	+					+				
Maltose	+	+	+	+				+	+	+	+							+	+	
Maltotriose			+					+		+	+							+		
D-Melibiose							+							+	+		+			
β-Methyl-D-glucoside	+	+		+				+		+	+						+		+	+
D-Psicose		+	+	+	+	+		+	+	+	+	+			+	+		+	+	
D-Raffinose							+							+						
D-Ribose	+		+	+						+	+	+	+			+			+	

All the isolates were able to use the following compounds as carbon source: glycerol, salicin, D-mannose, D-mannitol, α-D-glucose, D-fructose, cellobiose, arbutin, *N*-acetyl-D-glucosamine, sucrose, and trehalose. None of the isolates utilised α-cyclodextrin, β-cyclodextrin, dextrin, glycogen, inulin, mannan, Tween 80, L-arabinose, D-arabitol, L-fucose, *m*-inositol, α-D-lactose, lactulose, D-melezitose, α-methyl-D-galactoside, β-methyl-D-galactoside, α-methyl-D-glucoside, α-methyl-D-mannoside, L-rhamnose, D-sorbitol, D-tagatose, turanose, xylitol, D-xylose, acetic acid, and D-galacturonic-acid.

The overall low percentage of similarity of the isolates to the genus *Carnobacterium*, as found by the biochemical identification, and their high tolerance to NaCl led us to carry out further identifications aimed at gene characterisation.

The complete sequence of the 16S rRNA gene was therefore determined for isolate IV1. According to the alignments and the high scores obtained after BLAST analysis, the isolate IV1 matched at a level 99% identity with the 16S rRNA gene sequence (accession number AB083406) of *Marinilactibacillus psychrotolerans* M13-2^T (Ishikawa, Nakajima, Yanagi, Yamamoto, & Yamasato, 2003). Because of this high homology, sequencing of 500 bp hypervariable region located in the 5′ end of the 16S rRNA gene was sufficient to identify eleven strains of other isolates, as suggested by Hugenholtz, Goebel, and Pace (1998). The sequences obtained were aligned and compared with those of phylogenetically related bacteria and LAB from the public data libraries. The sequences of the isolates and the isolate M13-2^T were almost identical over the 500 nucleotides, with one or two (depending on the isolate) base differences. Therefore, all the analysed strains were identified as *M. psychrotolerans*. To this end, a cut-off <0.80% difference between the isolate and the database entries was chosen for species identification, as suggested by Patel et al. (2000). Moreover, as shown by the phylogenetic tree in the cluster, all the *M. psychrotolerans* isolates had very similar 16S rRNA gene sequences and corresponded to an independent lineage close relative to the LAB/carnobacterial group (Fig. 1).

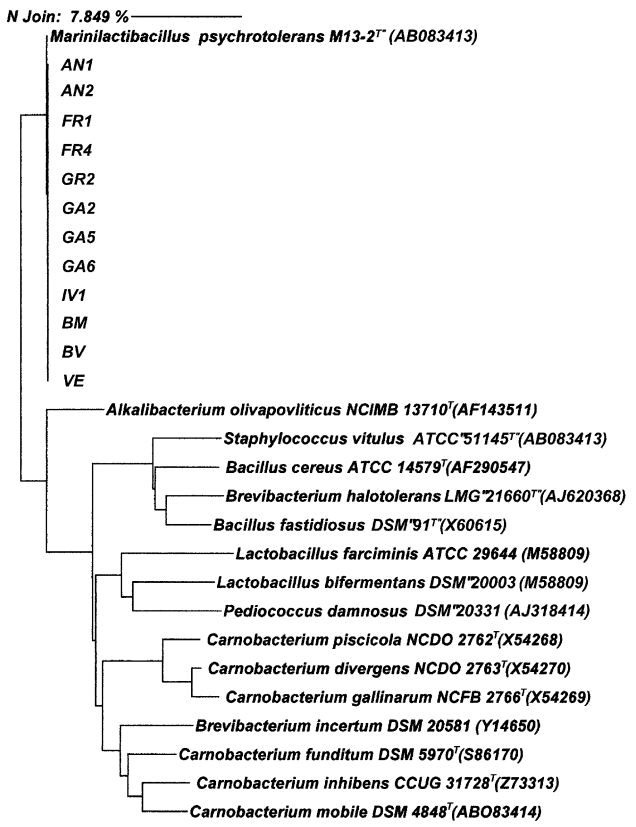

Fig. 1. Phylogenetic relationship between 12 meat halophilic isolates and some other related bacterial species belonging to the carnobacterial LAB group. The phylogenetic tree was based on the sequence of the hypervariable region of the 5′ end of the 16S rRNA gene using the neighbour joining method. Sequencing was performed using the MicroSeq 500 16S rDNA Bacterial Sequencing and Identification kit System (Applera Italia). Sequence alignment, distance matrix, and phylogenetic tree were calculated with the MicroSeq software. The sequences of the type strains of the main bacterial species belonging to the carnobacterial LAB group were downloaded from the EMBL database (EMBL accession numbers are indicated in parentheses).

3.3. Growth ability of isolates as affected by NaCl, pH and temperature

After preliminary observations and data comparison, *M. psychrotolerans* IV1 was chosen as representative

strain and subjected to survival and/or inactivation studies as well as growth evaluation tests under environmental conditions mimicking the salting and resting phases occurring in dry-curing ham manufacturing.

At 9 °C, *M. psychrotolerans* IV1 grew well at salt concentrations of 12% (at pH 7) and 10% (at pH 6). At temperatures as low as 0–3 °C, the bacterium decreased in number but still survived at all the salt–pH combinations (Table 4).

Even if higher inactivation rates were observed at salt concentrations as high as 18–25%, strain IV1 still survived after 20 days of incubation at all the three salt concentrations and at temperatures as low as 0, 3 and 9 °C. Results showed that the growth inhibition increased (about 1–3 decimal reductions) with increasing sodium chloride concentration combined with decreased temperature and pH (Table 5).

In spite of such a limited survival, *M. psychrotolerans* IV1 was able to resume its growing ability to grow within a week, once an incubation temperature of 20 °C was restored. The maximum NaCl concentration allowing growth at 20 °C was 12% at pH 6 and 14% at pH 7 (Table 6).

According to the above results, it appears that physico-chemical factors occurring at femoral vein level during ham curing, namely the high pH remaining after the leg bleeding together with the salt amounts typically applied in ham processing are key in growth pattern of *M. psychrotolerans*.

Marinilactibacillus psychrotolerans was described for the first time by Ishikawa et al. (2003), who isolated it froms marine subtropical areas of Japan. *M. psychrotolerans* was also recently found for the first time in dairy products as a part of the cheese ripening microbial

Table 4
Growth behaviour [$\log_{10}$ cfu/ml] of *Marinilactibacillus psychrotolerans* strain IV1, inoculum size 5.18 ± 0.07 log cfu/ml, after 20 days at the indicated temperatures, salt concentrations and pH values

NaCl % (w/w)	0 °C		3 °C		9 °C	
	pH 6	pH 7	pH 6	pH 7	pH 6	pH 7
8	4.17 (0.05)	4.85 (0.15)	4.17 (0.23)	4.80 (0.12)	**7.18 (0.20)**	**8.62 (0.13)**
10	4.51 (0.12)	4.65 (0.47)	3.93 (0.07)	4.36 (0.28)	**5.83 (0.01)**	**8.60 (0.04)**
12	2.64 (0.06)	4.26 (0.26)	2.30 (0.07)	3.92 (0.16)	4.23 (0.05)	**5.97 (0.03)**
14	2.64 (0.09)	3.60 (0.15)	3.32 (0.34)	3.38 (0.08)	2.91 (0.09)	3.68 (0.29)
16	3.60 (0.05)	4.08 (0.22)	2.72 (0.13)	3.89 (0.09)	3.34 (0.25)	2.48 (0.17)

Average values of five independent tests, SD in parentheses.
Bold values indicate factor combinations allowing growth.

Table 5
Growth behaviour [$\log_{10}$ cfu/ml] of *Marinilactibacillus psychrotolerans* strain IV1, inoculum size 6.18 ± 0.06 log cfu/ml, after 20 days at the indicated temperatures, salt concentrations and pH values

NaCl % (w/w)	0 °C		3 °C		9 °C	
	pH 6	pH 7	pH 6	pH 7	pH 6	pH 7
18	4.38 (0.48)	5.18 (0.10)	4.14 (0.26)	5.04 (0.06)	4.20 (0.06)	4.64 (0.10)
21	3.89 (0.08)	4.76 (0.10)	3.82 (0.10)	4.72 (0.08)	3.32 (0.13)	4.70 (0.01)
25	3.92 (0.07)	3.08 (0.11)	2.91 (0.02)	4.04 (0.13)	3.85 (0.01)	4.36 (0.05)

Average values of five independent tests, SD in parentheses.

Table 6
Growth behaviour [$\log_{10}$ cfu/ml] of *Marinilactibacillus psychrotolerans* strain IV1, stored 20 days at the salt concentrations, pH and temperatures reported in Tables 5 and 6, after 7 days at the resumed temperature of 20 °C

NaCl % (w/w)	From 0 °C		From 3 °C		From 9 °C	
	pH 6	pH 7	pH 6	pH 7	pH 6	pH 7
8	**8.00 (0.01)**	**7.30 (0.14)**	**8.26(0.08)**	**6.23 (0.06)**	nd	nd
10	**8.00 (0.05)**	**8.46 (0.04)**	**8.20 (0.00)**	**8.28 (0.11)**	**8.32 (0.03)**	nd
12	**4.66 (0.02)**	**8.46 (0.01)**	**4.26 (0.05)**	**8.30 (0.22)**	**6.90 (0.05)**	**8.20 (0.05)**
14	2.32 (0.00)	**5.83 (0.03)**	2.79 (0.03)	**4.25 (0.12)**	3.28 (0.02)	**5.14 (0.04)**
16	<2.00	3.68 (0.01)	2.66 (0.02)	3.43 (0.02)	<2.00	3.76 (0.01)
18	nd	2.00 (0.01)	2.98 (0.02)	4.38 (0.05)	nd	4.61 (0.01)
21	<2.00	3.15 (0.10)	2.30 (0.07)	3.49 (0.16)	<2.00	3.90 (0.02)
25	<2.00	3.63 (0.04)	<2.00	3.18 (0.11)	<2.00	3.32 (0.03)

Average values of five independent tests, SD in parentheses.
nd = not determined.
Bold values indicate factor combinations allowing growth.

consortia in red smear cheese (Maoz, Mayr, & Scherer, 2003). In both cases, the microorganism was isolated from rather salty media, as was the case with spoiled hams in this study.

Due to the halophilic character of *M. psychrotolerans*, the salt employed as ingredient in the curing technology of the raw ham can be expected to be a major contamination source by this organism.

4. Conclusions

While *Enterobacteriaceae* and other microbial groups have long been reported to be responsible for ham spoilage, highly halotolerant bacteria have never been isolated from the spoiled surface area around the femoral vein. Within those, *M. psychrotolerans* a halophilic lactic acid bacterium originally isolated from marine organisms, was found in Italian dry-cured hams affected by the vein defect. Since *M. psychrotolerans* is a recently discovered species, the Biolog system failed to identify this organism which is not included in its reference database. Therefore, the 16S rRNA gene was sequenced to obtain a reliable identification.

If such type of bacteria as *Marinilactibacillus* will be confirmed as a causative factor of ham spoilage, new manufacturing guidelines taking into account the behaviour of halotolerant bacteria will be needed. They should include: pH control of the vein and surrounding area by an appropriate pH modifier and thorough bleeding; adequate salt addition to ensure maximum salt intake at the vein level; and stringent hygiene standards by intensive sanitation procedures. In this respect, more in-depth research is required to ascertain the mechanism by which halotolerant bacteria can contaminate pork legs, since they do not appear to be common raw meat contaminants.

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