

ORIGINAL ARTICLE

Bifidobacteria as indicators of faecal contamination along a sheep meat production chainV. Delcenserie¹, D. Loncaric², C. Bonaparte², M. Upmann², B. China¹, G. Daube¹ and F. Gavini³¹ Food Sciences Department, Faculty of Veterinary Medicine, University of Liège, Liege, Belgium² Institute of Meat Hygiene, Meat Technology and Food Science, University of Veterinary Medicine Vienna, Wien, Austria³ Département Microbiologie et Chaîne Alimentaire, Institut National de la Recherche Agronomique, Villeneuve d'Ascq, France**Keywords***Bifidobacterium choerinum*, *Bifidobacterium pseudolongum*, *Bifidobacterium thermophilum*, DNA–DNA hybridization, faecal indicators, *hsp60* gene, meat, mupirocin, PCR, sheep, slaughter.**Correspondence**G. Daube, Food Sciences Department, Faculty of Veterinary Medicine, University of Liège, Sart Tilman, B43b, 4000 Liege, Belgium.
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Abstract**Aims:** The potential use of bifidobacteria as indicators for faecal contamination was studied along a sheep meat production and processing chain. The levels of bifidobacteria were compared with those of *Escherichia coli*. Total viable counts were followed along the chain (244 samples).**Methods and Results:** Forty-three per cent of the samples contained bifidobacteria, of which 15% were solely detected using a PCR method based on the *hsp60* gene and not by a culture-based method. Bifidobacteria were detected in only three of nine sheep faeces samples using one or the other method. However, carcasses (types C and E) were highly contaminated. These sample types (30% and 28%, respectively) were positive for bifidobacteria and negative for *E. coli*. The species *Bifidobacterium pseudolongum* and *Bif. thermophilum*, isolated from faecal samples, were predominant. *Bifidobacterium choerinum* were found in C, D, E and F sample types.**Conclusions:** Bifidobacteria were shown more efficient than *E. coli* in carcasses samples. The presence of *Bif. choerinum* suggested a faecal pork contamination. **Significance and Impact of the Study:** Detection and identification of bifidobacteria, in correlation with *E. coli* counting, should improve hygiene quality of mutton processing chains.**Introduction**

The presence of faecal material on meat can be used as an indicator for unhygienic treatment during meat production. Thus, surveillance of faecal contamination appears appropriate to verify the effective implementation and maintenance of Hazard Analysis Critical Control Point systems (Brown *et al.* 2000).

Numerous methods have been described to detect faecal contamination on carcasses or meat samples. There are fast and noninvasive methods, such as fluorescence real-time detection of degradation products from chlorophyll by intestinal digestion (Ashby *et al.* 2003), infrared spectroscopy measuring metabolites caused by the growth and enzymatic activity of micro-organisms (Ellis *et al.* 2002) and luminescence-based bacterial phosphatase assay monitoring (Kang and Siragusa 2002). Counts of enteric

bacteriophages have also been employed (Kodikara *et al.* 1991; Maciorowski *et al.* 2001; Hsu *et al.* 2002). However, *Escherichia coli* is still the most commonly used indicator of faecal meat contamination, using either culture-based methods (Biss and Hathaway 1996; Calicioglu *et al.* 1999; Gill *et al.* 1999) or molecular-based PCR methods (Warriener *et al.* 2002; Aslam *et al.* 2003; Johnson *et al.* 2004; Venieri *et al.* 2004).

Bifidobacteria were first proposed as faecal indicators by Mossel (1958) over 40 years ago. Recently, they have been implicated in faecal contamination studies of water (Lynch *et al.* 2002; Gilpin *et al.* 2003; Nebra *et al.* 2003), of raw milk and raw milk cheese (Beerens *et al.* 2000; Delcenserie *et al.* 2005) and also of meat (Beerens 1998; Gavini and Beerens 1999; Gavini *et al.* 2006). Bifidobacteria represent one of the most important bacterial groups in human and animal intestine (Matsuki *et al.* 1998,

1999), where they occur at concentrations of 10^9 – 10^{10} cells g^{-1} of faeces (Tannock 2001). Moreover, as the dominant *Bifidobacterium* species are different in human and animal gut flora (Gavini *et al.* 1991), it should be possible to determine the contamination source, i.e. whether of human or of animal origin.

A culture-based method comprising enrichment and isolation steps (using mupirocin as selective agent during both steps) and a PCR-based method have been previously developed and successfully tested to detect bifidobacteria in raw milk and raw milk cheese samples taken at different stages of production (Delcenserie *et al.* 2005). Both methods were used to assess bifidobacteria contamination levels of mutton and to compare them with those of *E. coli*. At the same time, total viable counts (TVC) were performed.

Materials and methods

Samples

Sampling procedure

Between May and November 2003, 244 samples were collected from different stages of a mutton production and processing chain in Austria (slaughter, cutting and deboning, retail trade). The following sample types were studied:

- A. Sheep faeces: after slaughter, nine intestines were placed in plastic bags and transferred on ice to the laboratory. Intestines were cut with sterile instruments and 10 g of faeces was placed in a sterile bag using a sterile spatula.
- B. Slaughtering environment: 20 meat contact or hand contact surfaces such as knives, door handles and walls were sampled during processing. The samples were collected with swabs using a wet and dry double swab technique either from a marked surface area of 100 cm^2 or from the entire surface (where the surface was smaller than 100 cm^2). The sampling technique is described below (see C).
- C. Freshly slaughtered sheep carcasses: four sample sites per carcass were monitored: flank, thorax lateral, brisket and breast, and also some other sites (such as the perineal region), likely to carry high levels of contamination. A total of 43 sites were sampled. An area of 100 cm^2 , delineated by a sterile teflon template, was sampled using a wet and dry double swab technique. Each 100 cm^2 was first swabbed with a 10 $cm \times 10$ cm sterile swab moistened in sterile dilution fluid (0.1% peptone, 0.85% NaCl) and then with a dry sterile swab. Swabs were placed in sterile bags and stored refrigerated at 4°C and transported to the laboratory for microbiological examination.

- D. Cutting environment (meat contact and hand contact surfaces): during cutting and deboning, 80 environmental swabs were taken from knives, sawblades, cutting tables and containers for transport. The sampling technique was described above (see B).
- E. Refrigerated sheep carcasses before cutting and deboning: 74 swabs of 100 cm^2 were taken from the same sample sites as described above (see C.). Sampling procedure and sample transport did not differ from the freshly slaughtered carcasses.
- F. Retail meat samples: 18 samples of retail meat cuts were taken at retail stage; 20 g of tissue was cut with sterile scissors and placed into sterile bags.

Samples preparation

From faeces and retail meat samples, a 1 : 10 dilution was prepared in buffered peptone water (Oxoid, Basingstoke, UK). Swabs were rinsed in 100 ml buffered peptone water. All samples were homogenized for 60 s in a Stomacher 400 (Seward Medical, London, UK). Subsequently, decimal dilution rows with Drop-solution (DIN 10161-2 1984) were prepared. For faeces samples, the 10^0 to 10^{-6} dilutions were analysed, for environmental and carcass swabs the 10^0 to 10^{-3} dilutions and for retail meat samples the 10^{-1} to 10^{-4} dilutions.

The samples were analysed for the presence of bifidobacteria, *E. coli* and TVC. Bifidobacteria were detected by PCR and by culture-based methods after enrichment of each dilution step. *Escherichia coli* and TVC were determined by culture-based methods.

The numbers of positive *E. coli* and bifidobacteria samples were compared. The comparison of their mean counts was performed on positive samples for both. Total viable bacteria researched as hygiene indicators were followed along the production chain in the total 244 samples.

Detection of bifidobacteria

Culture-based methods

The method comprises enrichment, isolation and confirmation steps in a semi-quantitative manner. For enrichment, 1 ml of the respective dilution was transferred into sterile tubes, mixed and homogenized with 9 ml of enrichment medium.

The enrichment medium (BHMup; Delcenserie *et al.* 2005) was composed of 37 $g l^{-1}$ of Brain Heart Infusion (BHI; Bio-Rad, Marnes-la-Coquette, France); 5 $ml l^{-1}$ of propionic acid; 0.5 $g l^{-1}$ of Fe-citrate; 0.5 $g l^{-1}$ of cystein chlorhydrate; 5 $g l^{-1}$ of yeast extract; 2 $g l^{-1}$ of agar and 80 $mg l^{-1}$ of mupirocin (GlaxoSmithKline, West Sussex, UK). Tubes were incubated anaerobically for 48 h at 37°C.

From each enrichment culture with visible growth, 0.1 ml was spread onto plates of isolation medium called CMup. This was composed of Columbia agar (Oxoid; 39 g l⁻¹) with the addition of cystein hydrochloride (Merck, Darmstadt, Germany) 0.5 g l⁻¹; glucose (Oxoid) 5 g l⁻¹; ferric citrate (Sigma, Vienna, Austria) 0.5 g l⁻¹ and Bacteriological Agar (Oxoid) 5.0 g l⁻¹. The final pH was adjusted with 1 mol l⁻¹ NaOH to pH 6.9 and autoclaved at 121°C for 15 min. After cooling to 45°C, 50 mg l⁻¹ of mupirocin (GlaxoSmithKline) was aseptically added. Inoculated plates were incubated anaerobically at 37°C for 96–120 h.

Colonies types with different morphologies were examined microscopically and transferred into Veillon tubes containing reinforced clostridial medium (RCM; Oxoid) in order to confirm aerobic tolerance. RCM was prepared with 38 g l⁻¹ RCM and 3.5 g l⁻¹ bacteriological agar. The final pH of 6.9 was adjusted with 1 mol l⁻¹ NaOH. The medium was dispensed in Veillon tubes, closed with cellulose and autoclaved at 121°C for 15 min. After inoculation, the tubes were incubated aerobically at 37°C for 24–48 h to eliminate aerobic cultures and to keep anaerobes, which were confirmed by fructose-6-phosphate phosphoketolase (F6PPK) activity (Scardovi 1986) to be bifidobacteria.

Samples were considered bifidobacteria-positive when bifidobacteria levels were superior to 1 CFU g⁻¹ in case of faeces samples, 10 CFU g⁻¹ in case of retail meat samples and 1 CFU cm⁻² in case of swab samples.

PCR methods based on the *hsp60* gene

DNA was extracted from culture broths obtained after the enrichment step of the culture-based method (from 10⁰ to 10⁻⁶ dilutions depending on the sample type). This enables a semi-quantitative evaluation of the sample's *Bifidobacterium* content.

One millilitre of each homogenized broth was transferred to a microcentrifuge tube and centrifuged at 12 000 g for 2 min. Pellets were transferred to sterile, demineralized water, and the DNA extracted using a Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) with the addition of lysozyme (10 mg ml⁻¹; Eurogentec, Seraing, Belgium), as recommended for Gram-positive bacteria. DNA concentrations were spectrophotometrically estimated (GeneQuant *pro*; Amersham Pharmacia, Roosendaal, the Netherlands). DNA samples were diluted with distilled sterile water to obtain a concentration between 25 and 50 µg ml⁻¹.

Detection of the *Bifidobacterium* genus was performed using the PCR procedure described by Delcenserie *et al.* (2005). The following primers were used: B11 up: 5'-GTS CAY GAR GGY CTS AAG AA-3', B12 down: 5'-CCR TCC TGG CCR ACC TTG T-3' (Sigma Genosys, Haverhill, UK), corresponding to a fragment of 217 bp of the *hsp60* gene.

An internal DNA control was included in each reaction. The PCR mix was composed of 0.2 mol l⁻¹ dNTPs, 400 pmol l⁻¹ of each primer, 1 U of FastStart TaqPolymerase (Roche, Mannheim, Germany), 1× buffer: 500 mmol l⁻¹ Tris-HCl, 100 mmol l⁻¹ KCl, 50 mmol l⁻¹ (NH₄)₂SO₄, pH 8.3/25°C (Roche), 4 µl DNA (50–100 ng), 1 µl internal control (1.1 ng) and H₂O in a total volume of 20 µl.

The PCR was run using the following conditions: 5 min at 95°C, 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by a final extension (5 min at 72°C). Amplified PCR products were then analysed by gel electrophoresis. After electrophoresis, gels were stained with ethidium bromide (1 mg ml⁻¹) and photographed under UV (302 nm).

As described for the cultural-based method, samples were considered bifidobacteria-positive when at least one dilution was found positive and semi-quantitative results were analysed.

Identification of bifidobacteria

Phenotypic characterization and numerical analysis

The phenotypic numerical analysis included 74 strains isolated from different samples in different slaughtering stages and sample types, and type and reference strains, representative of human, animal and environmental species (Table 1).

Strains were considered members of the genus *Bifidobacterium* or of the close genera *Scardovia*, *Parascardovia* and *Aeriscardovia* on the basis of their F6PPK activity (Scardovi 1986). Enzymatic and carbohydrates fermentation tests utilized the ID32A and 50CH kits of 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 (Scardovi 1986).

The levels of similarity between strains were calculated by using the Jaccard index. The aggregation method was the unweighted pair group method with averages (Sneath and Sokal 1973).

DNA–DNA hybridization

Experiments were carried out on 13 strains selected from the numerical analysis, 12 of which were phenotypically close to *Bif. choerinum* and on one strain close to *Bif. thermophilum*, *Bif. thermacidophilum* ssp. *thermacidophilum* and *Bif. thermacidophilum* ssp. *porcinum*.

DNA was prepared with the simultaneous use of achromopeptidase (5000 U/500 mg bacteria) and lysozyme (400 000 U/500 mg bacteria) as lytic enzymes. DNA extraction was carried out according to Marmur's (1961) method.

Degree of DNA–DNA binding was determined quantitatively by spectrophotometry from renaturation rates in

Table 1 List of type and reference strains used in the phenotypic numerical analysis and in DNA–DNA hybridizations

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

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

accordance with a modification of the method of De Ley et al. (1970), using a Cary 100 spectrophotometer connected to a Peltier temperature controller (Varian, France). The temperature of renaturation was 25°C below the midpoint (T_m) according to the G + C content of

type strains. DNA–DNA relatedness values were calculated after incubation for 21 and 24 min, following removal in calculation of the first 3 min of renaturation. Identification of a strain at species level was given when the DNA–DNA reassociation value between DNAs of the strain studied and of the species' type strain was equal to or more than 70%.

Escherichia coli and total viable counts

Escherichia coli were enumerated on Coli ID medium (BioMérieux, Marcy-l'Étoile, France). One millilitre from each dilution (faeces: 10^0 to 10^{-6} ; environmental and carcass swabs: 10^0 to 10^{-3} ; retail meat: 10^{-1} to 10^{-4}) was transferred into sterile Petri dishes, 15 ml of molten Coli ID agar was added and left to set on a cool horizontal surface after gentle mixing. When the agar had set, a second layer (approx. 5 ml) of molten Coli ID agar was added. Plates were incubated aerobically at 42°C for 24 h. Pink to violet colonies (diameter 0.5–2 mm), based on β -glucuronidase and β -galactosidase activity were considered presumptive for *E. coli*. Only agar plates with more than five colonies were considered as positive.

Samples were considered *E. coli*-positive when *E. coli* levels were superior to 5 CFU g⁻¹ in case of faeces samples, 50 CFU g⁻¹ in case of retail meat samples and 5 CFU cm⁻² in case of swab samples.

Total viable bacteria were enumerated on Plate Count Agar (PCA; Oxoid) using the same method without over-layer as described for *E. coli*. Plates were incubated aerobically at 30°C for 72 h.

Statistical analysis

The McNemar test was used to statistically evaluate the different methods (culture-based and PCR methods) and to evaluate the effectiveness of bifidobacteria to replace *E. coli*, as faecal indicators. All dilutions were tested as separate values in order to compare culture-based and PCR methods.

Results

Detection of bifidobacteria by culture-based and PCR methods

The presence of bifidobacteria was investigated in 244 samples using both PCR and culture-based technique. They were detected in 43% (Table 2) of the total sample number. A significant difference was observed between both techniques in favour of PCR ($P < 0.0005$ and $P < 0.005$) with 37% and 15% of bifidobacteria-positive samples by PCR but bifidobacteria-negative by the cul-

| Sample types | Sample nos. | Number of positive or negative samples (% per sample type) | | | | Total bifidobacteria-positive samples using PCR and culture methods (% per sample type) |
|--------------|-------------|---|-------------------|-------------------|-------------------|--|
| | | PCR+*/ culture+† | PCR-/ Culture+ | PCR+/ Culture- | PCR-/ Culture- | |
| A | 9 | 3 (33) | 0 | 0 | 6 (67) | 3 (33) |
| B | 20 | 1 (5) | 0 | 2 (10) | 17 (85) | 3 (15) |
| C | 43 | 18 (42) | 0 | 16 (37)‡ | 9 (21) | 34 (79) |
| D | 80 | 5 (6) | 4 (5) | 6 (8) | 65 (81) | 15 (19) |
| E | 74 | 25 (34) | 9 (12) | 10 (14) | 30 (41) | 44 (59) |
| F | 18 | 2 (11) | 1 (6) | 3 (17) | 12 (67) | 6 (33) |
| Total | 244 | 54 (22) | 14 (6) | 37 (15)§ | 139 (57) | 105 (43) |

A, faeces; B, slaughtering environment swabs; C, freshly slaughtered carcasses swabs; D, cutting and deboning environment swabs; E, chilled carcasses swabs before cutting; F, retail meat.

*PCR+ if ≥ 1 CFU g⁻¹ or cm⁻².

†Culture+ if in faeces (type A) >1 CFU g⁻¹, in meat (type F) >10 CFU g⁻¹, in swabs (types B, C, D and E) >1 CFU cm⁻².

‡Significant difference in favour of PCR, $P < 0.0005$

§Significant difference in favour of PCR, $P < 0.005$.

ture-based method for type C and for total samples, respectively (Table 2).

False-negative results by PCR (PCR negative/culture positive) were noticed in D, E and F samples (5%, 12% and 6%, respectively; Table 2).

Identification of bifidobacteria strains

Three species were predominant (Table 3). Forty-three per cent of the strains studied were identified as *Bif. pseudolongum*, 41% as *Bif. thermophilum* and 15% as *Bif. choerinum*. One strain isolated from sample type F (retail meat) was close to *Bif. thermophilum* (52% of DNA–DNA relatedness with the type strain of the species), to *Bif. thermacidophilum* ssp. *thermacidophilum*, and to *Bif. thermacidophilum* ssp. *porcinum* (50% and 59% of DNA–DNA relatedness with respective type strains).

| Sample types | Identified strain nos. | Strain nos. and % per sample type identified as | | | |
|--------------|------------------------|---|--------------------------|-----------------------|----------------------------|
| | | <i>Bif. pseudolongum</i> | <i>Bif. thermophilum</i> | <i>Bif. choerinum</i> | <i>Bifidobacterium</i> sp. |
| A | 3 | 2 (66) | 1 (33) | 0 | 0 |
| B | 1 | 1 (100) | 0 | 0 | 0 |
| C | 19 | 8 (42) | 9 (47) | 2 (10) | 0 |
| D | 15 | 4 (27) | 8 (53) | 3 (20) | 0 |
| E | 29 | 15 (52) | 10 (34) | 4 (14) | 0 |
| F | 7 | 2 (29) | 2 (29) | 2 (14) | 1 (14) |
| Total | 74 | 32 (43) | 30 (41) | 11 (15) | 1 (1) |

A, faeces; B, slaughtering environment swabs; C, freshly slaughtered carcasses swabs; D, cutting and deboning environment swabs; E, chilled carcasses swabs before cutting; F, retail meat.

Table 2 Numbers of bifidobacteria positive or negative samples with PCR or culture-based method, or with both methods on 244 samples

Comparison of the contamination levels of bifidobacteria, *E. coli* and total viable counts

Regarding the total number of 244 samples, similar numbers of bifidobacteria positive (using PCR method) and *E. coli*-positive samples (37% and 32%; Table 4) were detected. This is also valid for sample types B (15% and 15%) and D (14% and 21%).

However, in faeces (type A) and retail meat cuts (type F), numbers of *E. coli*-positive samples (89% and 33%) were higher than those of bifidobacteria positive (33% and 28%), although the differences were not significant because of the low numbers of positive samples. The opposite was observed on freshly slaughtered carcasses (type C, 79% bifidobacteria-positive, 53% *E. coli*-positive) and on chilled carcasses (type E, 47% bifidobacteria-positive, 28% *E. coli*-positive), with high numbers of bifidobacteria-positive/*E. coli*-negative samples (30% and 28%).

Table 3 Identification of 74 bifidobacteria strains isolated from the different sample types

Table 4 Number of bifidobacteria (Bif.) positive or negative (using PCR method) and *E. coli* positive or negative samples from 244 sheep samples taken in the different sample types

| Sample types | Sample nos. | Sample nos. and % per sample type | | | | | |
|--------------|-------------|-----------------------------------|-----------------------------|-----------------------------|-----------------------------|----------|-----------------------|
| | | Bif. +/ <i>E. coli</i> + | Bif. -/ <i>E. coli</i> + | Bif. +/ <i>E. coli</i> - | Bif. -/ <i>E. coli</i> - | % Bif. + | % <i>E. coli</i> + |
| A | 9 | 2 (22) | 6 (67) | 1 (11) | 0 | 33 | 89 |
| B | 20 | 2 (10) | 1 (5) | 1 (5) | 16 (80) | 15 | 15 |
| C | 43 | 21 (49) | 2 (5) | 13 (30)* | 7 (16) | 79 | 53 |
| D | 80 | 7 (9) | 10 (12) | 4 (5) | 59 (74) | 14 | 21 |
| E | 74 | 14 (19) | 7 (9) | 21 (28)† | 32 (43) | 47 | 28 |
| F | 18 | 2 (11) | 4 (22) | 3 (17) | 9 (50) | 28 | 33 |
| Total | 244 | 48 (20) | 30 (12) | 43 (18) | 123 (50) | 37 | 32 |

A, faeces; B, slaughtering environment swabs; C, freshly slaughtered carcasses swabs; D, cutting and deboning environment swabs; E, chilled carcasses swabs before cutting; F, retail meat. Bif. +, if ≥ 1 CFU g⁻¹ or cm⁻², using PCR. *E. coli* +, if in faeces (type A) >5 CFU g⁻¹, in meat (type F) >50 CFU g⁻¹, in swabs (types B, C, D, E) >5 CFU cm⁻².

*Significant difference in favour of bifidobacteria, $P < 0.005$.

†Significant difference in favour of bifidobacteria, $P < 0.01$.

These differences were significant in favour of bifidobacteria (type C, $P < 0.005$; type E, $P < 0.01$).

Bifidobacteria and *E. coli* mean counts were compared based on the 48 bifidobacteria-positive/*E. coli*-positive samples (Tables 5 and 6). Bifidobacteria counts were

Table 5 Comparison of bifidobacteria and *E. coli* mean counts (log CFU g⁻¹ or cm⁻², standard deviation) in the 48 bifidobacteria-positive (using PCR)/*E. coli*-positive samples of different sample types

| Sample types | Sample nos. | Bifidobacteria counts (PCR method) | Bifidobacteria counts (culture-based method) | <i>E. coli</i> counts |
|--------------|-------------|------------------------------------|--|-----------------------|
| A | 2 | 3.50 (0.71) | 6.00 (0.00) | 7.44 (0.23) |
| B | 2 | 1.00 (0.00) | 0.50 (0.71) | 1.08 (0.17) |
| C | 21 | 1.95 (1.02) | 1.14 (1.53) | 1.57 (0.73) |
| D | 7 | 1.00 (0.00) | 0.29 (0.49) | 1.98 (0.63) |
| E | 14 | 1.64 (0.63) | 1.07 (0.47) | 1.31 (0.38) |
| F | 2 | 1.00 (0.00) | 2.00 (0.00) | 2.22 (0.54) |

A, faeces; B, slaughtering environment swabs; C, freshly slaughtered carcasses swabs; D, cutting and deboning environment swabs; E, chilled carcasses swabs before cutting; F, retail meat.

Table 6 Mean total viable counts (TVC, log CFU g⁻¹ or cm⁻², standard deviation) in the 244 samples of different sample types

| Sample types | Sample nos. | TVC |
|--------------|-------------|-------------|
| A | 9 | 5.63 (1.07) |
| B | 20 | 3.96 (1.25) |
| C | 43 | 4.47 (0.87) |
| D | 80 | 4.97 (0.97) |
| E | 74 | 3.87 (0.78) |
| F | 18 | 5.60 (0.87) |

A, faeces; B, slaughtering environment swabs; C, freshly slaughtered carcasses swabs; D, cutting and deboning environment swabs; E, chilled carcasses swabs before cutting; F, retail meat.

always higher using the PCR method than the culture-based method, except in type A samples (PCR, 3.50 log CFU g⁻¹; culture-based method, 6.0 log CFU g⁻¹) and in type F samples (PCR, 1.00 log CFU g⁻¹, culture-based method, 2.00 log CFU g⁻¹). For type A samples, *E. coli* count was much higher than the two bifidobacteria counts (7.44 log CFU g⁻¹ of *E. coli* in the two *E. coli*-positive/bifidobacteria-positive samples, 5.40 log CFU g⁻¹ in the six other *E. coli*-positive/bifidobacteria-negative samples). In the other sample types, *E. coli* counts, comprised between 1.08 and 1.98 log CFU cm⁻², were close to those of bifidobacteria (between 1.0 and 1.95 log CFU cm⁻²). Mean counts of total viable bacteria (Table 6) were calculated on the 244 samples and were between 3.87 (type E) and 5.63 log CFU g⁻¹ (type A).

Discussion

Detection and identification of bifidobacteria by culture-based and PCR methods

Based on all samples, the PCR method was shown statistically more sensitive than the culture-based method, which additionally was more time-consuming. The significant differences in favour of PCR, expressed by the high number of PCR+/culture- samples, on type C and total samples (Table 2) can be explained by competition between bifidobacteria and other micro-organisms such as clostridia not inhibited by mupirocin in the culture medium. The absence of significant differences between both methods for types B, D and F samples could probably be explained by the low numbers of positive samples.

A larger number of bifidobacteria positive faeces samples (type A) were expected. Using the same culture-based method, Gavini *et al.* (2006) detected bifidobacteria, in

seven of nine sheep faeces samples from animals on farm, with mean counts in bifidobacteria-positive samples of $4.29 \log \text{CFU g}^{-1}$. In this study, only three of nine samples were positive using the culture-based method, with slightly higher mean counts of $6.0 \log \text{CFU g}^{-1}$. The low number of positive samples obtained with the culture-based method was not explained. Using PCR, negative results were at first related to matrix faeces being known to contain inhibitors (Radstrom *et al.* 2004; Thornton and Passen 2004). However, a 10-fold dilution of the DNA extract, with inhibition checking by internal control, enabled this phenomenon to be minimized. Another hypothesis for these low results could be the bad survival of bifidobacteria in faeces as sheep produce pelleted faeces which may dry rapidly after deposition (Wehausen *et al.* 2004). PCR and culture-based methods, both, need improvement for faeces samples.

In contrast to faeces, high numbers of positive samples were detected on carcass surfaces (types C, slaughtered carcasses and E, refrigerated carcasses before cutting and deboning) in, respectively, 79% and 59% (Table 2) of the samples. This high contamination rate separated carcasses from low contaminated environmental samples (types B and D). Retail meat (type F) took an intermediate position between both. Obviously, faecal contamination occurred mainly in the slaughter phase with subsequent reduction due to spreading of the microbes to new surfaces and due to bacterial death.

The species identified in faeces samples (type A) were *Bif. pseudolongum* and *Bif. thermophilum*. These two species were also shown as the most frequent species isolated from different animal faeces on farm and along beef and pork processing chains (Gavini *et al.* 2006). They were also recovered at the other different slaughtering stages, in types C, D, E and F samples, confirming faecal origin of the contamination along the sheep processing chain.

The presence of *Bif. choerinum* is somewhat surprising. This species was described by Scardovi *et al.* (1979) based on strains isolated from swine faeces. It was also isolated in pork faeces and on a chilled swine carcass by Gavini *et al.* (2006). A possible explanation for its presence in sample types C, D, E and F could be a cross-contamination with pig faeces during transport, lairage and/or slaughter due to improper cleaning measures as pigs were slaughtered at the same abattoir. The presence of one unidentified strain close to *Bif. thermophilum* and *Bif. thermacidophilum* suggested that strains from animal origin are still badly known and need further taxonomic studies.

The PCR method has been validated in a previous study (Delcenserie *et al.* 2005) on 127 pure bifidobacteria strains belonging to 14 different *Bifidobacterium* species. However, only one strain belonging to the species *Bif.*

choerinum was tested. Therefore, it could explain the presence of some false negative by PCR in samples types D, E and F if some *Bif. choerinum* strains present in these samples were genetically different in the primers area. The results of identification using culture-based and DNA-DNA hybridization methods show that this species should be researched in meat samples with the two predominant species *Bif. pseudolongum* and *Bif. thermophilum*.

Contamination levels of bifidobacteria, *E. coli* and total viable bacteria along the mutton production chain

It was not surprising to find high levels of *E. coli* and TVC in faeces samples, with mean counts of 7.44CFU g^{-1} for *E. coli* in the two *E. coli*-positive/bifidobacteria-positive samples (Table 5) and $5.63 \log \text{CFU g}^{-1}$ for TVC (Table 6). Moreover, six of the nine samples studied, which were *E. coli*-positive/bifidobacteria-negative (Table 4), present a mean count a little lower, equal to 5.40CFU g^{-1} . The lower level of bifidobacteria in bifidobacteria-positive faeces samples (3.50 using PCR and $6.0 \log \text{CFU g}^{-1}$ using culture-based method), such as the low number of positive samples compared with the 89% of *E. coli*-positive samples, was not explained using the culture-based method. Problem of inhibition could be put forward using PCR. Further faeces analyses should be carried out to revise and understand these results.

The mean *E. coli* counts ($1.57 \log \text{CFU cm}^{-2}$) and TVC ($4.47 \log \text{CFU cm}^{-2}$) of freshly slaughtered carcasses (sample type C; Tables 5 and 6), were in agreement with those observed in other studies on microbial quality of sheep carcasses. According to Biss and Hathaway (1996), *E. coli* counts ranged from 0.39 to $2.11 \log \text{CFU cm}^{-2}$ and TVC from 3.45 to $5.36 \log \text{CFU cm}^{-2}$. Zweifel and Stephan (2003) detected TVC of 2.5 – $3.8 \log \text{CFU cm}^{-2}$. Lower counts were determined by Sumner *et al.* (2003): *E. coli*, $0.27 \log \text{CFU cm}^{-2}$ and TVC, $2.59 \log \text{CFU cm}^{-2}$.

Environmental samples during slaughter (type B) gave TVC values below $4.0 \log \text{CFU cm}^{-2}$, which is a rather low contamination level during processing (Table 6). This is not surprising as many sampling sites had only irregular meat contact (walls, doorhandles). On the other hand, the high standard deviation reflects a large variation in surface cell numbers. Equally, environmental samples during cutting and deboning showed a high contamination variability, but on a rather high level. A mean TVC of $4.97 \log \text{CFU cm}^{-2}$ (Table 6, type D) was observed. This is surprisingly high, as the raw material (chilled sheep carcasses, type E) showed cell numbers, which were $1.1 \log$ steps lower. Upmann *et al.* (2000) proposed equilibrium between surface cell counts of raw materials and meat contact surfaces during cutting. Therefore, ineffective cleaning and disinfection

measures may account for the increased TVC before sheep cutting began.

Regarding total samples, bifidobacteria were not shown to be more sensitive as faecal indicators than *E. coli* as their mean counts were similar. However, in carcass meats (types C and E) 30% of the freshly slaughtered and 28% of the chilled carcasses were bifidobacteria-positive/*E. coli*-negative. Detection of bifidobacteria as faecal indicators was more efficient than *E. coli* for these samples.

Therefore, based on present knowledge of the *Bifidobacterium* species in faecal material from animals, semi-quantitative detection and identification of bifidobacteria as faecal indicators in correlation with *E. coli* counting should improve hygiene quality along meat processing chain.

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