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Original Article

Characterisation of *Bergeyella* spp. isolated from the nasal cavities of piglets

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ABSTRACT

The aim of this study was to characterise bacteria in the genus *Bergeyella* isolated from the nasal passages of healthy piglets. Nasal swabs from 3 to 4 week-old piglets from eight commercial domestic pig farms and one wild boar farm were cultured under aerobic conditions. Twenty-nine *Bergeyella* spp. isolates were identified by partial 16S rRNA gene sequencing and 11 genotypes were discriminated by enterobacterial repetitive intergenic consensus (ERIC)-PCR. *Bergeyella zoohelcum* and *Bergeyella porcorum* were identified within the 11 genotypes. *Bergeyella* spp. isolates exhibited resistance to serum complement and phagocytosis, poor capacity to form biofilms and were able to adhere to epithelial cells. Maneval staining was consistent with the presence of a capsule. Multiple drug resistance (resistance to three or more classes of antimicrobial agents) was present in 9/11 genotypes, including one genotype isolated from wild boar with no history of antimicrobial use. In conclusion, *Bergeyella* spp. isolates from the nasal cavities of piglets showed some in vitro features indicative of a potential for virulence. Further studies are necessary to identify the role of *Bergeyella* spp. in disease and within the nasal microbiota of pigs.

Introduction

The microbiota is composed of microorganisms that colonise the skin and mucosal surfaces and lumina, including the alimentary, respiratory and urogenital tracts (Budden et al., 2017). These organisms influence the development and maturation of the innate and adaptive immune responses (Günther et al., 2016). The microbiota can protect against colonisation by pathogens through direct inhibition of the growth of pathogens, modification of the microenvironment or competition for colonisation sites. Conversely, some microorganisms in the microbiota are able to cause disease under particular conditions (Reid et al., 2011).

Respiratory tract infections are among the leading cause of disease and death in human beings and animals throughout the world (Ayrle et al., 2016).[1] Respiratory disease in pigs is often polymicrobial and is influenced by adverse environmental conditions (Brockmeier et al., 2002). Some agents, such as porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV) and *Actinobacillus pleuropneumoniae*, can act as primary pathogens. Primary infections frequently are complicated by opportunistic bacteria, such as *Pasteurella multocida, Haemophilus parasuis* and *Streptococcus suis*, resulting in more severe respiratory disease (Brockmeier et al., 2002). The relationship between the components of the nasal microbiota, especially carriage of potential pathogens, and respiratory disease in pigs was reported by Bertschinger and Nicod (1970). A deeper knowledge of the composition of the microbiota has been achieved with the introduction of massive sequencing (Slifierz et al., 2015; Correa-Fiz et al., 2016; Niederwerder et al., 2016).

Differences in the composition and diversity of the porcine microbiota act as contributing factors to disease (Correa-Fiz et al., 2016; Niederwerder et al., 2016). Specifically, the nasal microbiota has been shown to influence the development of disease by *H. parasuis* (Correa-Fiz et al., 2016). In the study by Correa-Fiz et al. (2016), the phylum *Bacteroidetes* was abundant in the nasal microbiota of healthy piglets and *Weeksella* was one of the most abundant genera. Holmes et al. (1986) defined two species of *Weeksella* (*Weeksella virosa* and *Weeksella zoohelcum*); *W. zoohelcum* has been renamed *Bergeyella zoohelcum* (Vandamme et al., 1994).

Bacteria from the phylum *Bacteroidetes* that have been used as probiotics include *Prevotella bryantii* in cattle (Chiquette et al., 2012) and *Barnesiella* spp. in human beings (Ubeda et al., 2013).

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[1] See: http://www.who.int/gho/mortality_burden_disease/en (accessed 12 December 2017).

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Table 1
Bergeyella spp. isolates obtained from nasal swabs of piglets at weaning (10 piglets from each farm were sampled for this study).

Farm	Number of *Bergeyella* isolates	Country	Health status	Antibiotic treatment[a]
AR	4	Spain	Healthy	Amoxicillin
CR	6	Spain	Healthy	Amoxicillin
EJ	2	Spain	Glässer's disease	Enrofloxacin
GM	8	Spain	Healthy	Amoxicillin
GW	1	UK	Healthy	Amoxicillin
LG	4	UK	Healthy	None
LL	1	Spain	Nervous signs	Unknown
MT	2	Spain	Glässer's disease	Penicillin + Streptomycin
WB[b]	1	Spain	Healthy	None

[a] Antibiotic treatment in the perinatal period.
[b] European wild boars. Other farms have domestic pigs.

Although *Bergeyella* spp. are abundant in the nasal microbiota of piglets, the role of these bacteria in health or disease is poorly characterised. In this study, we determined virulence traits and antimicrobial susceptibilities of *Bergeyella* spp. isolated from the nasal cavities of piglets at weaning.

Materials and methods

Sampling and bacterial isolation

Procedures involving animals followed European Union (EU) Directive 2010/63/EU. Samples were collected from healthy piglets, 3–4 weeks of age, from farms with a range of health statuses (Table 1). In some cases, disease was observed in older animals on the farms at the time of sampling, but piglets were unaffected. Nasal swabs from sampled piglets were transported in Amies medium (Deltalab) to the laboratory and were processed for bacterial isolation. Nasal swabs were plated on chocolate agar (Biomerieux) to isolate colonies. After 24 or 48 h incubation at 37 °C in 5% CO_2, colonies were selected and subcultured on chocolate agar to obtain pure cultures for further analysis.

Bacterial identification and genotyping

DNA from each isolate was purified from bacterial suspensions using a Chelex-based Instagene Matrix (Bio-Rad). For preliminary identification, a small fragment of the 16S rRNA gene was amplified and sequenced. This fragment was amplified using oligonucleotide primers 358F (5′-CTACGGGAGGCAGCAGT-3′) and 907R (5′-CCGTCWATTCMTTTGAGTTT-3′) (Lane, 1991). The PCR reaction solution comprised 1.5 mM $MgCl_2$ (Promega), 0.2 mM deoxynucleotide triphosphates (dNTPs; Promega), 0.4 μM each oligonucleotide primer, 3 μL bacterial DNA and 0.75 U GoTaq DNA polymerase (Promega) in a final volume of 25 μL. The amplification cycle was 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, then 72 °C for 7 min (GeneAmp PCR System 2700; Applied Biosystems). After confirming specific amplification by agarose electrophoresis, the amplicons were sequenced with primer 907R. Sequences were analysed using the Ribosomal database Seqmatch[2] application to identify the bacterial genus or species.

Different genotypes of *Bergeyella* spp. were differentiated by enterobacterial repetitive intergenic consensus (ERIC)-PCR (Versalovic et al., 1991) using oligonucleotide primers ERIC-1F (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-2R (5′-AAGTAAGTGACTGGGGTGAGCG-3′). DNA concentrations were determined using a μLite (Biodrop). The reaction solution comprised 3 mM $MgCl_2$ (Promega), 1.2 μM each oligonucleotide primer, 0.23 mM dNTPs (Promega), 0.75 U GoTaq polymerase (Promega) and 25 ng DNA in a final volume of 25 μL. The amplification cycle was 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2.5 min, then 72 °C for 20 min (GeneAmp PCR System 2700; Applied Biosciences). PCR products (5 μL) were separated by electrophoresis at 80 V for 1 h in 2% agarose gels, stained with ethidium bromide and examined using an ultraviolet transilluminator (U:Genius; Syngene).

Final identification of selected isolates was performed by 16S rRNA gene sequencing using universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) (Weisburg et al., 1991). The PCR reaction solution comprised 2 mM $MgCl_2$ (Promega), 0.4 μM each oligonucleotide primer, 0.2 mM dNTPs (Promega), 0.75 U GoTaq polymerase (Promega) and 250 ng DNA in a final volume of 25 μL. The amplification cycle was 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, then 72 °C for 7 min (GeneAmp PCR System 2700 thermocycler; Applied Biosciences). Amplicons were sequenced with primers 8F, 358F, 907R and 1492R. Sequences were analysed using Fingerprinting II v3.0 software (Bio-Rad) and an unweighted pair group method with arithmetic mean (UPGMA) tree was constructed.

[2] http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp (accessed 12 June 2017).

Antimicrobial susceptibility

Susceptibility to gentamicin, erythromycin, enrofloxacin, tetracycline, doxycycline, trimethoprim-sulphonamide, amoxicillin, ceftiofur, amoxicillin-clavulanic acid and lincomycin-spectinomycin were tested using Neo-Sensitabs (Rosco Diagnostica) by measuring the diameter of growth inhibition. Diameters of the zones of inhibition were measured using a calliper and were compared with the manufacturer's breakpoint tables. Susceptibility to marbofloxacin, florfenicol and tulathromycin were tested by dilution in 96-well plates to determine minimum inhibitory concentrations (MICs). Plates were incubated overnight at 37 °C in 5% CO_2 and the bacterial growth was measured by determining the optical density at 600 nm (OD_{600}) using a spectrophotometer (VIS 7200; Dinko Instruments). Isolates with values greater than or less than the diameter or concentration of the breakpoint suggested by the manufacturer were designated 'sensitive' and 'resistant', respectively.

Biofilm formation

Bergeyella spp. isolates were cultured in 96-well plates (Bello-Orti et al., 2014) that were uncoated or coated with 5 μg/well mucin or bovine serum albumin (BSA) (Sigma–Aldrich). Bacterial suspensions were prepared in brain heart infusion (BHI; Merck) broth to an OD_{600} of 0.3. A 1:10 dilution of the bacterial suspension was made in BHI broth and 100 μL were dispensed in 96-well plates in quadruplicate. Plates were incubated at 37 °C in static conditions or by shaking at 100 revolutions per min. After the appropriate time of incubation, unattached bacteria were removed by vacuum suction and wells were washed once by immersion in water. Biofilms were stained with 0.1% crystal violet for 2 min. Stained biofilms were washed three times with water and then placed at 37 °C until the plates were dry. The dye was released from the biofilm with 100 μL 70% ethanol and quantified by determining the OD_{590} using a spectrophotometer (Powerwave XS Microplate; Biotek Instruments).

Cell adhesion assay

The cell adhesion assay was performed using lung adenocarcinoma A549 cells. Cells were plated in 96-well plates at a density of 500,000 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM; Lonza) supplemented with 10% foetal bovine serum (FBS; Euroclone) and 1% glutamine (Gibco). Cells were grown overnight at 37 °C in 5% CO_2 to form monolayers. Bacterial suspensions of selected isolates of *Bergeyella* spp. were prepared in phosphate buffered saline (PBS) to an OD_{600} of 0.3. Monolayers of A549 cells in 96-well plates were washed once with sterile PBS and inoculated into duplicate wells with 100 μL bacterial suspension, equivalent to ~10^9 colony forming units (CFUs)/mL. Plates were centrifuged for 10 min at 100 *g* to facilitate contact between bacteria and cells, then incubated for 1 h at 37 °C in 5% CO_2. After incubation, wells were washed twice with sterile PBS to remove unattached bacteria. To release the attached bacteria, wells were treated with 0.1% saponine (Sigma–Aldrich) in PBS followed by pipetting. Bacteria in the wells were quantified by dilution and colony counts.

Serum susceptibility assay

Serum susceptibility assays were performed using rabbit serum (Cerdà-Cuéllar and Aragon, 2008); bacteria (10^7–10^8 CFUs/mL) were incubated with 80% serum for 1 h at 37 °C. Bacterial counts were performed by dilution and plating before and after incubation with serum. The logarithmic reduction of bacterial counts after 1 h with serum was then calculated. Selected isolates were also tested with porcine serum using the same conditions.

Phagocytosis assay

Phagocytosis assays were carried out with porcine alveolar macrophages (PAMs) previously harvested from two piglets euthanased by intravenous sodium pentobarbital overdose following EU Directive 2010/63/EU (Olvera et al., 2009).

Table 2
Isolates with the same enterobacterial repetitive intergenic consensus (ERIC)-PCR genotype were considered identical and a representative strain was selected for further analysis.

Representative strain	Isolates with same ERIC-PCR genotype
LL-7	LL7, GM5-4, GM10-5
AR-9	AR-9, AR-12, AR-13, AR-14, CR-13, CR-14, CR-15,CR-16, CR-19
CR-17	CR-17
GM2-3	GM2-3, GM2-5, GM3-5, MT-248-4, MT-T35-4
GM2-4	GM2-4
GM5-6	GM5-6, GM10-4, EJ45-7B
EJ45-6	EJ45-6
LG4-7	LG4-7, LG4-6
LG9-4	LG9-4, LG9-5
GW4-3	GW4-3
WB6-3	WB6-3

The first two letters in the name of the isolates refer to the farm of origin.

Bacterial isolates were grown on chocolate agar overnight at 37 °C in 5% CO_2 and non-confluent growth was resuspended in PBS to ~10^8 CFUs/mL. Bacteria were stained with fluorescein isothiocyanate (FITC; Sigma–Aldrich) and bacterial counts were determined by serial dilutions and plating. PAMs were plated at a concentration of 5×10^5 cells per well in 6-well plates. After attachment, duplicate wells were inoculated with 50 μL FITC-labelled bacteria and incubated for 1 h at 37 °C in 5% CO_2. After incubation, wells were washed twice with PBS and PAMs were harvested using a cell scraper in 500 μL 0.1% BSA in PBS for analysis by flow cytometry (FC) using an EPICS XL-MCLTM Flow Cytometer (Beckman Coulter). Two reference strains of *H. parasuis* were included in the assays as controls; Nagasaki (virulent, phagocytosis resistant) and SW114 (non-virulent, phagocytosis sensitive).

Capsule staining

Maneval staining was performed with selected *Bergeyella* spp. isolates (Maneval, 1941).

Results

Bacterial identification and genotyping

Initial identification of bacterial isolates was carried out by partial sequencing of the 16S rRNA gene. Twenty-nine *Bergeyella* spp. isolates were identified, while *Weeksella* spp. were not detected (Table 1). Eleven genotypes were identified among the 29 *Bergeyella* spp. isolates by ERIC-PCR (Table 2). Genotypes were shared among farms with different heath statuses. One isolate representative of each genotype was selected for further analysis. On the basis of sequencing of 1374 base pairs of the 16S rRNA gene and UPGMA analysis, five nasal *Bergeyella* spp. isolates clustered with *Bergeyella porcorum* (Zamora et al., 2016), with ~99% similarity, while the other six isolates clustered with *B. zoohelcum*, with ~98% similarity (Fig. 1).

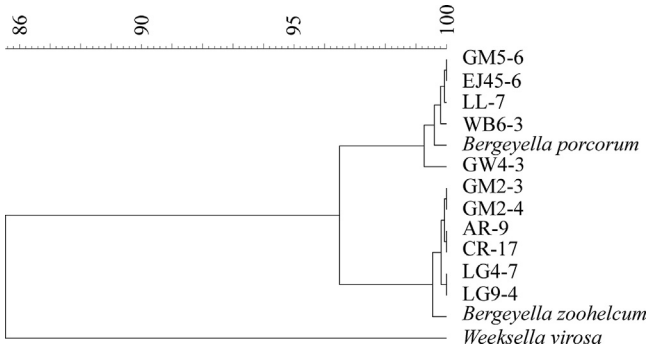

Fig. 1. Unweighted pair group method with arithmetic mean (UPGMA) tree with 16S rRNA gene sequences of *Bergeyella* spp. isolated from the nasal cavities of piglets at weaning. The sequences of *Bergeyella zoohelcum* HO357, *Bergeyella porcorum* 1350-03 and *Weeksella virosa* DSM 16922 were included in the analysis as references.

Antimicrobial susceptibility

Nasal *Bergeyella* spp. isolates exhibited diversity in antibiotic susceptibility (Table 3). All selected isolates were sensitive to doxycycline and ceftiofur, whereas there were high frequencies of resistance to erythromycin, trimethroprim-sulphonamide, tulathromycin and marbofloxacin. *B. porcorum* GW4-3 was resistant to only one antimicrobial agent, *B. zoohelcum* LG4-7 and LG9-4 were resistant to three antimicrobial agents, and *B. porcorum* GM5-6, EJ45-6 and WB6-3 were resistant to more than six antimicrobial agents.

Biofilm formation

Nasal *Bergeyella* spp. isolates had poor capacity to form biofilms on plastic after 48 and 72 h of incubation under shaking or static conditions, whether or not the wells were previously coated with mucin or BSA (Fig. 2).

Cell adhesion

In the cell adhesion assay using *B. zoohelcum* (CR-17) and *B. porcorum* (GM5-6), ~10^7 CFU/mL of both isolates exhibited adherence from original inocula of 10^9 CFU/mL (Fig. 3).

Serum resistance

The 11 nasal genotypes of *Bergeyella* spp. were tested for resistance to serum complement. The maximal sensitivity to serum was observed in *B. porcorum* GM5-6, which showed a reduction of 4 logs after incubation with serum, while the remaining isolates had reductions of 1–3 logs (Fig. 4). Strains GM2-3 and LL-7 were also tested with a porcine serum, with the same results (not shown).

Resistance to phagocytosis

On FC analysis, all 11 genotypes associated poorly with PAMs, indicating that *Bergeyella* spp. are relatively resistant to phagocytosis by PAMs (Fig. 5).

Capsule staining

Capsules were visible on Maneval staining in *B. porcorum* GM5-6 (Fig. 6) and *B. zoohelcum* CR-17.

Discussion

The genus *Bergeyella* (previously included in *Weeksella*) is abundant in the nasal microbiota of weaned pigs, but these

Table 3
Antimicrobial susceptibility of isolates of *Bergeyella zoohelcum* and *Bergeyella porcorum* from the nasal cavities of piglets at weaning.

Strain	COL	ENR	MARB	TET	DOX	S + T	AMOX	AMC	CEFT	GEN	LI + SP	ERY	TUL	FLOR
Bergeyella zoohelcum														
AR-9	S	S	R	S	S	R	S	S	S	S	S	R	R	R
CR-17	S	I	R	S	S	R	S	S	S	S	I	R	R	R
GM2-3	S	R	R	S	S	R	S	S	S	S	R	R	R	R
GM2-4	S	S	R	S	S	R	S	S	S	S	I	R	R	R
LG4-7	R	R	S	S	S	S	S	S	S	S	S	R	S	S
LG9-4	S	I	S	S	S	R	R	R	S	S	S	S	S	S
Bergeyella porcorum														
GM5-6	R	R	R	R	S	S	S	S	S	S	R	R	R	R
EJ45-6	S	R	R	S	S	R	S	S	S	S	R	R	R	R
LL-7	S	I	R	S	S	R	S	S	S	S	R	R	R	R
WB6-3	R	I	R	R	S	R	R	S	S	R	R	S	S	S
GW4-3	R	S	S	S	S	S	S	S	S	S	S	S	S	S

COL, colistin; GEN, gentamicin; ERY, erythromycin; ENR, enrofloxacin; TET, tetracycline; DOX, doxycycline; S + T, trimethroprim-sulphonamide; AMOX, amoxicillin; CEFT, ceftiofur; AMC, amoxicillin + clavulanate; LI + SP, lincomycin-spectinomycin; MARB, marbofloxacin; TUL, tulathromycin; FLOR, florfenicol.

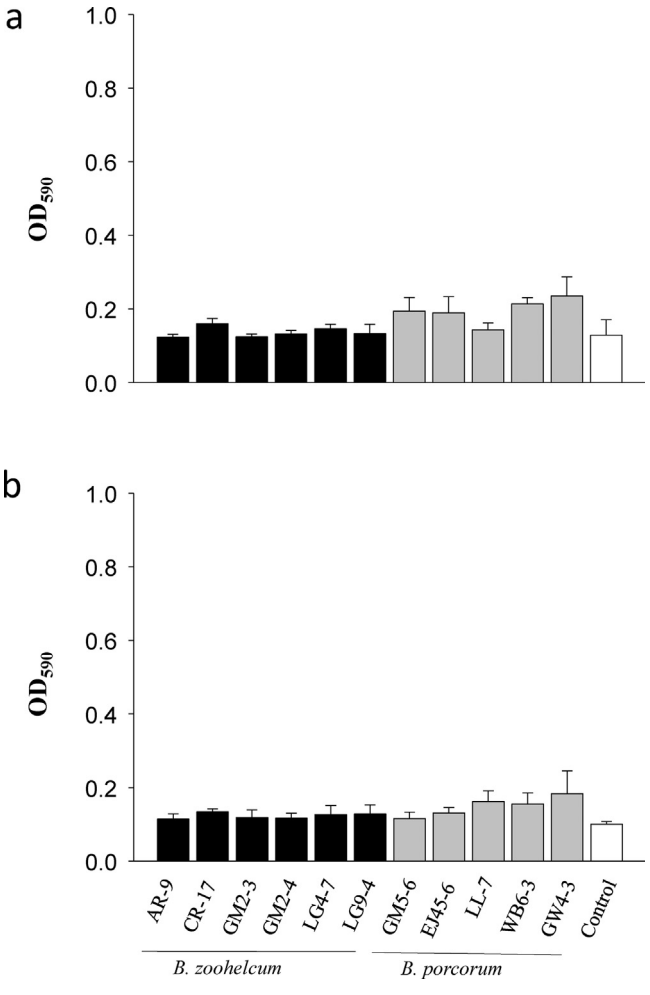

Fig. 2. Biofilm formation on plastic plates by *Bergeyella* spp. strains. Plates were incubated for 72 h under static (a) and shaking (b) conditions. Biofilm formation was measured by staining with crystal violet, and quantified by determining the optical density at 590 nm (OD_{590}) of the dissolved dye. A negative control with only culture medium was also processed and is included in the graphs (Control).

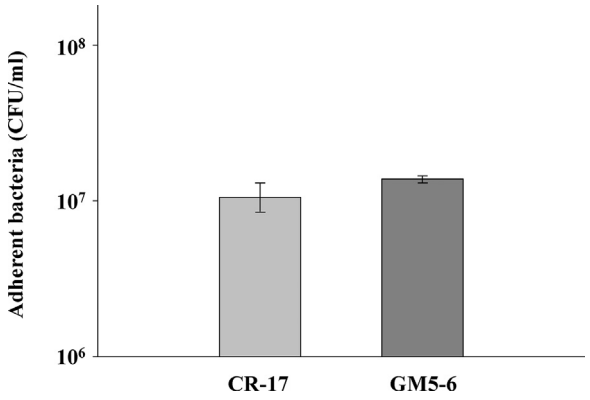

Fig. 3. Adhesion of *Bergeyella zoohelcum* CR-17 and *Bergeyella porcorum* GM5-6 to A549 cells. Bacterial suspensions of 10^9 colony forming units (CFUs)/mL of isolates GM5-6 and CR-17 were used to inoculate wells with A549 cell monolayers. Inoculated wells were incubated for 1 h at 37 °C, then, after washing to eliminate unbound bacteria, attached bacteria were quantified by colony counts.

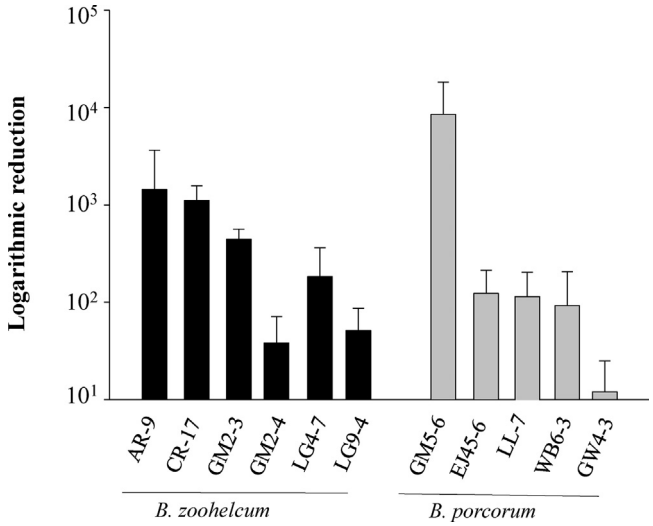

Fig. 4. Serum susceptibility of nasal *Bergeyella* spp. strains. Bacterial suspensions were incubated with fresh rabbit serum for 1 h. Survival before and after incubation was measured by colony counts. Results are expressed as the logarithmic reduction of bacterial counts after incubation with serum.

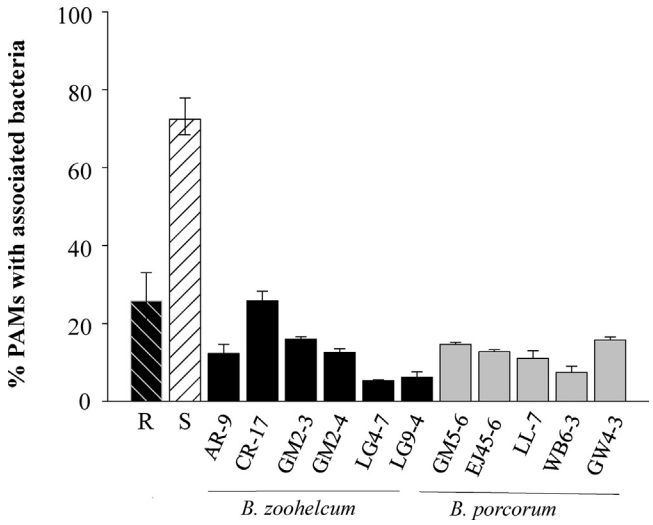

Fig. 5. Percentages of porcine alveolar macrophages (PAMs) with associated bacteria. PAMs were incubated for 1 h with different isolates of *Bergeyella zoohelcum* (AR-9, CR-17, GM2-3, GM2-4, LG4-7 and LG9-4) and *Bergeyella porcorum* (GM5-6, EJ45-6, LL-7, WB6-3 and GW4-3) previously labelled with fluorescein isothiocyanate (FITC). After washing to eliminate unbound bacteria, PAMs were analysed by flow cytometry to determine the percentage of macrophages with associated bacteria. Two *Haemophilus parasuis* strains were included as reference strains for phagocytosis resistance (R, Nagasaki) and sensitive (S, SW114).

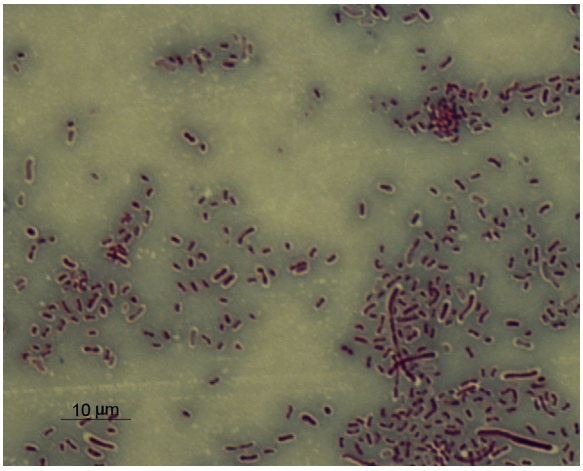

Fig. 6. Capsule detection by Maneval staining in strain *Bergeyella porcorum* GM5-6. One drop of bacteria suspended in phosphate buffered saline was mixed on a glass microscope slide with a drop of 1% aqueous Congo red stain. The mixture was spread into a thin film and allowed to air dry. After fixation, the films were counterstained with Maneval stain for 2 min, drained, blotted dry and examined microscopically.

bacteria are not well characterised (Correa-Fiz et al., 2016). Many members of the microbiota are beneficial bacteria, but some can be opportunistic pathogens. Studies of the oral community of dogs have indicated that the presence of *Bergeyella* spp. may be associated with improved oral health (Wallis et al., 2015). However, the in vitro assays used in the present study indicate that porcine nasal *Bergeyella* spp. have properties that suggest pathogenic potential.

Pathogens possess virulence mechanisms to evade host defence factors in order to cause disease. Alveolar macrophages constitute one of the respiratory barriers to avoid lung colonisation. Phagocytosis by these macrophages allows the clearance of bacteria that can reach the lower respiratory tract. When PAMs were exposed to nasal *Bergeyella* spp., there was no association, suggesting that these bacteria are resistant to phagocytosis. This finding is in agreement with the detection of capsule, a potential anti-phagocytic factor, along with a previous report of respiratory disease associated with *B. zoohelcum* in a cat (Decostere et al., 2002). In addition, serum complement had a poor ability to kill *Bergeyella* spp.; other members of the nasal microbiota, such as non-virulent *H. parasuis*, are not able to survive in the presence of complement under the same assay conditions (Cerdà-Cuéllar and Aragon, 2008). In our study, in vitro serum and phagocytosis resistance assays suggest that *Bergeyella* spp. isolated from the nasal cavity of normal piglets have pathogenic potential, in agreement with previous reports where bacteria from this genus were associated with disease (Decostere et al., 2002; Shukla et al., 2004).

Porcine nasal *Bergeyella* spp. had homogeneous adhesion capacities, with poor ability to form biofilms, even when the plastic substrate was coated with mucin, but were able to adhere to epithelial cells. However, *B. zoohelcum* has been found at high relative abundance in the biofilm on the enamel of dogs, together with other bacteria that can also be found in the nasal cavities of piglets (Holcombe et al., 2014; Correa-Fiz et al., 2016). These results may indicate the need for cooperation with other bacterial species for *Bergeyella* spp. to be associated with biofilms. Adhesion to epithelial cells could be the mechanism by which porcine *Bergeyella* spp. colonises the nasal cavity.

The antimicrobial resistance profiles of porcine *Bergeyella* spp. indicate that these bacteria can exhibit multiple drug resistance. However, there was no association between the specific antimicrobial treatment applied on each farm and the type or number of antimicrobial resistance phenotypes found in the corresponding isolates. As an example, WB6-3 had a high level of antimicrobial resistances, but no antimicrobial agents were used on the farm of origin. However, other factors could explain this result, as already reported for *Escherichia coli*, where the detection of resistant *E. coli* in piglets was associated with the presence of the resistant bacteria in the corresponding sows (Cameron-Veas et al., 2016).

Conclusions

Bergeyella spp. isolated from nasal cavities of weaned healthy piglets belonged to two species, *B. porcorum* and *B. zoohelcum*. Representative isolates from both species showed similar adhesion characteristics, supporting their capacity to colonise the nasal mucosa. Although *Bergeyella* spp. may be associated with improved oral health in dogs, phagocytosis and serum resistance results for porcine nasal isolates indicate a potential pathogenic capacity, which deserves further study, along with the role of these bacteria as carriers of potentially transferrable antibiotic resistance.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper

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