Experimentally induced intramammary infection with multiple strains of *Streptococcus uberis*

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ABSTRACT

The effect of infusing a mixture of 5 *Streptococcus uberis* strains into mammary quarters of 10 lactating cows was investigated. All 5 strains, which included 2 originally isolated from the dairy environment and 3 from clinical cases of mastitis, were capable of establishing an intramammary infection when infused individually. However, when the 5 strains were infused together, a single strain predominated in 7 out of 10 quarters. One strain in particular prevailed in 4 mammary quarters and was also found to inhibit the growth of the other 4 strains with deferred antagonism on esculin blood agar. The genes required for the production of bacteriocins nisin U and uberolysin were identified in this strain, whereas the other 4 strains contained only uberolysin genes. Direct competition may have occurred between strains within the mammary gland but competition was not apparent when cultured together in UHT milk, where no strain predominated. Although the mechanism is unknown, these results imply that a selection process can occur within the mammary gland, leading to a single strain that is detected upon diagnosis of mastitis.

Key words: *Streptococcus uberis*, mastitis, strain, bacteriocin

INTRODUCTION

The environmental pathogen *Streptococcus uberis* is one of the major causes of bovine mastitis in pasture-based dairy systems (Douglas et al., 2000; McDougall et al., 2004). It is ubiquitous throughout the dairy environment (Zadoks et al., 2005; Pullinger et al., 2006) and can be isolated from many sites on the cow, including the lips, rumen, udder, and teat skin, and from the feces (Cullen and Little, 1969; Buddle et al., 1988; Lacy-Hulbert et al., 2005). Despite the large number of strains in the dairy environment, infection with multiple

strains of *S. uberis* within the same mammary quarter is rare, with only 1, or at most 2, strains detected at each infection event (Phuektes et al., 2001; Zadoks et al., 2003; Pryor, 2008). Only a small number of studies have strain-typed multiple *S. uberis* isolates from each mastitis case. If a selection process were to take place, the dominant strain may be selected in the teat canal during penetration or within the mammary gland itself. To establish infection, *S. uberis* must out-compete other bacterial species that may also gain entry to the mammary gland.

Selection of a single strain within the mammary gland could be linked to several factors. A superior array of virulence factors could enhance the ability of a particular strain to grow in milk (Leigh, 1994; Smith et al., 2002), to adhere to and invade mammary epithelia or macrophages (Almeida et al., 2006; Denis et al., 2006; Dogan et al., 2006; Tamilselvam et al., 2006), or to resist the host immune response (Field et al., 2003). Direct competition between strains could occur through production of bacteriocins, small (<10 kDa) antimicrobial peptides that typically kill other bacteria of the same or closely related species (Tagg et al., 1976; Riley and Gordon, 1999). The two most recently discovered bacteriocins in *S. uberis* are nisin U (Wirawan et al., 2006), and uberolysin (Wirawan et al., 2007).

Streptococcus uberis has been recently identified as a highly recombinant organism, with 15 to 18% of the genome differing between strains (Lang et al., 2009). Many of these variable genes are involved in nutrient acquisition, energy metabolism, virulence, and adaptation to the environment. Because of this dissimilarity in gene content, each strain may have differing abilities to infect the mammary gland.

Very little information is available on the dynamics of bacterial growth, establishment, and infection during the development of mastitis and, particularly, on the selection pressures that lead to a single strain infecting the mammary gland at any one time. There are no published studies on the outcome of infusing more than one strain of *S. uberis* into the mammary gland. The aim of this study was to determine whether selection of a single infective strain could occur within the ud-

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der when the mammary gland was challenged with a mixture of 5 *S. uberis* strains.

MATERIALS AND METHODS

Animals

Ten Friesian-Jersey crossbred cows were selected from the DairyNZ Lye Farm research herd (Hamilton, New Zealand). Cows averaged 5.3 ± 1.5 yr (mean $\pm$ SD) and 248 ± 12 DIM and were milked twice daily. Bacteriology performed every 8 to 12 wk from calving on foremilk samples from individual quarters implied no history of *S. uberis* infection in the current lactation. Similarly, bacteriological analysis and foremilk SCC conducted 3 d prior, 8 h prior, and at the milking immediately preceding infusion, indicated that all cows were free from infection at the start of the study.

Selection of *Streptococcus uberis* Strains

Three *S. uberis* strains isolated from clinical mastitis cases and 2 from the dairy environment (farm race and paddock soil) of different multilocus sequence types were randomly selected from 253 isolates collected from Lye Farm (Lopez-Benavides et al., 2007). The ability of the 5 strains to grow in UHT milk was determined by inoculating individual vials of 5 mL of sterile UHT skimmed milk with 250 μ L of an 18-h brain-heart infusion (BHI) broth culture of each strain and incubating them at 37°C while shaking (180 rpm) for 20 h. Serial 10-fold dilution of the broth and milk cultures was performed in UHT milk, 100 μ L of sample was spread onto plates containing 0.1% esculin and 5% sheep's blood agar (EBA; Fort Richard Laboratories Ltd., Auckland, New Zealand), and plates were incubated at 37°C for 48 h before enumeration of colony-forming units.

Preparation of Inocula and Infusion

To generate inocula, cultures of the 5 strains (A to E) were prepared by transferring a single colony from BHI agar into BHI broth and incubating it at 37°C for 20 h. The BHI cultures were stored at 4°C while bacteria were enumerated by 10-fold serial dilution in UHT milk and plating on EBA as described above. Each prepared 10-fold dilution was stored at 4°C. On the day of infusion, individual strain inocula were prepared from the 10-fold dilution containing the most appropriate number of bacteria. If required, further dilutions were performed in UHT milk to obtain approximately 2,500 cfu/mL of each strain. One-milliliter volumes were aseptically aspirated into 1-mL sterile disposable syringes, capped with 18-gauge blunt-ended teat infusion cannu-

las (J-12, Jorgensen Laboratories Inc., Loveland, CO), and incubated at 37°C for 1 h to allow acclimatization directly before infusion into mammary quarters. The mixed-strain inoculum (SU5) was prepared by mixing appropriate volumes of strains A to E to generate inocula containing approximately 500 cfu of each strain in a total volume of 1 mL of milk.

Following an afternoon milking and thorough cleaning of the teat end with 70% ethanol-soaked swabs, SU5 was infused into the teat cistern of one quarter of each of 10 cows. Five cows received SU5 in a front quarter and 5 received SU5 in a hind quarter. At the same time, the second and third quarter of each cow was infused with 1 of the 5 strains such that each strain was infused, singularly, into one quarter of 4 cows and across an equal number of front and hind quarters. The fourth quarter of each cow was left uninfused.

Total bacterial numbers in each inoculum were estimated after the infusion process by plating 100 μ L of additional prepared syringes on EBA and were found to be 3,190, 1,970, 2,160, 3,580, and 3,400 cfu/mL for strains A to E individual inocula, respectively, and 3,337 cfu/mL for the SU5 inoculum.

Sample Collection and Postinfusion Analysis

Foremilk electrical conductivity (EC) was measured at both morning and afternoon milkings for 3 d postinfusion using a hand-held, digital mastitis detector (Technipharma, Rotorua, New Zealand). Following this, teats were scrubbed with cotton wool soaked in 70% ethanol and foremilk samples (approximately 30 mL) were collected aseptically for SCC, bacteriological analysis, and selection of colonies for strain typing by repetitive extragenic palindromic (REP)-PCR. Somatic cell count was determined using an electronic fluorometric cell counting technique (Fossomatic, Foss Electric, Hillerød, Denmark). Intramammary infection was diagnosed when EC was elevated (>15% above control quarter for each cow), SCC was greater than 2.0×10^5 cells/mL, and bacteria were detected in the milk, or when clinical signs were apparent, including heat, pain, swelling of the udder, or clots or discoloration of the milk.

Foremilk samples were collected and analyzed for 5 milkings after experimental infusion, after which antibiotic treatment commenced; treatment consisted of intramammary infusion with an extended course (up to 5 tubes) of Orbenin LA (Pfizer Animal Health, Auckland, New Zealand). All 5 *S. uberis* strains were susceptible to this antibiotic in vitro. Cows with systemic signs of inflammation were administered a nonsteroidal, antiinflammatory product (Metacam, Boehringer-Ingelheim GmbH, Ingelheim, Germany). Bacteriology

was performed 28 d after completion of the antibiotic treatment to ensure full bacteriological recovery, and dry cow antibiotics (Dryclox, Bomac Laboratories Ltd., Auckland, New Zealand) were subsequently administered at dry-off, approximately 12 d later.

Bacteriological Procedures

Identification of bacteria in milk was performed using procedures recommended by Hogan et al. (1999). For each quarter, 10 µL of foremilk was streaked onto one quadrant of an EBA plate and incubated at 37°C for 48 h. Presumptive identification of isolates was made on the basis of Gram stain, colony morphology, catalase test, patterns of hemolysis, esculin reaction, inulin fermentation, sodium hippurate reaction, growth in BHI broth with 6.5% salt, and the Christie, Atkins, Munch-Petersen (CAMP) test. Strain C had a notably different morphology from the other 4 strains when cultured on EBA.

To enumerate bacterial numbers in milk and obtain representative colonies for strain typing, 10-fold serial dilutions of milk samples were performed to 10⁻⁶ in 0.1% peptone (Fort Richard Laboratories Ltd.); 100 µL of sample was spread onto EBA plates and incubated as above. Total colony-forming units were enumerated and the number of colony-forming units per milliliter of milk was log₁₀ transformed before statistical analysis. For SU5-infused quarter milk samples, distinctive colony-forming units corresponding to strain C were also counted.

Between 2 and 5 cfu were selected at each milking from quarters infused with individual strains in order to confirm presence of the infused strain. For SU5-infused quarters, a representative sample of 5 to 10 cfu with a distinctive morphology corresponding to strain C, and 15 to 25 (average 17) other colony-forming units, were chosen for each sample where *S. uberis* was recovered. When a mixture of strains was identified in one sample after strain typing, additional colony-forming units were selected and strain-typed to verify the proportion of each strain present.

DNA Isolation and Strain Typing

DNA was isolated from selected colonies using the alkaline polyethylene glycol (PEG)-based method of Chomczynski and Rymaszewski (2006). Briefly, a single bacterial colony was mixed with 25 µL of an alkaline PEG solution consisting of 60% PEG 200 (Sigma-Aldrich NZ Ltd., Auckland, New Zealand) and 20 mM of KOH (pH 13.4), incubated at 95°C for 10 min and then mixed thoroughly. Crude DNA preparations were measured with a Nanodrop ND-1000 spectrophotom-

Table 1. Oligonucleotide primer sequences designed for anchored typing (*mtuA*-R) and forward (F) and reverse (R) sequences for identification of *hasA*, *hasB*, and *hasC* genes in *Streptococcus uberis*

Primer	Sequence, 5' to 3'
<i>mtuA</i> -R	CTGTCGCCGTTTTTTCCTTTC
<i>hasA</i> -F	CATAGGGGATGACCGTTGTT
<i>hasA</i> -R	GCAACACTTGGTGTGGCTAA
<i>hasB</i> -F	AACCCCTCTTTTGGCTATGGA
<i>hasB</i> -R	TCTTCATTCATCATTGGCTCA
<i>hasC</i> -F	CGTCCATTGAAGACCATT
<i>hasC</i> -R	CATCGTAGCGTTTGCCAGTA

eter (Nanodrop Technologies, Wilmington, DE) and diluted with Tris-EDTA buffer (10 mM of Tris, 1 mM of EDTA; pH 8.0) to obtain approximately 200 ng of nucleic acids.

Repetitive extragenic palindromic-PCR and a slight variation of this method called anchored typing were used to strain-type *S. uberis* colonies from milk of infused quarters. For REP-PCR, the BOXA1R, and ERIC1R, primers were used as described in Versalovic et al. (1994). For anchored typing, the primer REP-2 (Wieliczko et al., 2002) was used along with an oligonucleotide primer directed toward the metal transporter *uberis A* gene (*mtuA*, Table 1). This primer was designed using Genamics Expression 1.1 (Genamics, Hamilton, New Zealand) based on *S. uberis* gene sequence data for *mtuA* (GenBank accession AJ539135). Designed primer sequences were entered into NetPrimer (www.premierbiosoft.com/netprimer) to check melting temperatures and possible secondary structures. The primer was synthesized by Sigma-Aldrich NZ Ltd. and reconstituted in Tris-EDTA buffer to 200 µM.

Each REP-PCR or anchored typing reaction (25 µL) contained 1 × HotMaster buffer (Eppendorf AG, Hamburg, Germany) with 2.5 mM of Mg²⁺; 40 µM or 200 µM of deoxynucleotides (Invitrogen, Carlsbad, CA) for REP-PCR and anchored typing, respectively; 0.4 µM of BOXA1R and ERIC1R primers or *mtuA* and Rep-2 primers for REP-PCR and anchored typing, respectively; 1.25 U of Thermo-Start DNA polymerase (ABgene, Epsom, Surrey, UK) or 1 U of *Taq* DNA polymerase (1 U/µL; Roche Diagnostics Ltd., Auckland, New Zealand) for REP-PCR and anchored typing, respectively; and 200 ng of DNA. Amplification consisted of an initial step of 94°C for 7.5 (REP-PCR) or 2 min (anchored typing) followed by 40 cycles of 94°C for 20 s, 45°C for 30 s, and 68°C for 2 min. A final extension step of 72°C for 5 min was performed for REP-PCR. The same PTC-100 thermal cycler (MJ Research Inc., Waltham, MA) was used for all reactions, with the temperature ramp rate before and after annealing set to 0.5°C/s for REP-PCR and the default setting of the machine for anchored typing.

The PCR products (10 μ L) were electrophoresed in a 1.5% agarose gel prepared in sodium borate buffer (0.45 M of boric acid, $\sim$ 0.11 M of NaOH; pH 8.5) along with a 100-bp DNA ladder (Invitrogen). Band patterns for each strain-typed colony were visually compared with band patterns representing each of the 5 strains. The percentage of each strain type within the set of selected colonies was used to estimate the overall proportion of the 5 strains in each milk sample from SU5-infused quarters.

In Vitro Analysis of Strains

After the infection study, the 5 strains were cultured together in UHT skimmed milk to determine whether competition occurred between strains when grown in vitro. Experiments were performed using 5 replicates. Vials of 10 or 20 mL of UHT skimmed milk were inoculated with either 5-h or 24-h BHI cultures to obtain a mixture of approximately 300 cfu/mL of each of the 5 strains. Samples (0.5 mL) were removed both directly after mixing and after incubation at 37°C and 100 rpm for 74 h. This period was equivalent to the approximate length of time that strains were present within the mammary gland during the infection study. One hundred microliters each of undiluted time-0 sample and a 10^{-6} dilution (in 0.1% peptone) of the 74-h sample were spread onto EBA plates. Plates were incubated for 48 h before counting both total colony-forming units and colony-forming units with a distinct morphology corresponding to strain C. Five colony-forming units corresponding to strain C and 16 to 25 other colony-forming units were selected for DNA isolation and strain-typing by REP-PCR and anchored typing.

Polymerase chain reactions were performed to determine whether each strain contained the 3 genes (*hasA*, *hasB*, and *hasC*) required for production of a hyaluronic acid capsule. Oligonucleotide primers were designed and prepared for each gene, as described above, using gene sequence data for *hasA*, *hasB* (GenBank accession AJ242946), and *hasC* (AJ400707). Each 25- μ L PCR contained $1 \times$ HotMaster buffer with 2.5 mM of Mg^{2+} , 200 μ M of deoxynucleotides, 0.4 μ M of *hasA*, *hasB*, or *hasC* forward and reverse primers (Table 1), 0.625 U of HotMaster *Taq* DNA polymerase (Eppendorf AG, Hamburg, Germany), and 200 to 500 ng of DNA. Amplification included 94°C for 2 min, followed by 40 cycles of 94°C for 20 s, annealing at 55°C for 20 s, and extension at 68°C for 1 min. DNA from strain 0140J, known to contain all 3 *has* genes (Ward et al., 2001), was included as a positive control together with a negative control that lacked template DNA. The PCR products were verified by sequencing (Waikato DNA Sequencing Facility, Hamilton, New Zealand).

After the completion of the infection study, production of bacteriocin-like activity by the 5 strains was investigated using a modification of the deferred antagonism method of Tagg and Bannister (1979). Briefly, single colonies of strains A to E were cultured on EBA plates for 48 h or in BHI broth for 18 h. A sample of culture from EBA was swabbed in a 1-cm-wide streak diametrically across the surface of fresh EBA plates (test strains), which were then incubated at 37°C for 18 h. The macroscopic growth was removed and agar surface was resterilized by exposure to chloroform vapor for 30 min. After airing the plates for more than 30 min, BHI cultures of the 5 strains were streaked at right angles (indicator strains) across the original growth zone of the test strain using a cotton-tipped swab. After incubation at 37°C for 18 to 24 h, each plate was examined for inhibitory activity as evidenced by the absence of indicator strain growth in the proximity of the original test strain growth zone.

Polymerase chain reactions to identify the presence of structural genes for 2 known bacteriocins in *S. uberis*, uberolysin and nisin U, were performed by the Department of Microbiology and Immunology, University of Otago (Dunedin, New Zealand) using previously described methods (Wirawan et al., 2006, 2007). Identification of bacteriocins produced by the 5 strains was also investigated by this same group using producer typing (P-typing). This involved deferred antagonism, as described above, but using 9 standard indicator species detailed in Tagg and Bannister (1979).

Statistical Analysis

The total number of bacteria in milk, EC, and SCC were compared between infused and uninfused quarters and between individual strain-infused and SU5-infused quarters across the 5 milkings using GenStat (ninth edition, VSN International Ltd., Hemel Hempstead, UK) with linear mixed models that accounted for experimental design, animal and quarter effects, and repeated measurements. Specific comparisons of interest were evaluated using a nested treatment model. Examination of residuals showed that $\log_{10}$ transformed SCC could be safely compared using a normal distribution assumption. Changes in bacterial numbers over the 5 milkings were separately calculated for each cow for strains A to E and SU5. *T*-tests were performed on slopes to determine whether they significantly departed from zero. To analyze the predominance of one strain, confidence intervals were calculated for the proportion of cows yielding a mixture of strains (Clopper and Pearson, 1934; Owen, 1962) and a Chi-square test was performed to test whether the dominant strain occurred by chance. This was undertaken using maximum

likelihood by fitting a generalized linear model with a log link and a Poisson distribution using GenStat. To investigate whether each strain was present in SU5-infused quarters in different proportions from the other strains, the sample data were analyzed for each of the 5 milkings as multinomial data using log linear models.

RESULTS

All quarters infused with bacteria developed clinical mastitis. Twenty-four hours after infusion, foremilk EC and SCC of infused quarters were greater ($P < 0.01$) than those of uninfused quarters, with the average SCC of infused quarters greater than 4.5×10^6 cells/mL and average EC more than 15% higher than in uninfused quarters. The foremilk EC and SCC of uninfused quarters remained constant, with no significant difference between milkings and no bacteria detected in milk.

Viable bacteria were detected in milk from all infused quarters from the first milking after infusion, with numbers ranging from 300 cfu/mL (strain A-infused quarters) to 9.5×10^5 cfu/mL (SU5-infused quarters). Similar levels were maintained over 5 milkings, with no significant difference in bacterial numbers observed between quarters infused with individual strains or SU5. Despite differences in total bacterial numbers within each initial inoculum, there was no apparent difference

in the development of infection for strains A to E or SU5. Similarly, there appeared to be little difference in the time to establish infection or pathological changes in the milk for quarters infused with strains B and D, originally isolated from the dairy environment, compared with quarters infused with strains A, C, and E, isolated from clinical mastitis cases.

Strain Identification and Predominance

Band patterns generated by a combination of REP-PCR and anchored typing allowed identification of the 5 *S. uberis* strains present in milk (Figure 1). Quarters infused with individual strains yielded the infused strain throughout the study, whereas strains identified in SU5-infused quarters varied across milkings.

In the original mixed strain inoculum, strains A, D, and E were present in approximately equal proportions (22, 25, and 24%, respectively) of the total bacterial number, whereas strains B and C represented only 14 and 15%, respectively. Although each strain was present in slightly different proportions in the inoculum, this appeared to have little effect on the predominance of a particular strain over time. At the first milking after infusion, all 5 strains were detected together in only one SU5-infused quarter, whereas 2 to 4 strains were detected in other quarters (Table 2).

The number of strains recovered from each quarter changed over time. At the first and second milkings, multiple strains were identified in almost all quarters; by the third milking, a single strain was detected in 75% of the quarters available for analysis. Some strains must have been just below the detection limit of the bacterial enumeration method (<10 cfu/mL) because strains that appeared absent at the third milking were identified again at the fourth milking, when a single strain was found in only 50% of quarters. By the fifth milking after infusion, when the majority of quarters (73%) exhibited clinical signs, a single strain was detected in 70% of quarters.

A Chi-square test indicated differences ($P < 0.05$) in the proportion of quarters where each strain was dominant. At the fifth milking, strain C was the only detectable strain in 40% of quarters and was also dominant in one other quarter (quarter 2, Table 2), in which strain A was present in low numbers (data not shown). Strain D was the only detectable strain in 20% and strain E in 10% of quarters, whereas strains A and B were found only in combination with other strains. The quarter with a mix of 3 strains at the fifth milking had similar proportions of strains B, C, and D present.

When averaged across the 10 SU5-infused quarters, the proportion of strain C isolated from each quarter increased across the 5 milkings after infusion (Figure 2)

Figure 1. Repetitive extragenic palindromic-PCR and anchored typing band patterns for *Streptococcus uberis* strains A to E after electrophoresis on 1.5% agarose gels. Relative positions of the 100-bp DNA ladder bands are indicated to the right of the gel photos.

Figure 2. Average proportion (%) of *Streptococcus uberis* strains A to E in milk from 10 quarters (1 quarter/cow) at the first 5 milkings after infusion with a mixture of all 5 strains. *Indicates differences between strains at $P < 0.001$.

and, by the fourth milking, the proportion of strain C differed from the other 4 strains ($P < 0.001$).

Coculture of the 5 Strains in UHT Milk

All strains were individually capable of growth in sterile skimmed UHT milk and, when cultured together, no single strain prevailed. Total bacterial numbers ranged from 9.7×10^2 to 2.0×10^3 cfu/mL in the mixed culture before incubation, and 4.1×10^8 to 7.6×10^8 cfu/mL after an incubation of 74 h, which was equivalent to 5 milkings in the in vivo study. Although total bacterial numbers increased, the relative proportion of each strain remained similar before and after incubation (Figure 3).

Hyaluronic Acid Capsule and Bacteriocins

All 5 strains were capable of producing a hyaluronic acid capsule because the 3 genes *hasA*, *hasB*, and *hasC*

Figure 3. Average proportion (%) of *Streptococcus uberis* strains A to E ($\pm$ SEM) identified after combining all 5 strains together in UHT milk (0 h) and after incubation of the mixture at 37°C for 74 h, equivalent to 5 milkings.

were present; however, it is unknown whether a capsule was produced by all 5 strains during infection of the mammary gland or with coculture in UHT milk.

Production of bacteriocins was also investigated as a potential mechanism of direct competition between the 5 strains. Strain C completely inhibited the growth of all other strains on EBA when tested for deferred antagonism. Strain B also showed inhibitory activity toward strains A, D, and E, although not to the extent of strain C, with some resistant colonies of D and E observed. Following analysis by PCR, all 5 strains contained the structural gene for uberolysin, and strain C also contained the structural gene for nisin U. Strains B, C, and D strongly inhibited all indicator species when tested by P-typing and were assigned a P-type of 777, whereas strains A and E only weakly inhibited the same species (P-type 616 and 610, respectively). This indicated that uberolysin may be expressed by all 5 strains, but more strongly by strains B, C, and D.

Table 2. *Streptococcus uberis* strains (A, B, C, D, or E) identified in milk from 10 quarters (1 quarter/cow) for the first 5 milkings after infusion with a mix of all 5 strains

Quarter	Milking after infusion				
	1	2	3	4	5
1	CDE	C	C	C	C
2	BCE	BC	C	AC	AC
3	ACDE	ABCDE	— ¹	ABCE	C
4	ABCD	BCD	B	BD	D
5	DE	CE	E	E	E
6	CE	CE	E	E	— ²
7	ABCDE	ABCD	CD	CD	BCD
8	BCD	BCD	BC	C	C
9	ABCD	ABCD	C	CD	D
10	BCD	ABCD	— ¹	C	C

¹Results unavailable because of contamination of milk samples.
²Bacteria not detected in milk sample.

DISCUSSION

The results of this study demonstrated that the selection of a single predominant strain can occur within the mammary gland following intramammary infusion with 5 strains of *S. uberis*. Although each strain was capable of infecting the mammary gland when infused individually, a single strain predominated in 70% of quarters infused with a mix of 5 strains. Some strains appeared better adapted to infect the mammary gland under competitive conditions. In particular, strain C predominated in 4 of the 10 quarters, whereas strain A was detected only in the milk of one quarter in very low numbers by the fifth milking after infusion. This is the first study in which multiple strains of *S. uberis* were infused into the mammary gland and may explain why only one strain of *S. uberis* is usually detected when multiple isolates are strain-typed upon diagnosis of mastitis (Phuektes et al., 2001; Zadoks et al., 2003; Pryor, 2008). These results lend support to the general practice of strain-typing only a single isolate from each mastitis case for epidemiological studies.

Given that many different strains are evident within the dairy environment, it is likely that multiple strains gain entry to the teat canal and that selection occurs during the invasion process or within the mammary gland. Because *S. uberis* is the most frequently isolated environmental pathogen from mastitis cases in pasture-grazing systems (Pankey et al., 1996; McDougall, 1998), this species must also out-compete other environmental pathogens (including other streptococcal species and coliform bacteria; Smith et al., 1985) present on the teat skin or within the mammary environment.

Several factors may be involved in determining why certain species and strains survive and multiply instead of others. These include the ability to actively grow in milk (Leigh, 1994; Smith et al., 2002), adhere to mammary epithelia and resist the flushing effect of milking (Moshynskyy et al., 2003; Almeida et al., 2006), evade the host immune response (Field et al., 2003; Tamilselvam et al., 2006), and compete with other strains that may be present inside the mammary gland (Riley and Gordon, 1999).

Some bacteria may require a longer period of adaptation to the mammary environment, after which they are able to colonize and infect the mammary gland (Rambeaud et al., 2003). In the present study, all 5 strains of *S. uberis* were detected in similar numbers less than 24 h after they were infused individually into the mammary gland. Therefore, the ability to rapidly adapt and grow is unlikely to have created a selection pressure for the predominance of particular strains in this study.

Mechanisms for evading the host immune response have been investigated in *S. uberis*, and this survival property may be important in the selection of a predominant strain. Whereas a single strain was predominant in almost all quarters within 3 d of infusion with SU5, the same 5 strains coexisted and multiplied when cultured together in UHT milk for 74 h. Eliminating the influence of the immune response and other unique cow factors with culture in UHT milk may remove the selection pressure leading one strain to predominate.

Strains that differ markedly in their ability to infect the mammary gland have been reported to differ in their ability to resist phagocytosis by bovine neutrophils in vitro (Grant and Finch, 1997). Production of a hyaluronic acid capsule may aid survival (Hill, 1988), although other extracellular factors may be important in enabling resistance to phagocytosis (Field et al., 2003). All 5 strains contained the genes required to produce a hyaluronic acid capsule, but the capsular nature of the strains was not determined during infection of the mammary gland or during in vitro culture.

Adherence and internalization of *S. uberis* into mammary epithelial cells has been identified as a potential mechanism for evasion of the host immune response (Almeida et al., 1999; Tamilselvam et al., 2006). Dogan et al. (2006) observed that strains of *Escherichia coli* differed in their ability to invade cultured mammary epithelial cells, indicating a variation in virulence between strains. This may also be true for *S. uberis*, with strains differing in their ability to adhere to or invade cells within the mammary gland, thereby evading the immune response.

The differing ability of each strain to evade the immune response may have led to a predominant strain in SU5-infused quarters. However, when infused individually, all 5 strains were capable of evading the immune response and establishing infection, suggesting that mechanisms for survival within the mammary gland may be multifactorial.

Production of bacteriocins may have facilitated direct competition between the 5 strains when infused together in the mammary gland. Bacteriocins typically kill other bacteria of the same or closely related species (Tagg et al., 1976) and uberolysin, in particular, induces the lysis of metabolically active, susceptible target bacteria (Wirawan et al., 2007). Therefore, it is likely that bacteriocins would be produced in a competitive situation, such as within the mammary gland or when cultured together in UHT milk. All 5 strains produced uberolysin, and strain C alone produced nisin U; this may have contributed to the predominance of strain C in the mammary gland.

In conclusion, all 5 strains, including 2 originally isolated from the environment, were equally capable of causing mastitis when infused individually into the mammary gland. However, when infused together, a single strain predominated in 7 of the 10 quarters, indicating that multiple strains that gain entry to the mammary gland can be reduced to a single strain with development of infection. These results help explain why only one strain is usually detected in milk with the diagnosis of mastitis and highlight the complexity in the establishment of *S. uberis* mastitis and the interaction between pathogen and host.

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