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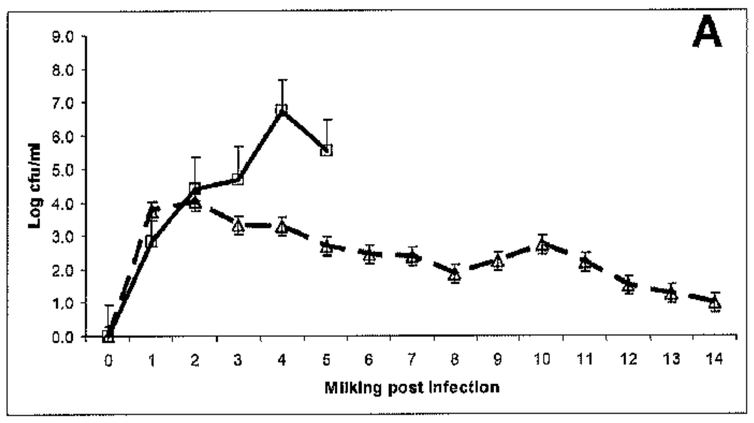
(54) **Titre : COMPOSITION COMPRENANT DES PROTEINES DE SURFACE ANCREES PAR LA SORTASE A STREPTOCOCCUS UBERIS**
(54) **Title: COMPOSITION COMPRISING SORTASE ANCHORED SURFACE PROTEINS OF STREPTOCOCCUS UBERIS**



(57) **Abrégé/Abstract:**
The present invention provides an immunogenic composition comprising one or more Streptococcus uberis sortase-anchored surface proteins, or an immunogenic part thereof, wherein the composition is capable of eliciting an immune response, when administered to a subject.

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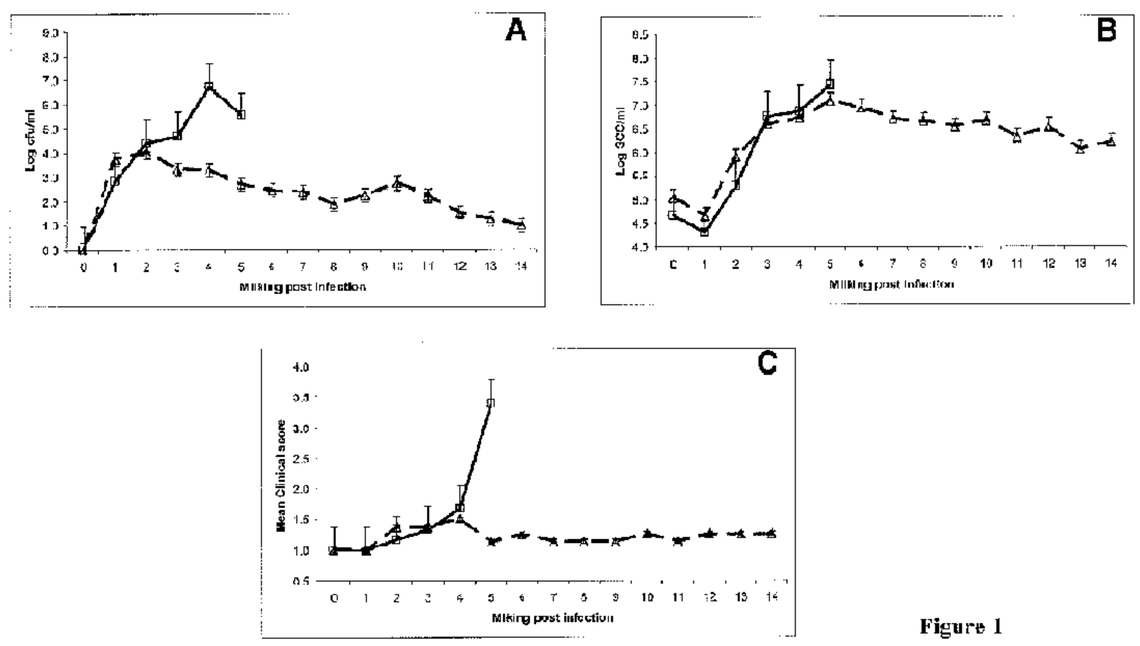

Figure 1

(57) Abstract: The present invention provides an immunogenic composition comprising one or more Streptococcus uberis sortase-anchored surface proteins, or an immunogenic part thereof, wherein the composition is capable of eliciting an immune response, when administered to a subject.

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COMPOSITION COMPRISING SORTASE ANCHORED SURFACE PROTEINS OF STREPTOCOCCUS UBERIS

The present invention relates to an immunogenic composition for use in eliciting an immune response to *Streptococcus uberis*, and in particular, to immunogenic compositions capable of eliciting a protective immune response.

Streptococcus uberis (*S. uberis)* is currently responsible for around 20-30% of all clinical mastitis cases in the UK and occurs at a similar incidence worldwide. Mastitis remains the most economically important infectious disease of dairy cattle throughout the world. The annual loss due to clinical mastitis in the UK has been estimated at approximately £170 million and between $1.5-2.0 billion in the USA. These losses can be attributed to a reduction in milk production, the associated costs of treatment and the culling of persistent and repeatedly infected cows. Micro organisms that cause mastitis can be divided into those that show a contagious route of transmission, such as *Staphylococcus aureus* and *Streptococcus agalactiae*, and those that additionally infect the udder frequently from an environmental reservoir, such as *Escherichia coli* and *Streptococcus uberis*. The application of various control measures over the past two decades, based on improved milking practices, post-milking teat disinfection and routine intra-mammary anti-microbial treatment after each lactation, has proved effective against pathogens with a solely contagious route of transmission, but has had little, if any, impact on the incidence of infection of the mammary gland from environmental reservoirs. The failure to control bovine mastitis caused by *S. uberis* is largely attributed to insufficient information on the pathogenesis of infection.

Bovine mastitis, which causes inflammation of the mammary gland (udder), usually arises as a result of intramammary infection by bacteria.

The signs of mastitis vary according to factors in the host and the invading pathogen and intramammary infection may result in sub-clinical or clinical disease. Sub-clinical mastitis, by definition, shows no obvious signs of disease. Infection associated with clinical disease can range from visible abnormalities in the milk (protein aggregates or clots) accompanied by pain and swelling in the affected gland, to production of a secretion which is composed solely of aggregated protein in a serous fluid. In severe cases, there may be systemic signs such as elevated temperature and loss of appetite, which may develop to bacteraemia, septicaemia and lead to death of the animal.

Milk from an uninfected mammary gland contains leukocytes, including macrophages, neutrophils and lymphocytes typically below 150,000 cells/ml. Infection usually results in a localised inflammatory response, characterised by the influx of neutrophils into the infected quarter of the mammary gland and milk. The resulting milk cell count is used internationally as a surrogate measure of infection of the mammary gland and as a measure of milk quality and udder health. Milk from sub-clinically infected quarters usually has a cell count in excess of 250,000 cells/ml but this figure may vary widely. Milk from clinically infected quarters usually contains in excess of 2,000,000 cells/ml. The interaction between bacteria and/or their products and the large number of neutrophils in the secretion has been considered to be the principal cause underlying the decreased rate of milk production, degradation of the secretion and the induction of widespread inflammatory changes characteristic of mastitis.

One aim of this invention is to provide one or more compositions which can be used to elicit a protective immune response to *Streptococcus uberis*, and thereby prevent or reduce the incidence of mastitis.

According to a first aspect, the present invention provides an immunogenic composition comprising one or more *Streptococcus uberis* proteins, or an immunogenic part thereof, wherein the composition is capable of eliciting an immune response, when administered to a subject.

Preferably the one or more *Streptococcus uberis* proteins are sortase-anchored proteins, or an immunogenic part thereof.

A *Streptococcus uberis* sortase-anchored protein refers to any protein which in wild type *Streptococcus uberis* is anchored to the surface of the bacteria by the action of the enzyme sortase.

The one or more sortase-anchored proteins, or the one or more *Streptococcus uberis* proteins, may be selected from the group comprising the proteins SUB0145, SUB1095 and SUB1154 or a protein with 50%, 60%, 70%, 80%, 90%, 95% or more, preferably 80% or more, sequence homology with one of the aforementioned proteins.

The immunogenic composition may comprise two or more *Streptococcus uberis* proteins, or an immunogenic part thereof.

The two or more sortase-anchored proteins, or the two or more *Streptococcus uberis* proteins, may be selected from the group comprising the proteins SUB0145, SUB1095 and SUB1154 or a protein with 50%, 60%, 70%, 80%, 90%, 95% or more, preferably 80% or more, sequence homology with one of the aforementioned proteins.

The immunogenic composition may comprise proteins SUB0145, SUB1095 and SUB1154 or a protein with 50%, 60%, 70%, 80%, 90%, 95% or more, preferably 80% or more, sequence homology with one of the aforementioned proteins.

The immunogenic composition may comprise proteins SUB1095 and SUB1154 or a protein with 50%, 60%, 70%, 80%, 90%, 95% or more, preferably 80% or more, sequence homology with one of the aforementioned proteins.

The one or more sortase-anchored proteins, or the one or more *Streptococcus uberis* proteins, may be selected from the group comprising the proteins SUB0135, SUB0145, SUB0207, SUB0826, SUB0888, SUB1095, SUB1154, SUB1370, SUB1730 and SUB0241 or a protein with 50%, 60%, 70%, 80%, 90%, 95% or more, preferably 80% or more, sequence homology with one of the aforementioned proteins.

The one or more sortase-anchored proteins, or the one or more *Streptococcus uberis* proteins, may be selected from the group comprising the proteins SUB0135, SUB0207, SUB0826, SUB0888, SUB1095, SUB1154, SUB1370, SUB1730 and SUB0241 or a protein with 50%, 60%, 70%, 80%, 90%, 95% or more, preferably 80% or more, sequence homology with one of the aforementioned proteins.

Reference to percentage homology relates to the percent identity between two aligned sequences. The percent identity refers to the residues in two proteins which are the same, when the protein sequences are aligned for maximum correspondence and when inversions and translocations are accounted for. Preferably the percent identity ignores any conservative

differences between the aligned sequences which do not affect function. The percent identity between aligned sequences can be established by using well-established tools (such as the BLAST algorithm - Basic Local Alignment Search Tool; Altschul et al., (1990) J Mol Biol. 215:403-10)

In one embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is SUB0135. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB0135.

In one embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is SUB0145. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB0145.

In one embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is SUB0207. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB0207.

In one embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is SUB0826. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB0826.

In one embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is SUB0888. In another

embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB0888.

In one embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is SUB1095. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB1095.

In one embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is SUB1154. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB1154.

In one embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is SUB1370. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB1370.

In one embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is SUB1730. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB1730.

In one embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is SUB0241. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB0241.

In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB0164. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB0348. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB1739. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB0206. In another embodiment one or more of the sortase-anchored proteins, or one or more of the *Streptococcus uberis* proteins, is not SUB0337.

An immunogenic part of a protein refers to a part of a larger protein which is capable of eliciting an immune response. Preferably the immune response elicited will recognise the part of the protein and the whole protein. Preferably the immunogenic part includes at least one epitope from the full length protein.

An immunogenic composition is a composition that is capable of eliciting an immune response to an antigen in the composition when the composition is administered to a subject. Preferably the immune response elicited is protective. Preferably the subject is a mammal, more preferably a ruminant, such as a cow, sheep or goat. The antigen in the immunogenic composition of the invention may be one or more proteins which are anchored to the surface of *Streptococcus uberis* by the enzyme sortase.

Preferably the immune response elicited by a composition of the invention is directed to the antigen in the composition and acts to prevent or reduce infection by *Streptococcus uberis* in a subject to whom the immunogenic composition has been administered. The immune response may recognise

and destroy *Streptococcus uberis*. Alternatively, or additionally, the immune response elicited may impede or prevent replication of *Streptococcus uberis*. Alternatively, or additionally, the immune response elicited may impede or prevent *Streptococcus uberis* causing disease, such as mastitis, in the subject. Preferably, the immune response elicited by the composition is also capable of being directed to strains of *Streptococcus uberis* other than that from which the proteins in the composition are derived.

The immune response generated may be a cellular and/or antibody-mediated immune response. Usually, an immune response includes, but is not limited to, one or more of the following effects, the production of antibodies, B cells, helper T cells, suppressor T cells and/or cytotoxic T cells, directed to the one or more immunogenic proteins in the composition.

The composition may also comprise a further one or more antigens, in addition to one or more *S. uberis* sortase-anchored proteins, or one or more *S. uberis* proteins. The further antigens may also be capable of eliciting an immune response directed to the pathogenic organism from which they are derived. The further antigens may be derived from *S. uberis* or they may be derived from a different pathogenic organism.

The composition may be used to elicit/produce a protective immune response when administered to a subject. The protective immune response may cause *S. uberis* to be killed upon infecting a subject, or it may prevent or inhibit *S. uberis* from replicating and/or from causing disease in a subject.

The composition may be used as a prophylactic or a therapeutic vaccine against *S. uberis*

According to a further aspect, the invention provides a pharmaceutical composition comprising one or more *S. uberis* sortase-anchored proteins, or one or more *S. uberis* proteins, or part thereof, in combination with a pharmaceutically acceptable carrier or excipient.

Preferably the pharmaceutical composition comprises a composition according to any aspect of the invention.

Preferably the pharmaceutical composition is capable of producing a protective immune response to *S. uberis*.

The phrase "producing a protective immune response" as used herein means that the composition is capable of generating a protective response in a host organism, such as a cow, to whom it is administered. Preferably a protective immune response protects against subsequent infection by *S. uberis*. The protective immune response may eliminate or reduce the level of infection by reducing replication of *S. uberis* by affecting the mode of action of *S. uberis*. Preferably the protective immune response reduces or prevents disease caused by *S. uberis*.

Suitable acceptable excipients and carriers for use in a pharmaceutical composition will be well known to those skilled in the art. These may include solid or liquid carriers. Suitable liquid carriers include water and saline. The proteins of the composition may be formulated into an emulsion or they may be formulated into biodegradable microspheres or liposomes.

The composition may further comprise an adjuvant. Suitable adjuvants will be well known to those skilled in the art, and may include Freund's Incomplete Adjuvant (for use in animals), and metal salts, such as aluminium or calcium salts,

The composition may also comprise polymers or other agents to control the consistency of the composition, and/or to control the release of the proteins from the composition.

The composition may also comprise other agents such as diluents, which may include water; saline; glycerol or other suitable alcohols etc; wetting or emulsifying agents; buffering agents; thickening agents for example cellulose or cellulose derivatives; preservatives; detergents, antimicrobial agents; and the like.

Preferably the active ingredients in the composition are greater than 50% pure, usually greater than 80% pure, often greater than 90% pure and more preferably greater than 95%, 98% or 99% pure. With active ingredients approaching 100% pure, for example about 99.5% pure or about 99.9% pure, being used most often.

The composition of the present invention may be used as vaccine against infections caused by *S. uberis*. The vaccine may be administered prophylactically to animals at risk of exposure to *S. uberis*, and/or therapeutically to animals who have already been exposed to *S. uberis*.

Preferably, if the composition is used as a vaccine, the composition comprises an immunologically effective amount of antigen (comprised of *S. uberis* proteins). An "immunologically effective amount" of an antigen is an amount that when administered to an individual, either in a single dose or in a series of doses, is effective for treatment or prevention

of infection by *S. uberis*. This amount will vary depending upon the health and physical condition of the individual to be treated and on the antigen. It is expected that the amount will fall in a relatively broad range that can be determined by routine trials.

The route of administration of the composition may vary depending on the formulation of the proteins in the composition. The composition may be arranged to be administered intramuscularly, intradermally, subcutaneously, intraperitonealy, intravenously or intramammaryly. Alternatively the composition may be arranged to be administered parenterally, such as by intranasal, oral, buccal, inhalation, epidermal, transcutaneous, topical, vaginal or rectal administration.

The composition may be arranged to be administered as a single dose or as part of a multiple dose schedule. Multiple doses may be administered as a primary immunisation followed by one or more booster immunisations. Suitable timing between priming and boosting immunisations can be routinely determined.

Compositions of the invention may be able to induce a serum bactericidal antibody response after being administered to a subject. These responses are conveniently measured in mice and the results are a standard indicator of vaccine efficacy.

The compositions of the invention may also, or alternatively, be able to elicit an immune response which effects proteins on the host cells to defend against infection by *S. uberis*, without necessarily destroying the bacteria.

According to a further aspect, the present invention provides the use of one or more *S. uberis* sortase-anchored proteins in the preparation of a

medicament for eliciting an immune response. The medicament may be used for the prophylactic or therapeutic vaccination of subjects against *S. uberis*. The medicament may be a prophylactic or a therapeutic vaccine.

According a still further aspect, the present invention provides a method of protecting a human or non-human animal, preferably a cow, from the effects of infection by *S. uberis* comprising administering to the human or non-human animal a composition according to any other aspect of the invention.

According to another aspect, the invention provides a method for raising an immune response in a human or non-human animal, preferably a cow, comprising administering a composition according to the invention to the human or non-human animal. The immune response is preferably protective. The method may raise a booster response in a subject that has already been primed. The immune response may be prophylactic or therapeutic.

The uses, methods and compositions of the invention are preferably for the prevention and/or treatment of a disease caused by *S. uberis*.

The skilled man will appreciate that any of the preferable features discussed above can be applied to any of the aspects of the invention.

Preferred embodiments of the present invention will now be described, merely by way of example, with reference to the following figures and examples.

Figures 1A, B and C – show the results of bacterial isolation, somatic cell count and clinical response following challenge with

wild type *S. uberis* 0140J and a *S. uberis* Srt mutant in dairy cattle. Figure 1(A) shows the bacterial recovery of *S. uberis* following challenge. Data are represented as the geometric means of the number of bacteria obtained from the milk of animals challenged with either strain 0140J (squares; n=4) or the SrtA mutant (triangles; n=8). Figure 1 (B) illustrates the inflammatory response following challenge with wild type and Srt mutant of *S. uberis*. Data are represented by the geometric means of the number of somatic cells obtained from the milk of animals challenged with either strain 0140J (squares; n=4) or the SrtA mutant (triangles; n=8). Figure 1(C) illustrates the combined clinical scores from clinical manifestations following challenge with wild type and Srt mutant of *S. uberis*. Data are represented by the mean of clinical scores given for the appearance of the quarter and appearance of the milk, as outlined in Figure 2 with either strain 0140J (squares; n=4) or the SrtA mutant (triangles; n=8);

Figure 2 – illustrates in tabular form the manifestation of a clinical response to infection with *Streptococcus uberis*. All quarters and milk samples were analyzed against these criteria at each milking following challenge;

Figure 3 – illustrates in tabular form the proteins found by bioinformatic examination of the S. uberis genome that were likely to be anchored by sortase; The genome of *S. uberis* was searched using the LPXXG motif for putative sortase-anchored proteins. The list of proteins identified was refined by using context and position of the motif, ie LPXXG toward the C-terminus and followed by a hydrophobic region and charged residues at a C-terminal position and the presence of a recognisable secretion signal peptide at the N-terminus;

Figure 4A: lists sortase anchored proteins identified in cell wall extracts of S. uberis 0140J cultured in THB media. [a] Gene and protein annotation according to the genomic sequence of Streptococcus uberis 0140J (Ward et al 2009); [b] Theoretical molecular mass values for protein precursors obtained from Artemis database from the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/); [c] Number of unique peptide hits for each protein; [d] Percentage of protein sequence covered by experimentally detected peptides; [e] 2 peptides identified in the SrtA mutant cell wall fraction. **Figure 4B**: lists sortase anchored proteins identified in cell wall extracts of *S. uberis* 0140J cultured in BHI media. [a] Gene and protein annotation according to the genomic sequence of Streptococcus uberis 0140J (Ward et al 2009); [b] Theoretical molecular mass values for protein precursors obtained from Artemis database from the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/); [c] Number of unique peptide hits for each protein; [d] Percentage of protein sequence covered by experimentally detected peptides; [e] 4 peptides identified in the SrtA mutant cell wall fraction;

Figures 5A and 5B – shows the identification of Sub1154 and Sub 1370 from extracts of *Streptococcus uberis* 0140J and srtA mutant. Figure 5A - rabbit antiserum to Sub1154 was used to probe immunoblots blots of protein detergent extracts from 0140J (lane 2) and SrtA mutant (lane 3), concentrated, precipitated media from 0140J (lane 4), SrtA mutant (lane 5) and Sub1154 mutant (lane 6). Molecular weight standards are shown in lane 1. Figure 5B - rabbit antiserum to Sub1370 was used to probe immunoblots blots of protein detergent extracts from 0140J (lane 1) and SrtA mutant (lane 2), concentrated, precipitated media from 0140J (lane 3),

SrtA mutant (lane 4) and Sub1370 mutant (lane 5). Molecular weight standards are shown in lane 6;

Figures 6A to 6O – are the amino acid sequences of *S. uberis* sortase anchored proteins;

Figure 6A is the sequence of SUB1370 (Seq ID No: 1) a zinc carboxypeptidase;

Figure 6B is the sequence of SUB0145 (Seq ID No: 2) a Lactoferrin binding protein;

Figure 6C is the sequence of SUB0135 (Seq ID No: 3) a frucan beta fructosidase precursor;

Figure 6D is the sequence of SUB1730 (Seq ID No: 4);

Figure 6E is the sequence of SUB0888 (Seq ID No: 5);

Figure 6F is the sequence of SUB0207 (Seq ID No: 6);

Figure 6G is the sequence of SUB1154 (Seq ID No: 7) a subtilin like serine protease;

Figure 6H is the sequence of SUB1095 (Seq ID No: 8) a collagen like protein;

Figure 6I is the sequence of SUB0826 (Seq ID No: 9) a putative surface anchored subtilase;

Figure 6J is the sequence of SUB0164 (Seq ID No: 10) a putative truncated surface anchored fibronectin binding protein (but is encoded by a probable pseudogene);

Figure 6K is the sequence of SUB0348 (Seq ID No: 11) a remnant of a putative collagen like protein (but is encoded by a pseudogene);

Figure 6L is the sequence of SUB1739 (Seq ID No: 12) a putative surface anchored protein (but is encoded by a pseudogene);

Figure 6M is the sequence of SUB0206 (Seq ID No: 13) a putative exported protein of unknown function;

Figure 6N is the sequence of SUB0241 (Seq ID No: 14) a putative surface anchored protein of unknown function;

Figure 6O is the sequence of SUB0337 (Seq ID No: 15) a putative surface located glutamine binding protein;

Figure 7 shows the bacterial colonisation following challenge with wild type (0140J) and attenuated mutant strains lacking sub0145, sub1095 or sub1154. Data is expressed as geometric means of Log_{10} cfu/ml detected in milk samples obtained at each milking after experimental challenge.

The data presented below demonstrates that proteins anchored to the surface of *S. uberis* by sortase, a transamidase, are important in virulence and further describes some such proteins (eg sub1095, sub1154 and sub0145) are essential for virulence, and thus are required to be functional in order for this bacterium to cause disease. Proteins are good immunogens. Immune responses to these proteins in the form of antibodies is likely to ablate their function, thus the identified protein would be useful inclusions within immunogenic compositions intended to reduce or prevent infection or diseases caused by *S. uberis*.

Example 1: Production and evaluation of a SrtA mutant of *S. uberis*.

Methods and Materials

Bacterial Strains and Reagents.

Streptococcus uberis strain 0140J, originally isolated from a clinical case of bovine mastitis in the UK, was used throughout this study. The bacterium was routinely grown in Todd Hewitt or Brain Heart Infusion broth.

Skimmed milk was produced from raw bovine milk collected aseptically from several cows from within the dairy herd at the Institute for Animal Health. Milk was collected from animals that were free of intramammary infection. Following centrifugation (3,000 x g, 10 min); skimmed milk was removed carefully from the upper fat-layer and the pellet of sedimented cells. The sterility of the skimmed milk was determined by plating 500μl of milk directly onto blood agar containing aesculin (1.0%, w/v; ABA) and by enrichment culture of 5 ml of milk in and equal volume of Todd Hewitt broth followed by isolation of single colonies on ABA. In both cases, plates were incubated at 37°C for 18 h. Skimmed milk was stored at 4°C and used within 72h.

Other bacterial strains and reagents were used as described in the text.

Isolation of srtA-mutant by genotypic selection.

The srtA (Sub0881) mutant was isolated following PCR screening of a *S. uberis* 0140J pGhost9::ISS1 mutant bank following a similar protocol to that described previously (Taylor, D. L. et al, 2003. J Bacteriol 185:5210-5219; Ward, P. N. et al 2001 Infect Immun 69:392-399). Briefly, overnight cultures from individual 96-well plates were pooled and genomic DNA was prepared for use as template in PCR amplification reactions containing a locus-specific primer, P261 (srtA) and an ISS1-specific primer, P247 or P250. Amplification was conducted using thirty-five cycles (95°C for 20 s, 54°C for 1 min, and 72°C for 3 min) and was performed with AmpliTaq Gold master mix (ABI). The products were visualized following gel electrophoresis, staining with ethidium bromide and transillumination with UV light. Following plate identification, a well location was similarly identified using genomic DNA pooled from the

columns and rows of the target plate. Following isolation of the mutant clone, excision of the plasmid vector was promoted by growth at the permissive temperature (28°C) without antibiotic selection. Loss of the pGhost9 vector and retention of ISS1 were confirmed by Southern blotting as described previously (Ward, P. N. et al 2001 Infect Immun 69:392-399). Presence of the insertion in srtA was confirmed by PCR amplification of the open reading frame and sequencing of the resulting product across the junction between ISS1 and the disrupted ORF. The PCR primers used are as shown in Table 1 below:

Designation	Sequence (5' - 3')	Application	Template	Annealing temp (°C)
P247 IS*S1* fwd	GCTCTTCGGATTTTCGGTATC	IS*S1* probe	pGh9::IS*S1*	58
P250 IS*S1* rev	CATTTTCCACGAATAGAAGGACTGTC	IS*S1* probe	pGh9::IS*S1*	61
P261	TGGTTGAAGCAGAAGCTGAA	Screening for IS*S1* within *srtA* ORF vs P247	pGh9::IS*S1*	55
P409	GAGCAATTGCAAAATGAAAAGC	Amplification of *Sub1154* ORF	*S. uberis* 0140J genomic DNA	58
P410	ATGTCAAAAGCCCGGTACCTTTACAG	Amplification of *Sub1154* ORF	*S. uberis* 0140J genomic DNA	58
P615	GAAATGATGATGAGAAATTGAGA	Screening for IS*S1* within *Sub1154* ORF vs P247	*S. uberis* 0140J ::pGhost9-IS*S1* genomic DNA pools	57
P630	AGCCACAAACACCATTCACA	Screening for IS*S1* within *Sub1154* ORF vs P247	*S. uberis* 0140J ::pGhost9-IS*S1* genomic DNA pools	59
P480	GAAGAAGTGGTAACTGCTACAAAC	Amplification of *Sub1370* ORF	*S. uberis* 0140J genomic DNA	60
P481	TACTAACTTCTTGTCATCTTGGTACCTTTT	Amplification of *Sub1370* ORF Screening for IS*S1* within *Sub1370* ORF	*S. uberis* 0140J genomic DNA	64
P621	CAACGAATCAACAAACTGAAAGC-3'	Screening for IS*S1* within *Sub1370* vs P250	*S. uberis* 0140J ::pGhost9-IS*S1* genomic DNA pools	59

Table 1: PCR primers. P247 IS*S1* fwd (SEQ ID NO: 16); P250 IS*S1* rev (SEQ ID NO: 17); P261 (SEQ ID NO: 18); P409 (SEQ ID NO: 19); P410 (SEQ ID NO: 20); P615 (SEQ ID NO: 21); P630 (SEQ ID NO: 22); P480 (SEQ ID NO: 23); P481 (SEQ ID NO: 24); P621 (SEQ ID NO: 25).

Extraction of chromosomal DNA from *S. uberis*

Genomic DNA was prepared using a variation of the method of Hill and Leigh as described previously (Hill, A. W. et al 1994 FEMS Immunol Med

Microbiol 8:109-117). Briefly, 1.5 ml of an overnight culture was centrifuged at 10,000 x g for 2 minutes and the cell pellet washed with 500µl of 10mM Tris-Cl, 5mM EDTA, pH 7.8. Bacterial cell walls were disrupted by resuspension in 375µl of 10mM Tris-Cl, 5mM EDTA pH 7.8 containing 30 units/ml mutanolysin and 10 mg/ml lysozyme (both from Sigma-Aldrich, St Louis, MO, USA) and subsequent incubation at 37oC for 30 minutes. Total cell lysis was achieved by addition of 20µl of SDS solution (20% w/v in 50mM Tris-Cl, 20mM EDTA, pH 7.8) and Proteinase K (Sigma) to a final concentration of 150µg/ml and a further incubation at 37°C for 1h. Cell wall material was removed by precipitation following the addition of 200µl of saturated NaCl and subsequent centrifugation at 12,000 x g for 10 minutes. The supernatant was extracted with phenol chloroform and DNA precipitated by addition of 2 volumes of absolute ethanol. DNA pellets were washed with 70% aqueous ethanol and air-dried prior to resuspension in TE buffer containing 20µg/ml RNAase-A (Sigma).

Challenge of lactating dairy cows with *S. uberis* 0140J and SrtA mutant

The role of SrtA in the pathogenesis of infection was determined by comparison of the virulence of strain 0140J and the mutant lacking SrtA (srtA mutant) in an intramammary infection model in the dairy cow. Bacteria were grown for 18 h at 37oC in Todd Hewitt broth. Cells were recovered by centrifugation (10,000 x g, 10 min), suspended in pyrogen-free saline (Sigma) and diluted in the same to provide the required cell density. Suspensions of each strain were held on ice prior to being used to challenge animals. The number of viable bacteria in identical aliquots of each suspension was enumerated both prior to and following challenge.

Six dairy cows, 2-10 weeks into their first lactation, were selected from the Institute's dairy herd for challenge. Criteria for selection were: absence of signs of mastitis, absence of bacteria in milk samples prior to challenge, no history of mastitis during the current lactation and no evidence of intramammary infection in milk samples taken at 7 and 14 days after parturition. Animals were challenged in mammary quarters by infusion of 1 ml of pyrogen-free saline (Sigma) containing *S. uberis*. Two animals were challenged in a total of four quarters with 6.0 $x10^2$ cfu of strain 0140J and a further four animals, were challenged in a total of eight quarters with a similar dose of the srtA mutant. Following challenge, animals were milked and inspected twice daily (07:00 h and 15:30 h) and those in which predetermined criteria for clinical end points (clotted and discoloured milk and/or udder quarter swollen or causing discomfort on palpation) had been reached were treated with proprietary branded antibiotics in line with the prescribed criteria outlined in Figure 2. Milk samples were taken and analysed for bacteria and somatic cells, as described below.

Analysis of milk samples

The number of viable bacteria present was estimated by direct plating of 1 ml and 100 μl of each milk sample on to ABA. Samples were also diluted in saline and 50 μl of each dilution plated directly onto ABA. In each case, the presence and/or number of S. uberis was determined and the genotype of the recovered isolates was determined by comparing restriction fragment length polymorphism (RFLP) of chromosomal DNA and amplification of the srtA locus, as described below. The number of somatic cells present in milk samples was determined using a coulter counter (Beckman Coulter, Ltd).

Preparation of proteins from bacterial growth media by methanol chloroform precipitation

Bacteria were grown in BHI (200ml) with cultures grown to an approximate OD600nm of 0.5 and harvested by centrifugation (16,000 x g, 20 min, 4°C) and bacterial growth media was filter sterilised through a 0.22 μM filter (Millipore). After the addition of complete protease inhibitor (Roche) at a 1X concentration, the bacterial growth media was concentrated approximately 100-fold using Amicon centrifugal filter devices (Millipore) with a molecular weight exclusion of 10 kDa. To precipitate proteins, 600μl of methanol and 150μl of chloroform (both from BDH) was added to 200μl of concentrated bacterial growth media. The preparation was vortexed and 450μl of MilliQ water was added prior to centrifugation (16,000 x g, 1 min). The upper phase was carefully removed and discarded and 450 μl of methanol was added to the remaining material which was vortexed and centrifuged (16,000 x g, 2 min). The supernatant was discarded and the remaining pellet air-dried before resuspension in SDS-loading buffer.

Extraction of non-anchored proteins with detergent

The bacterial pellets from the above cultures were washed 3 times in 40ml of PBS and resuspended in 500μl of PBS containing hyaluronidase (100U/ml, Sigma-Aldrich). Cells were incubated for 2 hours at 37°C and the hydrolysed capsular material removed by centrifugation (8000 x g, 6 min, 4°C). Cells were washed 3 times in 40ml of PBS and resuspended in 200μl of 0.1% (v/v) NonIdet P-40 (NP-40) in PBS. The detergent extract was harvested following removal of the bacterial cells by centrifugation (16,000 x g, 10 min, 4°C).

Production and purification of recombinant Sub1154 and Sub 1370 proteins

Primers p409 and p410 (see table above) were designed to amplify from *S. uberis* 0140J genomic DNA the predicted mature coding sequence (ie lacking N-terminal signal sequence) of Sub1154, a putative srtA substrate with homology to subtilase-like serine protease. A 3.4 kb amplicon was generated using Phusion™ high fidelity polymerase (New England Biolabs), purified using a MinElute PCR Purification Kit (Qiagen) and treated with KpnI (New England Biolabs) to facilitate directional cloning. Plasmid pQE1 (Qiagen) was prepared using using PvuII, KpnI and Antarctic phosphatase (all from New England Biolabs) and the construct ligated (T4 DNA Ligase, New England Biolabs) overnight at 20°C according to the manufacturers' instructions. Twenty microlitres of the ligation mixture was desalted using the method of Atrazhev and Elliott (Atrazhev, A. M., and J. F. Elliott. 1996 Biotechniques 21:1024). Approximately 10 ng of the desalted ligation mixture was transformed into Escherichia coli M15 pREP4 (Qiagen) and recombinant clones selected on LB Kan25μg/ml Amp50μg/ml agar plates. Recombinant (6 x His-tagged) Sub1154 protein commencing at residue Asp34 was purified by dilution (1/30) of overnight culture into 1600 ml LB broth containing 50 μg/ml of ampicillin and 25 μg/ml of kanamycin and growth at 20°C without shaking for 2h. Recombinant Sub1370 was similarly prepared, but using the primers P480 and P481, and was grown similarly in 800 ml of culture medium. Protein expression was induced by addition of IPTG to a final concentration of 0.2 mM. Cultures were incubated for a further 2-4 hrs and then centrifuged at 8,000 x g for 20 min to harvest the bacterial cells. Approximately 1mg and 0.3mg of soluble 6 x His tagged Sub1154 and Sub1370 proteins respectively was purified in the presence of protease inhibitors (Complete-EDTA free; Roche) using CelLytic and HisSelect high flow cartridges (both from Sigma) according to the manufacturers' instructions.

Production of Sub1154 and Sub1370 antiserum in rabbits and immuno-blotting

Five aliquots of approximately 50μg freeze dried purified recombinant Sub1154 and Sub1370 proteins were supplied to Davids Biotechnologie (Germany) for serum production in rabbits. Anti-serum (50ml) was supplied filter sterilised and containing 0.02% sodium azide as a preservative.

Detergent and media extracts from cultures of wild type *S. uberis* and a SrtA mutant were separated on 10% sodium-dodecyl sulphate polyacrylamide (SDS-PAGE) gels and then transferred onto nitrocellulose membranes (Amersham) for immuno-detection, or alternately Coomassie stained using InstantBlue (Novexin). Transfer was performed at 170 mA for 1 hr in a (Biorad) Transblot apparatus in transfer buffer consisting of 25mM Tris-base, 192mM glycine and 20% (v/v) methanol, pH 8.1-8.4. Following transfer, membranes were incubated in a blocking solution of 1% skim milk powder in PBS at 4°C overnight. Membranes were washed three times for 5 min in PBS containing 0.1% Tween 20 (PBST) then incubated with rabbit antisera at 1/12,000 dilution for the Sub1154 antiserum and 1/16,000 dilution for the Sub1370 antiserum in blocking solution for 1 hour. Membranes were washed three times for 5 min in PBST then incubated with goat anti-rabbit immunoglobulin G conjugated to HRP at a 1/1,000 dilution (Southern Biotech) for 1 hour. Membranes were washed again as above and HRP conjugate detected using a solution of 4-chloronaphthol (0.5mg/ml) in PBS containing 16.7% methanol and 0.00015% (v/v) of H_2O_2, incubated for 1 hour in the dark, before membranes were washed in PBS and allowed to dry.

Isolation and genetic characterization of srtA mutant

Analysis of the complete genome of *S. uberis* 0140J confirmed the presence of a single sortase homologue, sortase A (srtA) (Ward, P.N. et al (submitted 2008) BMC Genomics). A mutant clone was isolated with the IS*S1* element inserted between base pairs 248 and 249 of, and in reverse orientation to, the sortase coding sequence. The translation product of this mutated srtA gene consisted of the first 82 residues of the 252 amino acids encoded in the srtA ORF together with a further 18 residues in the IS*S1* element before a stop codon was reached.

Infectivity and virulence of the wild-type and srtA mutant *S. uberis* following experimental challenge in the bovine mammary gland.

The infectivity and virulence of the srtA mutant, compared to a wild type strain, was determined by challenging the bovine mammary gland of a number of diary cows. All challenged quarters of animals that received 600 cfu of wild type *S. uberis* became infected and shed bacteria at around 10^6 to 10^7 cfu/ml by 48 - 60 h post challenge (Figure 1A). Following challenge of eight quarters on four animals with a similar dose of the srtA mutant, all showed evidence of infection and the srtA mutant was detected in milk at levels similar to those for the wild type for up to 24h post challenge. However, subsequent bacterial colonization declined, from a maximum of 10^4 cfu/ml of milk by 24h post challenge, such that by the end of the experiment (7days post challenge) the mean bacterial number present was around 10 cfu/ml (Figure 1B). By this time only two of the eight quarters continued to shed bacteria, the remainder having eliminated the infection (< 1 cfu/ml milk).

The cellular infiltration into the mammary gland in response to infection was identical in both groups of animals and was not dependant on the challenge strain. In each case, this was similar to that reported

previously in this model and reached a maximum of approximately 10^7 cells/ml of milk by 48-60 h post challenge. In animals challenged with the wild type strain this coincided with the appearance of acute clinical signs of mastitis (Figure 2 and Figure 1C) which required administration of antibiotic therapy to eliminate infection and to alleviate signs of disease. In stark contrast, animals that had received the srtA mutant showed little, if any, signs of mastitis (Figure 2 and Figure 1C).

The results presented in Figure 1 demonstrate that *S. uberis* requires the sortase protein, encoded by srtA, for the full expression of virulence by this bacterium. Although initially able to colonise the bovine mammary gland similarly to the wild-type *S. uberis*, the mutant lacking SrtA was unable to colonise the gland to high levels; with bacterial maximal numbers remaining approximately 1000-fold lower than those detected in milk from animals challenged with the wild type strain. This corresponded with the failure of the srtA mutant to induce progressive clinical signs of disease.

It is understood that srtA anchors one or more proteins to the surface of the bacterium that are responsible for virulence, that is for high level colonisation and /or induction of severe inflammatory reactions associated with clinical disease.

Detection of sortase anchored proteins in *S. uberis*

To identify proteins anchored to the cell wall of *S. uberis* by sortase, the cell wall proteins of wild type *S. uberis* were compared to those of a SrtA mutant of *S. uberis*.

The methodology used to isolate tryptic peptides of anchored cell wall proteins is as follows. Bacterial cultures were grown in either THB or BHI to both exponential and stationary phases of growth. Exponential cultures were grown in 1.5 litres of broth to an optical density of 0.6 at OD550nm whilst stationary phase cultures were grown in 1 litre of broth overnight. Bacterial cell pellets were harvested by centrifugation (16,000 x g, 10 min, 4°C) and consecutively washed with PBS, 0.1% (v/v) Nonidet P40 (NP40) in PBS, and PBS and harvested by centrifugation as above. Cell pellets were resuspended in PBS containing 1X Complete protease inhibitors (Roche) and disrupted by bead beating in screw capped microfuge tubes containing 0.1 mm zirconia/silica beads at 5 x 1 min intervals at maximum speed, with interspersed resting periods on ice. Unbroken cells and beads were removed by centrifugation twice (8,000 x g, 10 min, 4°C) and supernatants then subjected to high speed centrifugation (125,000 x g, 30 min). The resulting pellets were resuspended in 4% SDS/PBS and heated at 80°C for 4 hours, then centrifuged (200,000 x g 30 min). Resulting pellets were washed 4 times with MilliQ water at 30°C and centrifuged as above. Pellets were then resuspended in 50 mM ammonium bicarbonate containing 1 μg of proteomics grade trypsin (Sigma) and incubated shaking overnight at 37°C. Peptides were harvested from the supernatant following centrifugation (16,000 x g, 10 min) and digestion was stopped by the addition of formic acid at a final concentration of 0.1%.

Peptides were separated and analysed by nanoLC-MS/MS using a reverse-phase liquid chromatography system. The interpretation and presentation of MS/MS data was performed according to published guidelines, with searches performed using Mascot software (Matrixscience, London, UK) using a genomic database generated for *S. uberis* 0140J.

The sequences of the tryptic peptides were aligned with the translated genomic sequence of *Streptococcus uberis* to identify the proteins present

The nine proteins listed in Figures 4 and B were found to be present on cell walls prepared from *S. uberis* 0140J, but were absent from equivalent preparations made from cultures of the isogenic srtA deficient mutant of *S. uberis*, demonstrating the proteins to be sortase-anchored proteins.

The sequence of the nine sortase anchored proteins are given in Figures 6A to 6I. Figures 6J to 6O are the sequences of the putative sortase anchored proteins identified by proteomics.

Detection of Sub1154 and Sub1370 protein in wild type and Srt A mutant *S. uberis* protein extracts

Recombinant Sub1154 and recombinant Sub1370, two examples of *S. uberis* sortase-anchored proteins, were both generated from amplified genomic *S. uberis* DNA and the product cloned in *E. coli* using the pQE1 vector, which incorporated a 6 x His tag at the N-terminal of each protein. The recombinant protein was purified utilizing the 6 x His tag and used for the production of anti-sera.

Rabbit anti-Sub1154 and anti-Sub1370 were then used to detect the Sub1154 and Sub1370 proteins by immunoblotting of detergent and media extracts of *S. uberis* 0140J and the srtA mutant. Media extracts from Sub1154 and Sub1370 mutants grown in BHI were also probed with the antisera. Detection of Sub1154 was confirmed in the srtA detergent extract, and also in the Sub1154 mutant media extract. In the latter case the predicted truncated form of the protein was detected (Figure 5A). The protein corresponding to Sub1370 was detected only in the growth media

obtained from the srtA mutant (Figure 5B). The presence of the Sub1154 and Sub1370 proteins only in extracts from the srtA mutant indicate that in the wild type strain the proteins are anchored to the cell wall of *S. uberis* by sortase.

Example 2: investigation into the requirement for specific sortase anchored proteins for virulence of *S. uberis*

Following on from the identification of sortase anchored proteins, each of the genes encoding these was mutated within the wild type strain. The mutants, each lacking an individual sortase anchored protein, were used in a challenge model in dairy cattle to assess virulence. The proteins missing from those that show reduced or ablated virulence are involved in pathogenesis/pathology of disease. Induction of a neutralizing immune (antibody) response to any and preferably all of these proteins would result in less disease following infection with wild type strains. Thus vaccines containing any or all of those identified as having a role in virulence would be useful in the prevention of mastitis in cattle.

Methodology

Production and isolation of mutant strains of S. uberis lacking SrtA anchored proteins (Sub 0135, Sub 0145, Sub0207, Sub0241, Sub 0826, Sub0888, Sub1095, Sub1154, Sub1370, Sub1730)

Insertionally inactivated mutants were located within a random insertional mutant bank by PCR screening of a *S. uberis* 0140J pGhost9::ISS1 mutant bank following a similar protocol to that described previously (Taylor, D. L. et al, 2003. J Bacteriol 185:5210-5219; Ward, P. N. et al 2001 Infect Immun 69:392-399). Briefly, overnight cultures from individual 96-well plates were pooled and genomic DNA was prepared for use as template in PCR amplification reactions containing a locus-specific primer for each gene of interest and used in conjunction with primer specific to *IS*S1. Following isolation of the mutant clone, excision of the plasmid vector was promoted by growth at the permissive temperature (28°C) without antibiotic selection. Loss of the pGhost9 vector and retention of ISS1 were confirmed by Southern blotting as described previously (Ward, P.

N. et al 2001 Infect Immun 69:392-399). Presence of the insertion in the appropriate ORF was confirmed by PCR amplification of the open reading frame and sequencing of the resulting product across the junction between ISS1 and the disrupted ORF.

Attempts to isolate an insertion mutant from the 0140J random mutant bank with IS*S1* located appropriately near to the start of the SUB1154 coding sequence proved unsuccessful. A targeted deletion strategy was used to ablate production of the SUB1154 gene product. Briefly, two fragments located at either end of the 3432 base pair open reading frame were amplified from genomic DNA. The two fragments were purified and then used as template in equal proportion in a further PCR amplification reaction to generate a single Δ1154 product lacking 3169 base pairs from the 3432 base pair SUB1154 coding sequence. This amplicon was subcloned into the multiple cloning site of the low copy pG$^+$h9 temperature sensitive plasmid. The plasmid construct was amplified by transformation of *E. coli* TG1 RepA with selection on 200μg/μl Erythromycin at 37.5°C and 10ng of the subsequently purified plasmid used to further transform *S. uberis* 0140J with selection on 1μg/ml Erythromycin at 28°C. *S. uberis* 0140J / pG$^+$h9::Δ1154 transformants were grown to OD_{550}0.5 in Todd Hewitt broth culture at 28°C, the growth temperature was then raised to the non-permissive plasmid replication temperature of 37.5°C to force single cross-over chromosomal integration. Integrants were selected on THA containing Ery at 1 μg/ml at 37°C and subsequently grown in THB lacking antibiotic at 28°C to promote excision of the pG$^+$h9 replicon by a second cross-over event. Resulting bacteria were plated on to THA and colonies picked following overnight growth at 37°C. Deletion of the Sub1154 locus was determined by PCR amplification of the Sub1154 locus.

Challenge of lactating dairy cows with *S. uberis*

The requirement for individual SrtA substrates for virulence was determined by experimental challenge in a well established intramammary infection model in the dairy cow. Bacteria were grown for 18 h at 37°C in Todd Hewitt broth. Cells were recovered by centrifugation (10,000 x *g*, 10 min), suspended in pyrogen-free saline (Sigma) and diluted in the same to provide the required cell density (500-1500 cfu/ml). Suspensions of each strain were held on ice prior to being used to challenge animals. The number of viable bacteria in identical aliquots of each suspension was enumerated both prior to and following challenge.

Dairy cows, 2-10 weeks into their first lactation, were selected for challenge. Criteria for selection were: absence of signs of mastitis, absence of bacteria in milk samples prior to challenge, no history of mastitis during the current lactation and no evidence of intramammary infection with *S.uberis* in milk samples taken at 7 and 14 days after parturition. Animals were challenged in mammary quarters by infusion of 1 ml of pyrogen-free saline (Sigma) containing between 500-1500 cfu of *S. uberis*.

Following challenge, animals were milked and inspected twice daily (07:00 h and 15:30 h) for a period of 4 days. Those in which predetermined criteria for clinical end points (clotted and discoloured milk and/or udder quarter swollen or causing discomfort on palpation) had been reached were treated with proprietary branded antibiotics. Milk samples were taken at each milking and analysed for the presence of bacteria and somatic cells, as described below.

Analysis of milk samples

The number of viable bacteria present was estimated by direct plating of 50 µl of each milk sample on to ABA. Samples were also diluted in saline and 50 µl of each dilution plated directly onto ABA. In each case, the presence and/or number of *S. uberis* was determined and the genotype of the recovered isolates was determined by amplification of the appropriate locus. The number of somatic cells present in milk samples was determined using DeLaval portable cell counter according to the manufactures instructions.

Results

Mutants lacking one of Sub 0145, Sub1095, or Sub1154 were used to challenge mammary quarters to determine if the mutation had resulted in major attenuation of *S. uberis*. In all cases, the strains were recovered from milk post challenge and each was genotyped to show the presence of the correctly mutated gene. challenge with strains (lacking either sub1095, sub0145 or sub1154) resulted in relatively poor colonisation for the duration of the experiment (Fig. 7) and in contrast to the wild type strain, in no instance was any of these strains able to induce clinical signs of disease. Consequently, the function of these proteins in pathogenesis of infection can be considered essential and non-redundant. Induction of a neutralizing immune (antibody) response to any and preferably all of these proteins would be predicted to result in less disease following infection with wild type strains.

CLAIMS

1. An immunogenic composition for eliciting an immune response in a subject comprising a carrier or excipient and one or more *Streptococcus uberis* sortase-anchored proteins selected from SUB1154 (SEQ ID NO: 7), SUB1095 (SEQ ID NO: 8), SUB0145 (SEQ ID NO: 2), and proteins with 95% or more sequence identity thereto, wherein the composition is suitable for eliciting an immune response directed to an antigen of the one or more *S. uberis* sortase-anchored proteins and acts to prevent or reduce infection by *Streptococcus uberis* in the subject.

2. The immunogenic composition of claim 1 wherein the subject is a mammal.

3. The immunogenic composition of claim 2 wherein the mammal is a ruminant.

4. The immunogenic composition of any one of claims 1-3 wherein the composition further comprises one or more antigens in addition to the antigen of the one or more *S. uberis* sortase-anchored proteins.

5. The immunogenic composition of any one of claims 1 to 4 wherein the composition is a prophylactic or a therapeutic vaccine against *S. uberis*.

6. The immunogenic composition of any one of claims 1 to 5 wherein the composition further comprises an adjuvant.

7. The immunogenic composition of any one of claims 1 to 6 wherein the composition further comprises polymers or other agents to control the consistency of the composition, to control the release of the one or more *S. uberis* sortase-anchored proteins from the composition, or to control the

consistency of the composition and the release of the one or more *S. uberis* sortase-anchored proteins from the composition.

8. The immunogenic composition of any one of claims 1 to 7 wherein the composition comprises the *Streptococcus uberis* sortase-anchored protein SUB1095 (SEQ ID NO: 8) or a protein with 95% or more sequence identity thereto.

9. The immunogenic composition of any one of claims 1 to 8 wherein the composition comprises the *Streptococcus uberis* sortase-anchored protein SUB1154 (SEQ ID NO: 7) or a protein with 95% or more sequence identity thereto.

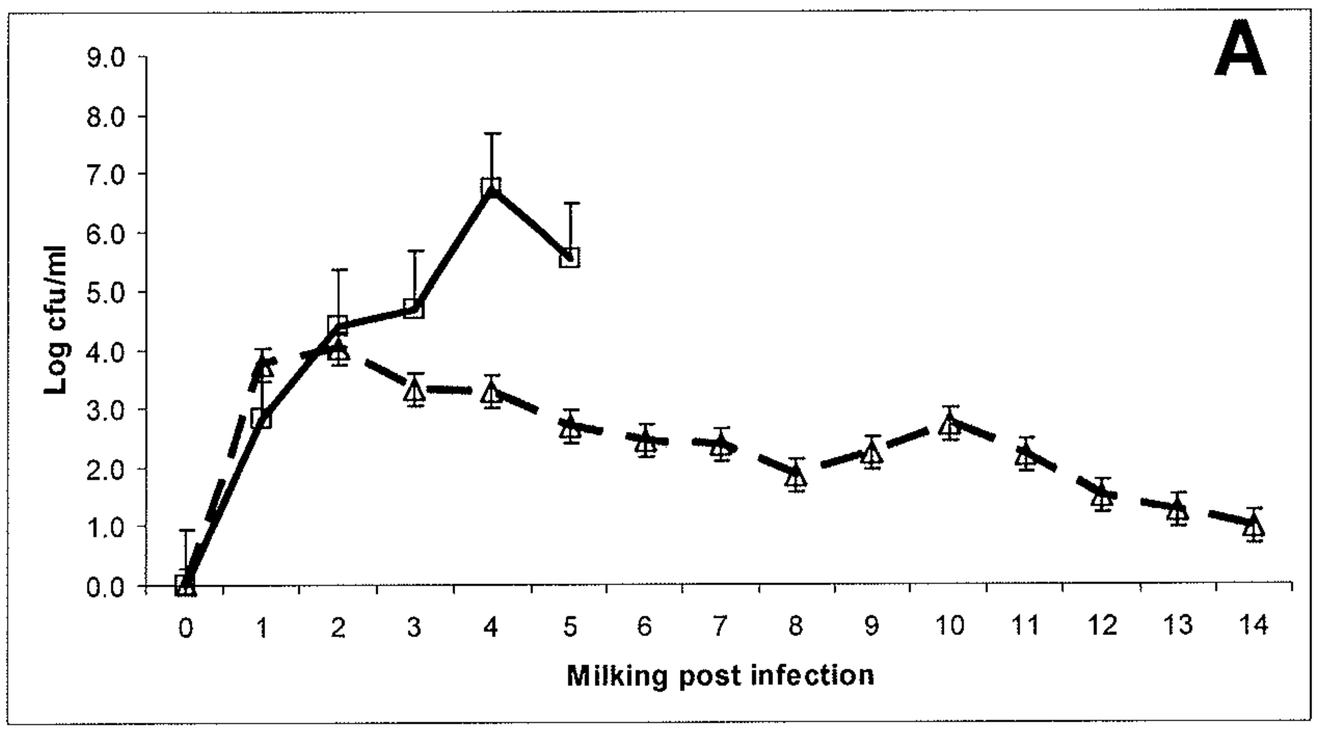
10. A pharmaceutical composition comprising the immunogenic composition of any one of claims 1 to 9 in combination with a pharmaceutically acceptable carrier or excipient.

11. Use of one or more of *Streptococcus uberis* sortase-anchored proteins SUB1154 (SEQ ID NO: 7), SUB1095 (SEQ ID NO: 8) or SUB0145 (SEQ ID NO: 2), or a protein with 95% or more sequence identity thereto in the preparation of a medicament for eliciting an immune response in a subject, wherein the medicament is suitable for use for prophylactic or therapeutic vaccination of the subject against *S. uberis*.

12. Use of one or more of *Streptococcus uberis* sortase-anchored proteins SUB1154 (SEQ ID NO: 7), SUB1095 (SEQ ID NO: 8) or SUB0145 (SEQ ID NO: 2), or a protein with 95% or more sequence identity thereto for eliciting an immune response in a subject, wherein the one or more *S. uberis* sortase-anchored proteins or the protein with 95% or more sequence identity thereto are suitable for use for prophylactic or therapeutic vaccination of the subject against *S. uberis*.

13. The composition of any one of claims 1-10 for use for prevention, treatment, or prevention and treatment of a disease caused by *S. uberis*.

14. The composition of any one of claims 1-10 for use in the manufacture of a medicament for prevention, treatment, or prevention and treatment of a disease caused by *S. uberis*.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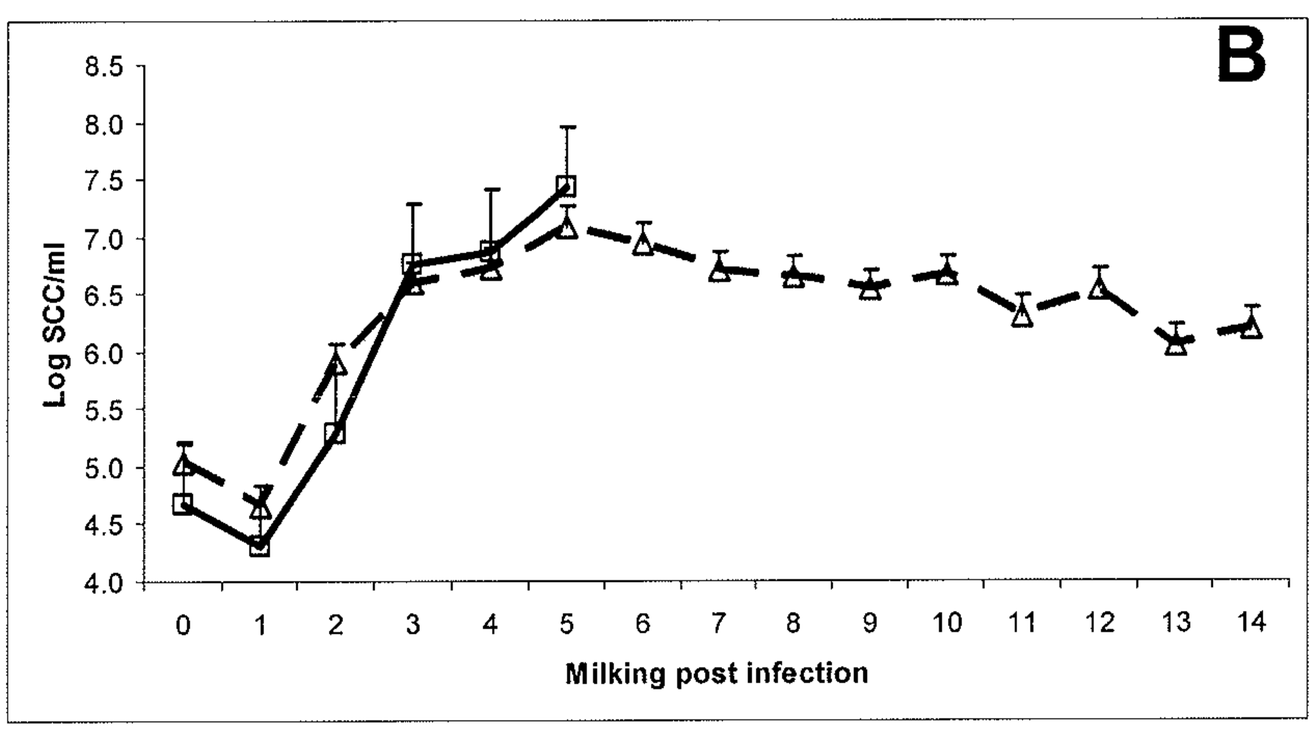
Milking post infection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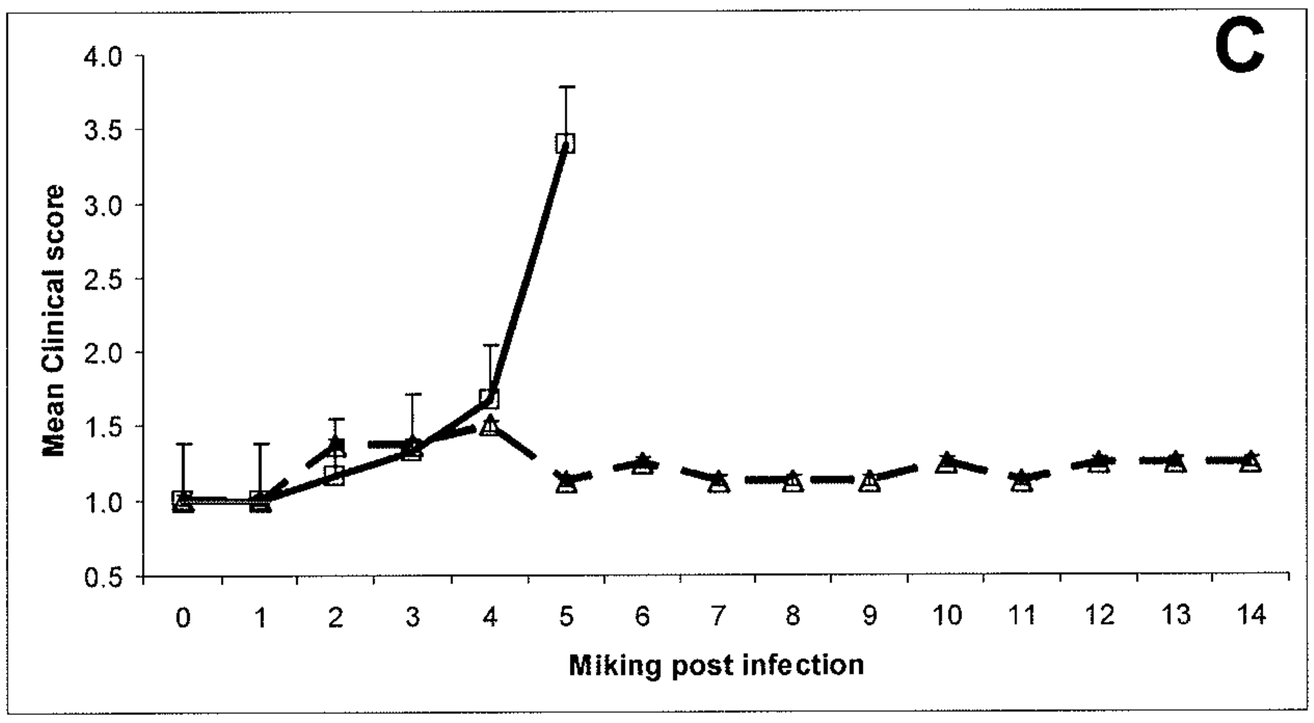
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Mean Clinical score
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3.5
3.0
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2.0
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1.0
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0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Miking post infection

Figure 1

SCORE	APPEARANCE OF QUARTER	SCORE	APPEARANCE OF MILK
1	NORMAL	1	NORMAL
2	ABNORMAL (eg. Hardness)	2	SLIGHT ALTERATION (eg. A few flakes)
3	ABNORMAL (eg. Heat, tenderness,)	3	ABNORMAL (eg. Clots &/or clumps)
4	SWOLLEN (eg. Distended & discomfort on palpation)	4	ABNORMAL (eg. Changes in colour &/or composition)

Figure 2

Gene	Putative Anchoring motif	Additional comments
SUB0145	LPXTG	Lactoferrin binding protein
SUB0164	LPXTG	Pseudogene
SUB0207	LPXAG	
SUB0241	LPKAG	2',3'-cyclic nucleotide 2'-phosphodiesterase
SUB0337	LPATG	ABC transporter amino acid-binding protein
SUB0348	LPHTG	Pseudogene
SUB0888	LPPTG	
SUB1095	LPSTG	Collagen-like protein
SUB1730	LSPTG	
SUB1739	LPTTG	Pseudogene

Figure 3

Gene[a]	Protein annotation	Putative sortase anchor	Molecular mass (Da)[b]	Exponential growth phase MASCOT score	Exponential growth phase No. of peptides matched[c]	Exponential growth phase Coverage (%)[d]	Stationary growth phase MASCOT score	Stationary growth phase No. of peptides matched[c]	Stationary growth phase Coverage (%)[d]
sub0135	Putative fructan beta-fructosidase precursor	LPMTSDS	142,956	41	(1)	1.4	541	10	11.3
sub0145	Putative lactoferrin binding protein	LPSTGDK	57,829	1138	15[e]	29.2	695	18	31.8
sub0207	Putative surface anchored protein	LPMAGER	53,765	39	2	5.4	117	2	5.4
sub0826	Putative surface anchored subtilase family protein	LPETRDS	168,468	ND	ND	ND	50	2	1.2
sub0888	Putative surface anchored protein	LPPTGSQ	29,230	105	(1)	12.9	164	2	18.0
sub1095	Putative collagen like surface anchored protein	LPSTGDK	47,642	59	(1)	1.7	88	2	4.3
sub1154	Putative C5a peptidase precursor	LPKTVDS	127,957	68	(1)	1.7	111	6	4.8
sub1370	Putative zinc-carboxypeptidase	LPALADG	116,563	507	7	10.6	2614	17	25.5
sub1730	Putative surface anchored protein	LPSTGED	40,439	165	2	6.8	238	4	13.6

Figure 4A

Gene[a]	Protein annotation	Putative sortase anchor	Molecular mass (Da)[b]	Exponential growth phase MASCOT score	Exponential growth phase No. of peptides matched[c]	Exponential growth phase Coverage (%)[d]	Stationary growth phase MASCOT score	Stationary growth phase No. of peptides matched[c]	Stationary growth phase Coverage (%)[d]
sub0135	Putative fructan beta-fructosidase precursor	LPMTSDS	142,956	286	5	5.8	1032	18	23.2
sub0145	Putative lactoferrin binding protein	LPSTGDK	57,829	3047	17[e]	27.0	746	15	28.8
sub0207	Putative surface anchored protein	LPMAGER	53,765	50	(1)	2.4	163	4	11.2
sub0888	Putative surface anchored protein	LPPTGSQ	29,230	209	2	18.0	118	2	18.0
sub1154	Putative C5a peptidase precursor	LPKTVDS	127,957	153	3	3.4	237	10	14.4
sub1370	Putative zinc-carboxypeptidase	LPALADG	116,563	146	3	4.0	1079	12	22.3
sub1730	Putative surface anchored protein	LPSTGED	40,439	81	(1)	4.1	167	4	13.8

Figure 4B

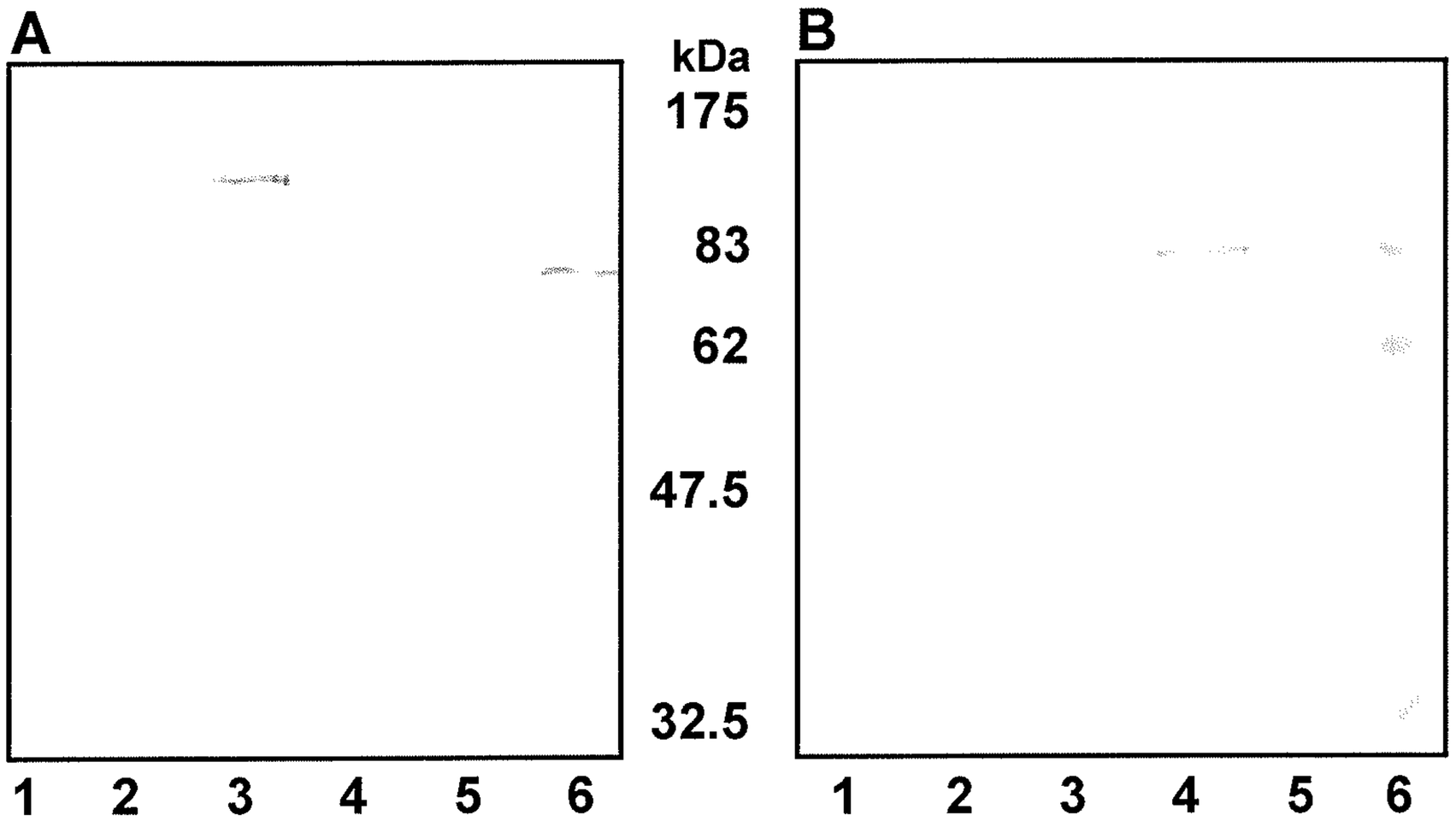

Figure 5

MTKNRSSHSTYADKVIKGLSASCFILGAFVFAQQVSAEEVVTATNTSLTA
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TNTSTTSSDTTSSPIPVTLNKAAIASPTSQTETLASQEIYMDKVNQVTINTTV
NPATPMTWTIENYPNQTYNMQTGDFTGSPSYTVTSTSPNNSSVQIEIPPLFG
TDLSLRWPNNIRRTYRDYMGSYTLKGISEDGLTIVTKELILRPYADYMTHEE
LLNELNAIEANHATDRLVTIETIGQSALGNAIKMGIVAKDQASLDTYLNQTT
PMMLMDPDQALNLLAQGKFDYKLPILINNTHADEQPGIDVVRGLFKTFATE
SVINYQTVDAANNPTTVQIDIKALLDKVILLFNFTENPDGDIANTRALNNGL
DPNRDTGYQTNPETRAIVEQINKWNPISIFDVHGFVKEFLIEPCTPPHDPNFE
YDLFDASLVEGAREMGNAGITNSVYDSYIIPKFDYGSGWDDSFSGYTAVYG
LYQGILGHTIEIPETNQESYNAGYFAVLAGINYDLANSDQLMKNKLTFFSRG
IHKAEVAAAEEALLTVDGSVKGRIKDGHDTFFPDYYMIPMTLSTESDTDQA
FKMIDYFRRNGVILNELTADVAGYHKGDLVIDMAQAKRGFANHVLYKGAN
ESEWPAMYAELVMNFPAMRGFKADAIYADSLFAGNLGAVTLTSAPRTAPS
DKEYYIVSNNSLAAVQAVNAAIRAGKNVYLTNDGYVMDKATYESVIGTYP
LFAQATCMKPVGDTLKAIKVYAPGNPNLYLGFNSPSEVSLALNQMGFDVV
PSVDQADVIVLDNDQFDASILGKKPVIILGGSAMAKLESLGILTGFDAAMTS
ESDGSSYEGLMKISLDANSPYTSGYAANSLYYSNSGSWIEGVPTGFMTLANI
SASDFYVSGWWPNHEGLANKTVAISGLYQGQPMFIFAGNPVNKTHTINFYR
WVSNAIFGTNLTSFIEGQCTIPTDSETQVVRVNHNGQTVAVYQQVANKEVN
GTVSQNSLPALADGSHKDDSKLFWVTGLLVASGGLFAALKRRED

Figure 6A

MRKFYYKEKKMEIKQKHGKHALRKAVTAAVLAGTAFSSLGGFAGAVT
TVKAEDLFTINNSEVQDKLESKVKQLLEAQRKGEDISEKLRELLSELPTDILK
DIMLSNIEADYLLGFLKPAVEEMVRRSEQNDERWKDITEKTLALEALKDSE
REIRKEKEKLEDEVQLAKVKIETKESELNDLKKDYIDTREELADTIEELDEV
KNSIVEKEAKVKGLEEKLRDLEKELGDYDKKLSEAAKQNSDLSNENKELKE
NLDTAENITVELQKKSHELEKTKKEVELELKAEKEALEAEKVKLAEANEAN
DKLSEERDAAKKEAEKVPELEGQVEKLVEEITAAKKEAEELQAKAEGLEKD
FEAVKAEKEALEAEIAKLKEDHQKEVDALNALLADKEKMLKSLQEQLDKA
KEEAMKNEQMSQEEKAKLQAELDKAKQELAEKIKDMPNKVAPQAEGKAN
AGQAAPNQNQNNQAQANQAKNANNLPSTGDKPVNPLLVASGLSLMIGAG
AFVYAGKRKKG

Figure 6B

MKQEKKCVNWFMRKRGKQWIYGCGILICGLVFGVEATSVAAETIPTTA
TVETLNSDVTSKTSQETQKTTEIATPVSEIVMPSQQKVVEEVTQEVSVQNQE
TVINMPVLTQGVNIAGPNETAILTDSIVQNNVQPIDRVEKMETSFSTELTKK
AESSYNTNLQDLNYDPNVWEVREDGLYSNAVGKGDNFLFSASTGENFIFQT
DVTFLQNTGAASLVFRSNNDPENLNGYVVNLDGNSHKARLWRWAEANLI
NDKEILASPDNKYFLKVVATNGWISYYINGILIANLSDYTIQRDDLGQTTYIK
DGHFGLLNWNGEMVFQNTFYRELTNEELPLLNDVTVTSKNGPVEPKGQFF
SESSVYIQYVSNDASTVDLSFDANNSDALITVTDAHGKVYSNPSAIPVTVGP
NYLTVTSTYTTTDGYVIPSTYRINVHRRQPQSVYYNENFRDQYHYSVKDGW
ANDPNGLVYYNGVYHMFYQFYDDTKWGPMHWAHATSTDLIHWEDQPIAF
YPDYNGTMFSGCIVADVNNSSGLFDSENGGLVALITINGEGQRIKLAYSTDE
GKTWQKVDEIVADWTTDPLQTRDFRDPKVFRWENKWFMVIAGGPLRLYSS
DDLKNWTVESTYPDLHTECPDLYPVLAEDQTVKWVLSRGGRYYKVGDLQ
QADGNWKFIPDANYQETDSIMNFGKDSYAAMTYYVQDFGTKANPTIPKIIE
LNWMNTWDNYCNLVADRLGQSFNGTFNLNLELGLVKEGDKYVLTQTPVE
AYESLRDNDNKVEYKNVVVGKENDLFKDFSGDTYEIVAHFKPSDKTTKVG
FNLRVGSGEMTKVYYDLIAGRIIIDRSQSGIILTELFSNIDSQAVTPNIDGSIDL
HIFVDRASVEVFSKNHTVAGANQIFTSAQSLGLEVLIDGEDAKADIVLYPLK
SIWKNKIIDTTPQIVIPASEPKVRMNVGDSTTVKAYVSPVGASQDLIWNISNP
SLVLDQISGNQVFLKAIKKGQVIVRAQSQSNPAVYQDFIIDILEDNFNTNVKD
VNVFSGDWYVDGESLKVANHNSNDIYMSADKIPYENYQMDLDIKYGRGIV
NIFFASGNPDANNAYTIQFGSNNSVRLFRFYRDTIFEAPMIDVINDNQFHHV
RLVKSANVIHVYVDNEMVMSYTFDQVEEFFNNPYLGLGLWDGELAVQNF
YVIDLDAQKPVFVEEHEKEKLLSELKKSVVKTSSYSTLKTIETSSKTNSENLE
APTVSKKNLPMTSDSNNNLEELGILVILTTLGAFLGRVILKKEK

Figure 6C

MKKKQEMKYYLRKSAYGLAAVSVAVLAVGSPVSAQEKAASTEATPKVA
PKVPEKPSKEVIKKALKKTDEETKEKEKEAKEKVENSEESTAMVSELSSTNE
ETSSEEENNTDEEETDGLESEESEETESEVKEESEEEKEDDPSESDTEVENVE
AINLSEAEGNDSSKPETSEEVTAEEDRQETDRLAEVKTEESAKEGDEDADK
KDEAEEKAKKGAELSRVKAEALAKLEALNASRLMKKIVESGKTVEGILSFM
KESLPQLEAARASEQAKAPEVTQSPDHLPSEKKAVHNPVQVAKRSESLEQK
AENAKTSTNLQNTQIPVQEAKRTQAQLPSTGEDYQAYLVAAAMALIASSGM
VAYGSYRKKKQK

Figure 6D

MSKPMTKKKKAISIQKSVKPILGFTFGALLLSTVFTPSVFAEEVVSSLGHA
TSGLLSVSVPKELTSLETTTYLMASESPSNTLTSDTISSDNGGTASNPNEIVTT
ETTSEAIPFDTEVIQNPDLPIGEIKVVQEGVAGEVTVTKTTTTITLNGVSQSTT
TESRVPVKKPINKIIEVGTKEISTSPSSSDVITVSPSPSSTSSESNQQGSLTPAPK
SRQNSQEKKGSQTKKSKDDAKEKEGDKKELPPTGSQESGIFSLFSALISTALG
LFLLKSNKND

Figure 6E

MKSYLKRRYGLITTSVLAATVLATGWQSTSVLAENPTTSPTTTVTSNGFN
FNATLLDHNGKTVSGKTVSLYDITDGNRTLVQSAVSDQNGIASFSQLPLNR
NLSVFVDNVAQGYTTRTSESGQVRSSAFYIDGQGTNTPKYSDKTITISVLNE
EAEPLANQKVTLTNPLKEVVGEAMTDADGHVVFKDKLLEGVFYNYAVNG
KAIDSAQPDSKRSVFLESNQLAKEGFTFTATILGKNGKTVAGKTVSLYDITD
GNRTLVQSAVSDQNGIASFSQLPLNRNLSVFIDDVAQGYTTRTSENGQVRSS
AFYVDGQGTNTPKYSDKTITISVLNEEGEPLANQKVTLINPLKEVIGEANTD
ANGKVIFTDKLLDGVFYTYAVNDQTIDATQPDTSRNVFLRADQILKESPKN
TASEAATNLEKTTESKEGNMPQQNQSEAKEKAPEKQVDANAANKKAPGHG
EAKKGLPMAGERGSRLFTFIGLSLILGIAGYLLKHKKVKS

Figure 6F

MVKNNIHSRKKHILKISLLATSVLTTTVSTVSAEQLQNEKQSDLLSKMTET
STPHTIISSEDLSNSNQEANQKDETASKSLQPMIEKVDPSHIQALWEKVGTGE
GDVLAVIDSGIETKHSMLQLPEDADKMYTDQASIDSKKQLLGIERGQWIND
KLPFYHDYTQGEESIDRNTYHGTHVAGIATASGLTQKENKEQMQGIVPNAQ
LLFLKVGQPSVEGEREKHYAMAIKDAIALGATAINMSFGQVGKASHELNDD
FKKALALAADKGVAIVVAAGNDYAMGGSQTKPLAKNPDTGVIGTPATTEE
VFTVAAYVAPHYWSRVLSVTDGSTSKALALEMASPFAENKDYELIFLEKGL
ETEENAERLKNKVLVLNYDFVTNSKEVAEKVEALGAAGVLVHNNQAKKPL
IPLAYNGPLPMGFISKEDADWLKTMTSPQFRLKKEKQLVEVPGGRQMTNFS
SWGLSVDGNMKPDFAAPGYEIYSPTPGNDYSKMSGTSAASPHAMGIIHLVR
KHIQKEYPHLSAKEQLQLVKNLLMSTASPIYSELDHSYYSPRVQGAGALDA
KKALETDVYVTAADGLSKIQLGDVNNQFELRVTLHNLSNQEKNFTYFARV
LTDKVEKGRILLRPQELYQTRPLQVKLAPNQKQEVVIKVDISNFDQQLKAQ
MPNGYFLDGFVVFQSKEGAQKDLSIPFIAFKGKFADLEALDSPIYRNLDGTF
YYSPKEGQDPYDFEVDSIQQIKEQYMTGLITTFTPWSLVEGSKIDGFSPEMA
SEFSTTDYLGSYNKEGDNTVRRFRFVEGKPYLALSPNGDDNMDKVGFRGV
FLRNVRDIKAQVFASDDLQHPIWESPIKAFAKKDVNTNDIKESMLENTVWE
GKDASGNPVTEGLYRYRVTYTPLAEGAKEQFIDFDILVDLTPSKLPQSAILM
LAERRIELTESRDYLSHDTYRDRLYYKYGTDDINFTTFEKDDMGHFVIPNQ
VEDELSGEKITINLDKTDHFFFVREDFSGNFSVISLSQLLNNHSDQMHSLEES
KSDRKESNTGDIRHEKQENLSQQTLLSTPSIDGQKQNDQLMVEKEKDIMDE
SKSERSEKNKFPKVPASITLKDGTLYPQSISQKTSLPKTVDSQKTMTFLGIAM
LFGGILQVLWSYFKKRD

Figure 6G

MTHMNNNGRYKQRFSLRKYKFGAASVLLGTIFALGMTGTTAQAQMPSH
SHPGGVYPGGIIPGAPGAIPGIPGGGSGFDFDPSGYPAGPHGYLPSYGPGGVG
MLQGPPGPAGPIGPNGIPGERGPVGPAGAEGPRGPKGDKGETGQQGPRGEA
GIAGPSGPQGPAGVAGPAGPQGVAGRDGRDGRDGRPGEAGLDGLDGLNGL
NGIDGTDGKDGKDGKDGQNGQDGKNGRNGQDGQKGKDGQNGRNGQDGK
AGKDGKSGTDGKNGKAGTDGKNGKSGKDGKAGTDGKNGRAGTDGKNGK
AGQDGKDGKNGTDGLNGRDGRDGRDGRDGRDGLDGKDGKNGKDGESPII
NVKDNGNGSHTITFLNPDGSRKEVTISNGKDGQPGRDGKDGRDGKDGMPG
RDGMNGKDGQAAAGNTAGKGNASDMKPKAMAAPAAMTNQNAHANNNG
PAKAQLPSTGDKANPFFTAAALAVMASAGMVAVSRKRKED

Figure 6H

LRYKKMTRSSNSRIVLQSTLIMIFASSCVNHFKGTIHADEKVINGSEASIQV
DYTLNTASENRQIPEEKVTEEATNDQPELLEKQAAFLHEGREKNTENLPLD
GRGSLIASIDSGVDIKHEAFANNDDNHDFHKETEVSEGSTSKIPFVYDFLSGD
TSVRDDEEEHGMHIAGILVGDSKKGFKGMAPKAQLIAYRTWSKNNSEGYQ
EANQFFAMKDAIKRGADVISLSIGEIGSGQNDDIWAKVLEEAKKKNVVVVA
AMGNYGTSATSNTFDQVVDETFPQTDSSTLLSVSANPEVIGVGSIFEKEMYL
PTLKIDTLEVPYENINWQNYYLFKQEKQERISFNEMLITLNQSKEEGSLKDK
VVIIERQAENIFPQLKEVMKKGAKGVILINQSGPTTYGNYETVPELRNTLLD
DEDGDFKKTWAVSISANDGKALKDYLQKQDKKKSYSLVFNTKPQLKHVFK
YPGVSGFSTWGPGLDLTLKPDIVAPGENIYSTGNDNSYFISSGTSMSAPKVA
GASAMFLPVTKKWQKKWEKQNVSMSIPQLTKLLFQNTADILYDHSVPNGK
PILPYSPRRQGAGALNVKKAAQTNVFVTSADNKGAILLKDFKESRKEFDIVI
RNFSDQVRRFKIEPGSVLGKILYSKDRKNYDKNETIQTVHSRVIKDSAIESPL
YVQIAPNSSMILPLKLNVGKAVENEFVEGFIKLRSLEKDQPDLNIPMGFYGD
WNSENILDPVAWQEGSKTRLTGIVHPYGLGEDKFDIVPWGVDYEKWKQDP
KALDADQRFYVMQSQAGIANHAKMRLRLIFMRHAKDYRVDILNSQKDKVL
KTLKTGHQAPKYMESALLEHGDQYQMQFADFDPDLEWDGSVYNPKTNTE
DPLPDGNYFIRVSSRISKNRPYQEHIIPFAIDNQKPKVKIEEKTALQVVFHVD
DAHLQGIRLVKDNKIIQTLETDTQGRFRLNLADFQGKGFELEAIDFAENKTII
DLDSLKEKEVGYLFGASSSYNKSRYRSPRSVAHKNAEDILHENSEESEEIASA
LTFEDGSDFHDGKKTNAYSEINKSNDNSVHLKDNTYYRDYYIHLKEGQRLL
VTTTNAFHNSKQGNDITAPTWQANYTYDPSTNQGQYYRKIAIPIYQGSNTIN
VKAFYKDKLIFNKGYAVKLDTEVPQLTFDNPNISFTSDKWQNLSDDEYDDD
NIVGTITIPNNTLRLSGKIRDGLDGWRMFINGDMVDSDIKLGEYDDIFQQNR
RQWKYEKQVENDDYVLIKLSDHVKNSRSYLFKVKIDPTVSEYHFTNKNDII
DDDKTLLTLNTLTDSSLGYANKLLNMPKDLVKSTDDLFKAMTMLFKKESF
FLYPLKNDLNTNGISMMTSLVQFQAKDVKENIPLEWEIKTKASDSRQLLYQ
NLKNEKERLDQVSTNPLAHQLPLENSNQENGQDAILTSTKVLPMSKSSIFRD
SLRETSLPETRDSSSMANWSLAFFLSAVICFFKGRRKRLNKL

Figure 6I

MENQNQLTLQGILGKCLKWFLLLSISLFTAFPFIWMLISSLKTKAEVMNTEV
IWPHIPQWGNYLEIFTQSPIPKYIWNSLWTSVVIVLIQIVTGAMLAYALVFLR
FKGRQLIFAIVMGTYMLPAAATYIPSYIILSKGGMLNTLTGLIVSSTISIFGIFL
LRQAFMQVPRSLIEASRMDGASHFRVLWEIVCPMTKSSFITFGLMSFIAAYN
SGKETSIGDVTLTFSKELVPVPNIDEEIVIPSIPEKPLVEPEVDSILPLIPLQPSL
PIYPSPDLPEMEQPDQDSPEISGQSQIVDIVEDTLPGVSGQQSSSEETEITEDT
RPESDNEIIIGGQSELVDIVEDTQSGMSGQHSSSEETEISEDTRPESDNEIIVGG
QNELVDIVEDTQPSLSGHQSESQETVTVEDTQPNQTNILIGGQSEIVDIIEDTQ
AGMTGQYSSTDQLTIVEDTLPEQMEETDEIKSDSQVMDIPKVNDTNNDKGA
KASVAFDVEESKVVTTQDIKPSTYVKGDNQLPQTGDDDNVNAFFTLAALSV
IGATGLRQNKRREKERN

Figure 6J

VVKVTVDGSDNSTIDSGFVKVEQPTPGSNSSSESLSQSTTQSSSQSSSAKPVA
SQTAAELPHTGQAENNGLYGSAALAILAALGLAGKKRDEK

Figure 6K

MFKTKKEIFSIRKTALGVGSVLLGVILTTQVASANEVSLMTPSVDKSLTTTSP
VLESTSSQLAATTAPTTTDTTSNVTATSPVLAAATTAPSVTTSPIASPIRYVS
DPNQPVGYRATQVQGTDGSIITTQTGALDANGNPIVTVERIEPTKTVIVLGT
KSTSQVTSTQAATTTYSIDVTKPVGTDVVIPAVDGQTTTTTTYKIETATTAP
VSPSVLSEGYKWIDQPFYHVDTTQTLPSDRISIDQLFVPMPVLTPDYNTTEST
VREYAQYAENYMYDYITVTNPDGSTTRQQVIRPVTSEMLDPTNTRLRTLTG
LTDDNAFYSRLFDASQQDLWNTSAQDYGLEIVPEDLSTSTDDFLRYHSSNII
NDALYVDIKADYLRAQLAYDLVSLGTLTSDQQSAMDMMTSQFESLTLRYN
NYKDSVAIVVDYSNTTMSATQADFEAKLAALPVEVQRAISELTIYDGQIPG
MGETTLGLANSADQTIALKYEANNLNLVSTVLHEMTHIIDFKSGLYSETTD
RNTDGSLSTVMAFSDTQEFLDVYHTYFDRPDVWSYYRDNSEEAFAEGLSQ
YIMHRLFGTPYSTYIANPYTGDAYNPGDGSGYSPFAETEFYFASLYNRLFEY
PRTAQVVPYLVTTTTTAPVNGQVIYGAMPEETTTTTPYTTVYVGDTSFAYD
PTGQTDRVQAGVDGTETIRTTYSLDSNNQLVATQTVISSTPVQNQIITKGTQ
PTVVDTSVPMTIVYQEVTDGSLGDWQVNVLDAGQDGLIRSTTTYSVDPVTG
IVTPSTTEATITAMRPMIVQYQVGSEKVTAIPYQTRYVIDTSLATGTQVIVQE
GVNGSSTESVQSYNFIQDGSNSRFDAIVYASPVVVAAQDQVIAVGGQDQVT
DQAVAKTIFYQEVTDGSLGDWQVKVLDAGQDGLVRTTTSYSVDPVTGIVT
PSTTEATITAMKPMIVQYQVGKSKLSAIPFLTEYITDDSLAVGLEKVIQEGVG
GTQIETVQSFNFIQDGANSHFENIVYSSPTIVVAAVDQVIARGTKVVEVVVA
VPEVVTPKPETSEVISPEKGQTAPTITVEAIKAPAQKKAKVEVVTTPKESLPT
TGDDQNLLVTLMSSLLLMSLGLGLKKKEDE

Figure 6L

LLALSQLPDKLLEKIDITCFDDPKHFGRGIPFQEDSSTAWINSPIDAISYDYHD
MNDFQNWMEQKGLDTDQSYVPRSLYGRYMTERAHDLLQKLKASVIHEKV
TQLNYEPDSQKWNIGTSQRTIPTRFDEVHLTCGELPVLDPYHLQGNPNYIAD
PYPLKNLPKQPGKKDRIAIIGTGLASIDTLKWLLKNSQADLLAFSPSMTFPTV
RILKKETIDWQFLTDTNKQKLFEENSFNFKSLENLFLSELQALGFQNWEETC
RQFLAEGIPGISLSLAFPAQLFLLQQLASHLVDWLTDFWPQMTLSDRQYYK
ENYGKAIINLRNPMPEEAGRLLIEATAQGRLQIIEAVTDIEAGNYGFVLKRE
VGKELSVATVINATGYHLKESNVHQARTLIQQVIRDGLVQINPEGGLSILPQ
TGQVISPKYGILATLYAHGSLVNGVIYQNNSTIKIQQMAERAIGNVIKKPTI

Figure 6M

MRKHYVSKSAIFLAMLVATGSAQFVKAETPTTTTSPATSLTDASASTTPTTN
TTSTVTPALDPNTNFTVDSSATTSTTTPSPVEAAAISPVIATAQPTTNVTSAS
LAPAANTMATTPVEGQTVDVRIISTTDLHSNLVNYDYYQDKASQTIGLAKA
AVLIDQAKAENPNAVLVDNGDILQGTPLGTYEALIDPLQPGEVHPMYAALD
KLGFDASTLGNHEFNYGLTFIENAIASAGLPILNANVFDAATGEYLFQPYAI
VTKSFTDANGQAVDLKIGITGIVPPQIMLWDKANLEGKVTVKDAVQAVTEII
PTIKNAGADIVLVLAHTGIGDDVYETGEENVGYQIASLAGVDAVVTGHSHA
EFPSGQDTGFYESYNGVDGVSGLINGTPVTMAGKYGDHIGIIDLNVSYTGG
KWTVNRDKNHAEIRKIDTKSTIADADILALAQASHLGTIDYVRQTVGETTAP
INSYFALVKDDPSVQIVNNAQLWYAKQQLAGTPEADLPLLSAAAPFKAGTR
NDPTAYTDIPAGPIAIKNVADLYLYDNVTAILKLTGADIKEWLEMSAGQFN
TIDPNVAGPQNLVNTDYRTYNFDVIDGVTYEFDVTQPNKYDAKGNLLNPN
ASRVRNLKFQGKEIDPNQEFMVVTNNYRASGSFPGVKNATINRLLNLENRQ
AIINYIVSEKTINPSADNNWYFADTIQGLDLHFLSADTSKNLIGDKADISYTG
PSTIEGFGDFVFTYVKPELPVATPETPQETGSQLTENRRQEIHQLATRVYNQ
TKATSSSTTKAELPKAGSQESKGLFFMGLSLLGLAGLITKKEERQ

Figure 6N

MRKFLMSCFAALLLLFAGVSQADADQYLRVGMEAAYAPFNWTQDDNSNG
AVPIEGTNQYANGYDVQVAKKVAKSLNKKLLVVKTSWTGLIPALTSGKIDM
IAAGMSPTEERKKEIAFSDSYYTSEPVIVVKADSKFAKAKSLDDFAGAKITA
QQGVWHVNLIPQINGVKAQTPMGDFSQMRQALSSGVIDGYISERPEAMTAE
NANSAFKMVVLKKAFTVNESDAAIAVGMRKDDPRIVQVNTVLADLSANDR
LDLMDKMVTLQPKEKKAENGVQPSFLDQMWSIVTKNWKQFLRGTGLTLLI
STIGTIVGLIIGLLIGIYRTAPKSKHKVLAFFQKLFGWFLNVYIEVFRGTPMIV
QSMVIYYGTAQAFGISIDRTLAAIFIVSINTGAYMTEIVRGGIFAVDKGQFEA
ATALGFTHGQTMRKIVLPQVVRNILPATGNEFVINIKDTSVLNVISVVELYFS
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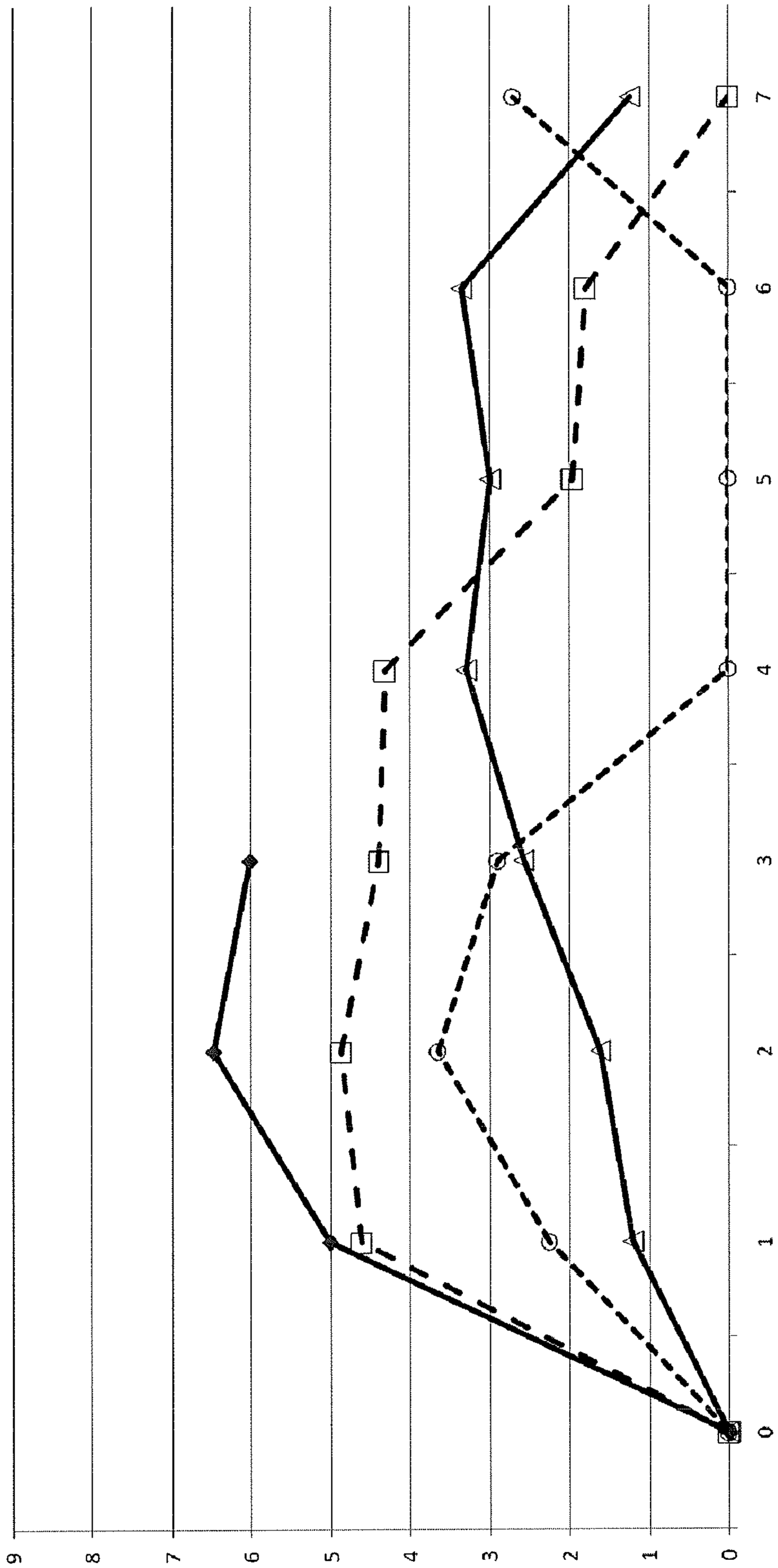
Figure 6O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cfu per ml
Miking post challenge
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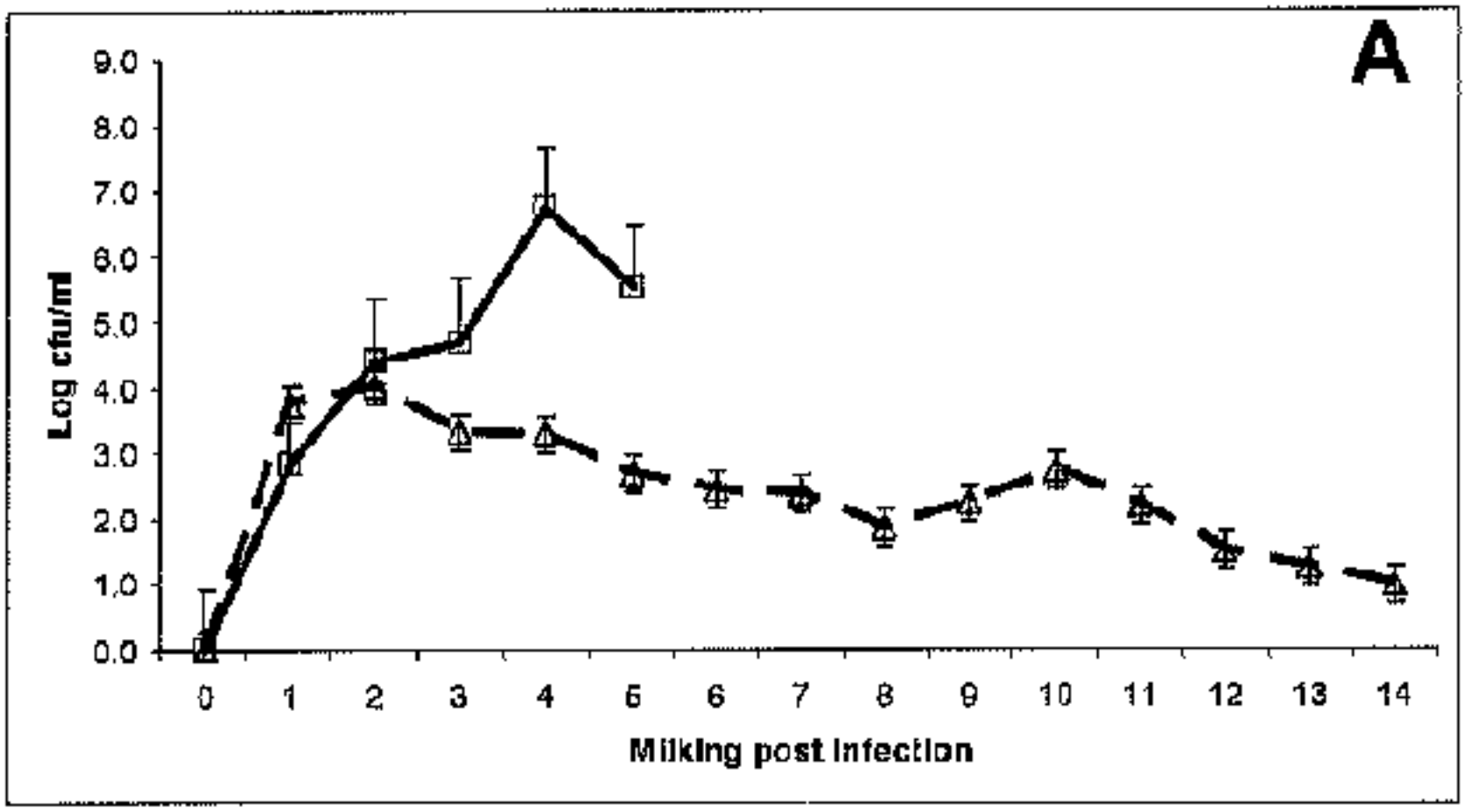
1
2
3
4
5
6
7
8
9

Figure 7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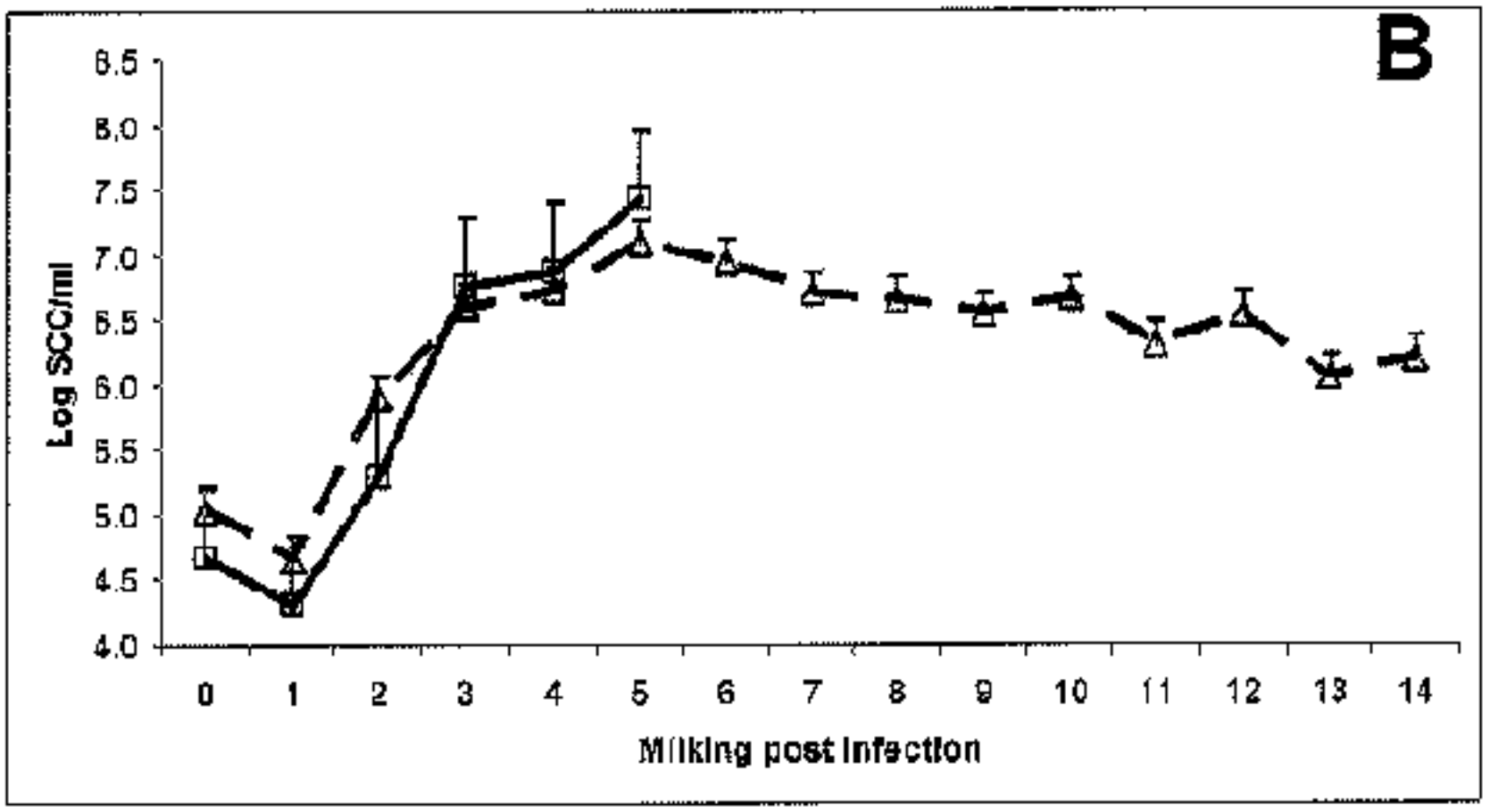
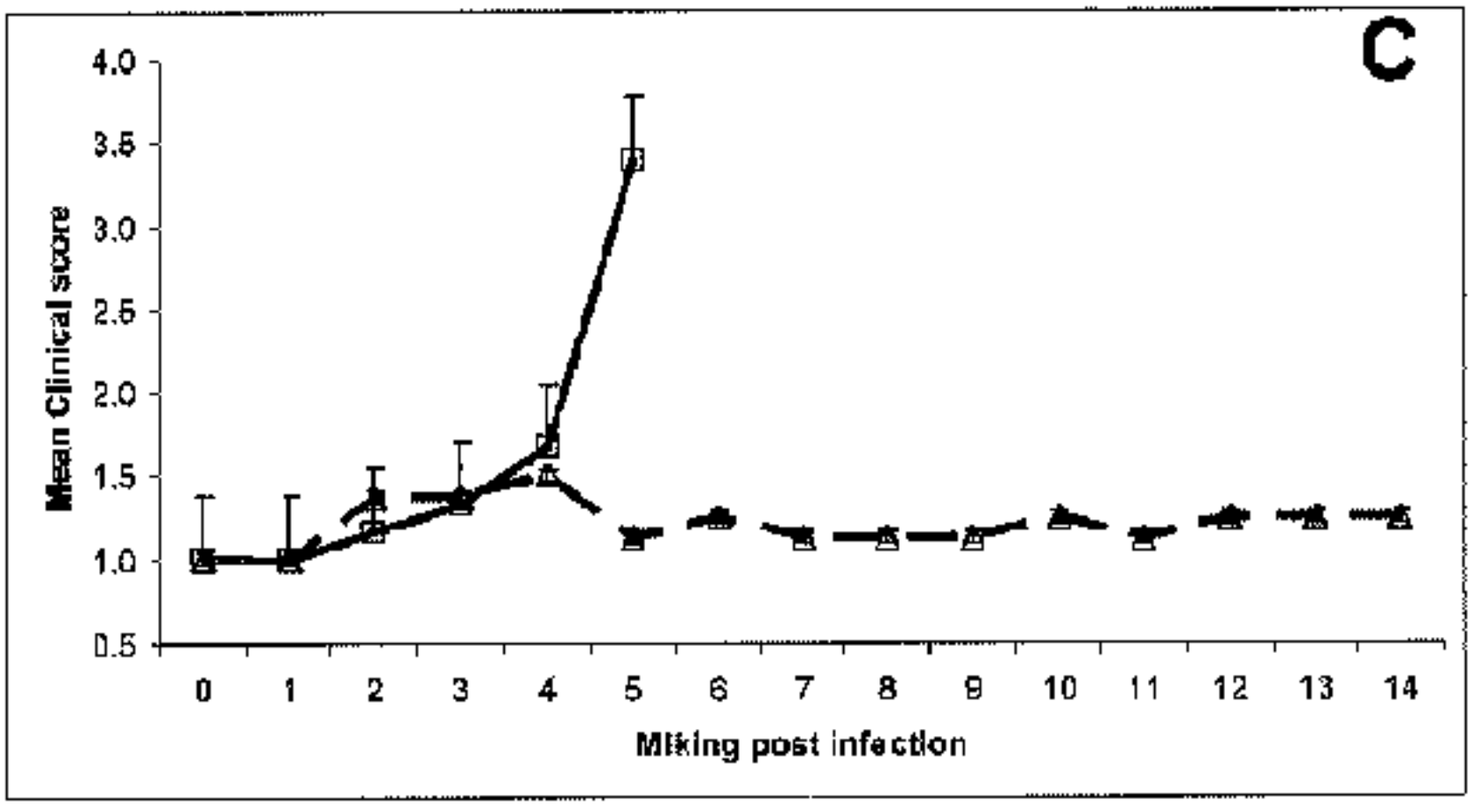
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Log cfu/ml
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Milking post infection


B
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
Log SCC/ml
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Milking post infection


C
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Mean Clinical score
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Milking post infection