Investigating alternative methods such as bacteriophages and bacteriocins to control mastitis organisms

(PRJ-0092-2016)

University of KwaZulu-Natal

Quarter 1 2016 (January 2016 till March 2016)

Project goals

Goal 1 - Obtain Staphylococcus aureus strains of interest representative of the interior of the country from Dr Martin van der Leek.

Achievements

Dr van der Leek has sent through to the UKZN research team 20 different strains of bacteria - both *Staphylococcus* spp. and *Streptococcus* spp. These samples are representative of the interior of the country. These bacterial strains have been re-cultured and samples placed in long-term storage. Furthermore, the *Staphylococcal* spp. are in the process of being screened against the phages that have been applied in the *in vivo* trials, as well as against the current phage bank that the UKZN research team is maintaining and expanding.

No Non-achievements / underperformance has been reported

Goal 2 - Isolate bacterial strains of interest from clinically infected dairy cows from the KwaZulu-Natal region and provide Dr van der Leek with these.

Achievements

More than 50 different strains of microbes have been isolated and stored for both further research at UKZN scheduled to start in June-2016, as well as for culture exchange with Dr van der Leek. Specific species include *Staphylococcus aureus*, *Streptococcus uberis*, *Strep. galactiae*, *Strep. dysgalactiae,* coagulase-negative staphylococci and *Escherichia coli*. We are awaiting confirmation from Dr van der Leek as to when to send strains to his research team.

No Non-achievements / underperformance has been reported

Goal 3 - Isolate and classify phages active against the S. aureus strains from (1) and (2). The same milk samples used for isolation of bacterial strains will be used for isolation of phages.

Achievements

Phages have been successfully isolated for all of the *Staphylococcus aureus* strains that were isolated from raw milk. To date, we have a phage bank of 130 strains. These phages have been screened for their lytic activity and host-range using spot-plating. However, further classification in terms of phage nomenclature, multiplicity of infection, and titre has not been determined. For the current study, we are looking at those phages where these factors have already been confirmed and we are using those phages in the *in vivo* trials.

We are in the process of screening phages against those staphylococcal strains obtained from Dr van der Leek.

No Non-achievements / underperformance has been reported

Goal 4 - Isolate bacteriocins from Staphylococcal and Streptococcal strains, and coagulase-negative Staphylococcus spp. from raw milk. Futhermore, isolate bacteriocins from Bacillus spp.

Achievements

Bacteriocin isolation has been scheduled to take place in the middle of Year 2016. Protocol development is complete. However, bacteriocins will only be extracted from staphylococcal and streptococcal species as these microbes are directly within the scope of the project that has been funded. Extraction from the other microbes (CNS, *Bacillus* spp., *E. coli*) will be adjunct to these, and will be undertaken at a later stage (envisaged for November 2016).

No Non-achievements / underperformance has been reported

Goal 5 - Run in vitro screening of the phages and bacteriocins to investigate their efficacy and required lethal doses against S. aureus, before proceeding with in vivo trials in Years 2 and 3.

Achievements

In vitro screening has been completed for phages. However, bacteriocin screening is only envisaged to take place in July 2016 during the *in vivo* trial off-season. It is envisaged that bacteriocins will be incorporated into *in vivo* trials in the latter part of Year 2016 - September to December.

The third *in vivo* trial using phages only is currently underway.

Non-achievements / underperformance

Phages and bacteriocins have not been screened together either *in vitro* or *in vivo*, for combined activity effects.

Reasons for non-achievements / underperformance

It was discussed and decided upon during project meetings that bacteriocin research be included in the study as a secondary to phage research in these initial stages of the project. This is due to the time that it would have taken to run the *in vitro* bacteriocin screening, which would have delayed progression of the *in vivo t*rials that are currently underway.

Planned remedies for non-

achievements / underperformance

The final decision was that the initial rounds of *in vivo* trials proceed using the most effective phages only. Thereafter, bacteriocin extraction and *in vitro* studies will take place from May-July 2016. This will be followed by subsequent inclusion of bacteriocins in *in vivo* studies in a new set of trials scheduled to begin in the latter part of Year 2016 (September - December 2016).

Goal 6 - Optimise protocols for large-scale production of phages and bacteriocins, in vitro, for use in vivo in Years 2 and 3.

Achievements

Optimisation of phage production protocols to satisfy the requirements for *in vivo* trials for up to 30 experimental cows has been successfully carried out.

Bacteriocin upscaling is only envisaged to take place from July 2016, in preparation for bacteriocin inclusion into *in vivo* trials starting in September 2016.

Non-achievements / underperformance

Protocols for large-scale production of bacteriocins has not been undertaken.

Reasons for non-achievements / underperformance

It was decided upon during project meetings that bacteriocin research be included in the study as a secondary adjunct to the phage research in these initial stages of the project.

This is due to the time that it would have taken to run the *in vitro* bacteriocin screening, which would have delayed progression of the *in vivo t*rials that are currently underway.

Planned remedies for non-achievements / underperformance

Bacteriocin extraction, *in vitro* screening and production optimization will take place from June-August 2016.

This will be followed by subsequent inclusion of bacteriocins in *in vivo* studies in new trials scheduled to start in the latter part of Year 2016 (September - December 2016).

Goal 7 - In addition to S. aureus, isolate strains of Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, and Escherichia coli from both the KwaZulu-Natal region as well from the interior (Dr van der Leek) and isolate phages and test bacteriocins against these pathogens.

Achievements

Strains of *Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis,* and *Escherichia coli* from the KwaZulu-Natal region have been undergoing with a total of 50 different strains in storage. Strains from the interior (Dr van der Leek) will be obtained between February-March 2016. The isolation and testing of phages against the staphylococcal strains is currently underway.

Non-achievements / underperformance

Phages and bacteriocins have not been isolated and screened against streptococcal species and *E. coli*. This will take place in the latter part of 2016. This will be carried out only once the primary *in vivo* trials testing phage and bacteriocin activity against staphylococcus-induced mastitis has been completed.

Reasons for non-achievements / underperformance

Project meetings lead to the decision that staphylococcus-induced mastitis was to be the priority for these initial stages of the project. It was decided that once we get concrete proof-of-concept data that is applied in large-scale trials, can we move onto control of the other problematic microbes. Our timelines show that the late part of Year 2016 (Nov-December 2016) will the most opportune time to initiate these studies.

Planned remedies for non-achievements / underperformance

Isolation and screening of phages and bacteriocins against the greater array of mastitis-causing microbes will take place from November 2016. This will only be once the large-scale *in vivo* trials testing phages against staphylococcus-induced mastitis have been completed.

Goal 8 - Explore alternative diagnostic methods for the detection of mastitis in raw milk, i.e., methods that differ from SCC alone.

Achievements

The student that has been working on this project has performed satisfactorily. Key achievements include:

Validation and Optimization of Reference Methods.

Reference methods for near infrared spectrometer were validated and optimized. These methods include milk fat assay, protein assay and lactose assay.

Milk Fat Assay using a UV method: A milk fat standard curve was successfully generated using prepared standard milk samples of milk fat content between 5-60 mg/mL, ratio between absolute ethanol and milk sample, 1:25, and 1 hour incubation time required for cold protein precipitation and absorbance was measured at 205 nm in a UV instrument. The standard curve had a correlation of 0.991 between points.

Protein Assay: 10 mL of commercial milk was used for the optimization of this reference method for proteins in milk, focusing on caseins and whey proteins present in milk. Optimization was carried out by centrifuging at 5000 g for 10 minutes at 4 degrees Celsius to remove fats followed by acid precipitation using 1M hydrochloric acid and centrifuging again at 5000 g for 10 minutes at 4 degrees Celsius to remove precipitated caseins. Caseins were removed from the supernatant, washed using 95% ethanol and dried for 48 hours and weighed using an analytical balance. Whey proteins in the supernatant were estimated using a Pierce BCA assay kit using bovine albumin serum as a standard protein.

Lactose Assay: 1 mL of commercial milk was clarified to remove proteins and fats using reagents potassium hexacynanoferrate (Carrez solution 1), zinc sulphate (Carrez solution 2) and 100 mM sodium hydoxide. The solution was then filtered and 0.2 mL of filtered sample was used in a lactose and D-galactose kit (Megazyme) according to manufactures instructions to estimate the amount of lactose in samples.

Optimization of Isolating media for mastitis causal pathogens

An isolation milk agar media was optimized where 6.25 mL, 12.5 mL and 25 mL volumes of added milk were investigated on their effectiveness in isolating as many mastitis causal pathogens as possible. Mastitis infected milk samples from local dairies were used. The results indicated that 12.5 mL of added milk was optimal in isolating as many pathogens as possible from infected milk.

pH and Electrical Conductivity (EC) Changes during Staphylococcus aureus inoculation on sterile milk.

Staphylococcus was inoculate on tryptone soy agar (TSA) and incubated at 37 degrees Celsius overnight after which a loopful amount from TSA plate was inoculated onto tryptone soy broth (TSB) and incubated at 37 degrees Celsius for 24 hours.

1 mL representing 10e9, 10e8 and 10e7 CFU/mL amount of *S. aureus* was used to inoculate autoclaved milk and parameters pH and EC were monitored from the onset of incubation at 37 degrees Celsius until 32 hours. As expected 109 CFU/mL amount of inoculum of *S. aureus* showed a high increase in EC and high decrease in pH compared to lower *S. aureus* inoculum size 10e8 and 10e7 CFU/mL.

Current Work

Calibrating the near infrared instrument using optimized reference methods, in order to obtain predictive models which will enable the NIR instrument to be used to predict milk fats, lactose and proteins in milk.

Molecular and morphological identification of mastitis causing pathogen isolates to be used in gas chromatography mass spectrophotometry (GC-MS) analysis.

Obtaining volatile organic compounds (VOC) from milk inoculated with isolated mastitis causal pathogens using GC-MS, the ultimate goal for the obtained VOC profiles for each pathogen is to use them to facilitate pathogen identification in cows infected by mastitis disease on-site.

No Non-achievements / underperformance has been reported

Income and expenditure statement

Income and expenditure statement	Income & Expenditure Q1 2016.pdf

Unnecessary spending during period	No

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Additional documentation

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Statement

Levy funds were applied only for the purposes stated in the contract	Yes
Levy funds were applied in an appropriate and accountable manner	Yes
Sufficient management and internal control systems were in place to adequately control the project and accurately account for the project expenditure	Yes
The information provided in the report is correct	Yes