**The intramammary microbiota: relic or real\***

*\* the better word is “intact” but that does not make a good alliteration, and part of the goal of these experiments is to answer the challenge presented by Pascal Rainard, so I am riffing on his title “fact or fiction”*

Quantifying relic DNA in the milk microbiome

Background:

The existence of an intramammary microbiome\*\* (“unculturable” intact intramammary bacteria ǂ) is controversial. *Here add sentence contrasting statements from Pascal Rainard’s Vet Res opinion piece (*Rainard P. Mammary microbiota of dairy ruminants: fact or fiction? Vet Res. 2017;48(1):25.

650 doi: 10.1186/s13567-017-0429-2 ) *and the recent review from Herman Barkema’s lab (*Derakhshani H, Fehr KB, Sepehri S, Francoz D, De Buck J, Barkema HW, et al. Microbiota of the bovine udder: Contributing factors and potential implications for udder health and mastitis susceptibility. J Dairy Sci. 2018;101(12):10605-25.doi: 10.3168/jds.2018-14860 ) and maybe the Addis paper from 2016)

Add sentences about PMA treatment and relic DNA

\*\* *The “proper” terminology remains controversial (at least for me).*

ǂ *In any of the reviews or primary papers does anyone actually imply or specifically indicate that the unculturable microbiota or microbiome is actually composed of viable bacteria? – yes, for sure, the Addis review does this, and we can construct a summary statement of their assumptions*

Justification:

General Hypothesis:

Specific aims:

Experiment 1:

Goal – to establish methods of PMA treatment for removal of relic (extracellular and non-viable cell associated) DNA from milk.

Methods – “simple” dilution experiment with crude quantification (binary, yes/no) of effect of PMA treatment of milk - checkerboard dilution series diluting both concentration of milk suspended (diluted in molecular grade DNAfree water or PBS) and amount of PMA per ml of milk

Paired PMA treated versus untreated whole milk samples

End-point – PCR amplification of 16S rRNA gene sequence, visualized PCR amplicons by gel electrophoresis

Other measures – controls:

1) confirm no viable bacteria in milk sample by aerobic culture of bacteria from samples by

a) direct plating 1 ml x 10 plates and count total cfu observed

b) enrichment and recovery by primary culture in a non-selective enrichment broth and transfer to culture of enrichment broth to plates

2) measure of sample turbidity by spectrophotometric assay – determine if there is an optimal ratio of PMA to sample quantity and of sample dilution concentration (turbidity) for UV activation of PMA

Limitations:

1. aerobic culture alone does not eliminate possibility of anaerobe presence – we could culture under anaerobic conditions as well
2. PCR amplicon presence/absence measure is somewhat crude (limited sensitivity) in determining DNA template “accessability”

Outline of specific experimental procedures:

1. Thaw lab pasteurized whole milk sample (these samples are stored in ~40 ml aliquots in 50 ml conical vials)

2. Culture aliquot to confirm aerobic culture negative – culture a total of 1 ml (10 plates x 100 uL per plate) under ultra-careful conditions (handle samples in hood)

Experiment 2:

Parallel/Secondary method for demonstrating quantity of relic DNA

So, presuming in the lab pasteurized samples there is no live and only relic DNA, in this second experiment we expect to generate bacterial DNA from 4 samples: 1) DNA from lab-pasteurized milk with a known number of live bacterial cfu, not treated to kill live bacteria and PMA treated; 2) DNA from lab pasteurized milk with a known number of live bacterial cfu, treated to kill live bacteria and PMA treated; 3) DNA from lab pasteurized milk with a known number of live bacterial cfu, treated to kill live bacteria and not PMA treated; and 4) DNA from lab pasteurized milk with no added live bacterial cfu, treated to kill live bacteria and not PMA treated.

Sample 1 – provides an estimate of only live bacteria DNA abundance and diversity (should be a fraction of sample 3)

Sample 2 – provides an estimate of live bacteria DNA abundance and diversity that survives heat treatment if heat treatment not 100% effective (should be less than all other samples; i.e. approaching “0”) – this treatment might be redundant, depending on design and results of experiment 1

Sample 3 – provides an estimate of total bacterial DNA abundance and diversity in spiked lab-pasteurized milk (effect of spiking with S. aureus in pure culture – should be greater than sample 4)

Sample 4 – provides an estimate of total bacterial DNA abundance and diversity in un-spiked lab-pasteurized milk sample (basal diversity and abundance in original milk sample; should be less than sample 3).

Rough outline of specific experiment procedures:

1. Thaw lab pasteurized whole milk sample (these samples are stored in ~40 ml aliquots in 50 ml conicals)

2. Culture aliquot to confirm aerobic culture negative – culture a total of 1 ml (10 plates x 100 uL per plate) under ultra-careful conditions (handle samples in hood)

3. To some volume of this culture negative milk (back calculated amount needed for all dilutions) add a live bacterial suspension (in molecular grade water) at some target cfu/ml (let’s use a S. aureus culture, because we can easily distinguish this organism from possible contaminants based on gross colony morphology on a non-selective media plate)

4. Conduct serial dilution counts on spiked milk sample to estimate cfu/ml in spiked sample.

Experiment 3:

Alternative method of removing relic DNA – DNase digestion? Does this actually work?

PMA vs DNase: I suspect the reaction parameters differ greatly. Therefore, a comparison could yield large differences. That's important understanding results across methods. If your trying to saturate the system qPCR, but below a threshold plus minus should work.