Title:

Diagnosing intramammary infection: a scoping review and meta-analysis on frequency and udder-health relevance of microorganism species retrieved in bovine milk samples.

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Introduction:

Mastitis is a major welfare and economic concern on dairy farms (Aghamohammadi et al., 2018). Mastitis is mainly caused by intramammary infection (IMI). In veterinary medicine, the term IMI is often misused to describe the simple presence of microorganisms in milk. However, according to the Merriam-Webster Medical dictionary (Merriam-Webster), the term infection rather describes "*The invasion and multiplication of microorganisms such as bacteria, viruses, and parasites that are not normally present within the body. An infection may cause no symptoms and be subclinical, or it may cause symptoms and be clinically apparent. … Microorganisms that live naturally in the body are not considered infections. …*". Therefore, when bacterial, algae, or fungal cells or DNA are retrieved from a milk sample, we cannot necessarily conclude that this constitutes an IMI. Nevertheless, in many instances, retrieval of a microorganism not normally present in milk (or usually present, but in very small concentrations) will coincide with measurable or visible signs of inflammation of the udder (mastitis). Such cases would match the proposed Merriam-Webster infection definition and could be considered IMI.

Mastitis is a disease that is characterized by inflammation of the mammary gland. Mastitis is classified as subclinical (SCM) or clinical (CM). According to Glossary of mastitis terms (NMC, 2016), SCM is the most prevalent form of udder inflammation, but it cannot be detected visually. Conversely, CM is an udder inflammation characterized by visibly abnormal changes in the mammary gland tissue or milk. While CM can be detected solely based on clinical observation, diagnosis of SCM is more challenging and requires the use of diagnostic tests. Most informative will be an evaluation of specific milk characteristics, such as conductivity, elevations in concentrations of milk enzymes and salts, decrease in percentages of fat,

lactose, and markers of inflammation such as somatic cell count (SCC) (Adkins and Middleton, 2018). Both forms of mastitis result in important economic losses on dairy farms, due mainly to milk yield reduction, culling, and treatment costs (Aghamohammadi et al., 2018).

A very large number of microorganisms, mainly bacteria, fungi, and algae have been retrieved from milk samples of apparently normal milking quarters and from quarters with clinical mastitis. Some microorganisms have been clearly associated in the literature with SCM and/or CM, and when they are retrieved from a milk sample, most will conclude that an IMI is present. For instance, Current Concepts of Bovine Mastitis (NMC, 2016) listed the following contagious and environmental microorganisms as important mammary gland pathogens: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis, Streptococcus dysgalactiae* and other *Streptococcus* spp*, Mycoplasma bovis* and other *Mycoplasma* spp, *Corynebacterium bovis*, *Escherichia coli*, *Klebsiella* spp, *Enterobacter* spp, *Citrobacter* spp, *Enterococcus faecalis*, *Serratia* spp, *Enterococcus faecium*, non-*aureus* staphylococci (*S. chromogenes*, *S. hyicus*, *S. warneri*, *S. epidermidis*, *S. cohnii*, *S. simulans*, *S. xylosus*, *S. sciuri*, *S. saprophyticus*), *Pseudomonas aeruginosa*, *Trueperella pyogenes*, *Nocardia* spp, *Mycobacteria*, *Serratia* spp, *Bacillus cereus*, yeasts (*Candida*), molds, and algae (*Prototheca*). However, for many microorganisms, the literature is scarce regarding their udder health relevance. To complicate the matter, improvement of the methods for microbial identification in diagnostic laboratories by matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry technology has led to the reporting of several "new" microbial species in cow's milk. These species should be considered relevant, from an udder-health perspective, if and only if they can be associated with measurable inflammation (either an increased SCC or clinical mastitis). To support the interpretation of milk bacteriological analyses conducted using MALDI-ToF technology, guidelines are needed regarding the udder health relevance of the different species that are commonly reported using this method.

The objectives of the current study are, therefore, to: 1) map the current knowledge on cow udder health relevance (mainly associations with a measure of inflammation) of the microbial species identified in milk samples using MALDI-ToF and identify knowledge gaps regarding their relevance; and 2) report the relative frequency of the different microbial species in bovine milk from apparently normal milking quarters and from quarters with clinical mastitis.

Methods:

Our protocol was developed and described using guidelines from the Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for Scoping Reviews (Tricco et al., 2018). The final version of the protocol will be made available on an open access web depository (SYREAF; Systematic reviews for animals & food; http://www.syreaf.org/contact/).

Objective 1) Describing current knowledge on udder health relevance

We aim to identify the microorganisms for which there is no or very little peer-reviewed literature on their association with udder health or inflammation. To achieve this objective, a scoping review methodology is chosen. This type of review is usually conducted not just to identify key characteristics related to a specific topic, but, most importantly, to identify and analyze knowledge gaps (Munn et al., 2018). The research questions will be formulated as: 1) what is the current knowledge about the udder health pathogenicity (mainly inflammation) of microbial species found in milk samples from apparently normal milking bovine quarters and from milk samples from bovine CM cases? What are the microbial species for which knowledge regarding udder health relevance is absent?

The first step of the review will be to develop a comprehensive list of the microbial species (bacteria, algae, and fungi) found in cow's milk from apparently normal milking quarters and from quarters with CM that have been identified using MALDI-ToF. To achieve this, we will contact several authors that had published research using milk samples analyzed using MALDI-ToF (mainly located in North and South America, Europe, New-Zealand and Australia). They will be asked to provide any research or diagnostic original databases where either: 1) quarter-milk samples were collected on apparently normal milking quarters and without any pre-selection criteria for quarters sampled (e.g., not only from high SCC quarters, or only from quarters selected because of a known IMI history); or 2) quarter-milk samples were collected from CM cases. The databases, which will be obtained, will then be merged in two datasets, one for studies on apparently normal milking quarters and one for CM. Each microbial species reported in one of the datasets will be initially considered for the review. Microorganisms reported in these databases, but only identified at genus-level (e.g., *Streptococcus* spp) or reported as a group of microorganisms (e.g., other Gram-positive) will be not considered for the subsequent search of the literature.

Moreover, all the groups and species of microorganisms already described as important mastitis pathogens in Current concepts of bovine mastitis will be excluded from the subsequent literature search (NMC, 2016). This latter reference synthetizes the current knowledge on the mammary gland health of dairy cows. The microorganisms mentioned in this document are already well-studied contagious or environmental mastitis pathogens. We will make an *a priori* assumption that a lot of scientific literature confirming their udder health relevance was already available and that no knowledge gap would be identified for these microorganisms.

A general search strategy will be developed to identify peer-reviewed manuscripts reporting on a given microbial species (the exposure) and a measure of inflammation (the outcome) in dairy cows (the population). The search terms used will be genus and species of the microorganism (for the exposure), somatic cell* OR intramammary infection OR mastitis (for the outcome), and cattle OR cow OR *bos taurus* OR bovin* (for the population). For instance, the search strategy for *Lactococcus lactis* will be:

> *(Lactococcus lactis)* AND ((somatic cell*) OR (intramammary infection) OR (mastitis)) AND ((cattle OR cow OR (bos taurus) OR bovin*))

Since the goal of the scoping review is to map the relative amount of knowledge available on each microbial species, only one bibliographic database, Medline (PubMed), will be searched. To find potentially relevant papers, Medline will be initially searched between day/month/year and day/month/year (to be determined). Each microbial species will be searched independently, by two reviewers, in parallel. When this initial search will be completed, the bibliographic search will be updated for all species on a same day (day month year; to be determined). Documents from 1976 and after, written in English, French, or German will be considered for the review.

Again, since the goal is to identify knowledge gaps (*vs.* reporting all the literature on a microbial species), the search strategy will be refined whenever the search strategy for a given microbial species yielded more than 30 articles. As a first attempt to refine the search strategy, we will ensure that the microbial species was mentioned in the title or the abstract (*vs.* the text) of the articles. The search strategy will thus be modified as follows (using the previous example):

> *Lactococcus lactis* [Title/Abstract] AND ((somatic cell*) OR (intramammary infection) OR (mastitis)) AND (cattle OR cow OR (bos taurus) OR bovin*)

Whenever this more specific search strategy will still generate more than 30 articles, we will specifically look for review, meta-analysis, or systematic reviews types of articles. If such articles types are available, only these will be reviewed, otherwise all listed articles will be reviewed.

The abstracts and full texts of the listed articles will be assessed independently by the two reviewers to make sure that the study was conducted using milk samples obtained from dairy cows. All data reporting on SCC (SCC, somatic cell score, CMT, or other measurements) of the affected quarter or cow, or on SCC difference between infected and healthy quarters or cows, or on presence of clinical signs will be extracted. Regarding presence of clinical signs, when the authors simply used wording such as: samples from "mastitic cows" or from "mastitis", we will not consider that these were necessarily CM, because these terms are also often used for SCM.

The output from each of the two reviewers will be compiled for each microbial species: 1) the number of articles initially retrieved; 2) the number of articles with the microbial species mentioned in the title or abstract (when > 30 articles were retrieved in the initial search); 3) the number of review, meta-analysis, or systematic reviews available (when the secondary search also yielded >30 articles); and 4) the presence or not of SCC and/or CM information and the actual SCC and CM information contained in each article. Then, they will compare the results obtained between reviewers and will achieve a consensual decision whenever discrepancies will be found between their respective extraction. With this extraction, we will be able to report the microbial species for which literature was nil, those for which associations with inflammation was not or uncommonly reported despite some literature on the subject, and, finally, those for which a substantial amount of literature was available.

Objective 2) Relative frequency of the different microbial species in milk from apparently normal milking quarters and from quarters with clinical mastitis

To achieve the second objective a meta-analysis approach will be used. Random effects meta-analysis models will be used to report prevalence of species of microorganism in each type of samples (samples from quarters with apparently normal milk *vs.* samples from CM cases) using the databases provided by our collaborators.

In each study, for each sample type and for each microorganism species, prevalence will be computed as:

$$P = \frac{Frequency}{n - c}$$

Where: Frequency = # of times the species was reported,
n = # of samples in the study,
c = # of contaminated samples or uninterpretable samples in the study.

Venn diagrams and other descriptive figures will be generated to illustrate the distribution of microorganisms across studies for apparently healthy quarters and in CM cases' samples. Then, as a first step to select the most important microbial species for meta-analyses, for each sample type, an overall prevalence combining results from all studies will be computed and the 50 most frequent microorganisms will be identified. A random effect meta-analysis will be conducted for each of these species and for each sample type to summarize the species prevalence among all studies.

Covariates that could potentially explain difference in species prevalence between studies will be investigated using meta-regression. The main covariates considered could be: sample size, effect of breed,

number of herds included, and region where study was conducted. Alpha threshold for statistical significance will be set at 0.05. Meta-analysis and meta-regression will be performed using the meta package (version XX, to define) of R (version XX, to define). The inverse variance approach will be used to assign weights to the studies, and the prevalence will be estimated using a logit distribution.

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